

RESEARCH ARTICLE

Bone regeneration in a rat cranial defect with delivery of PEI-condensed plasmid DNA encoding for bone morphogenetic protein-4 (BMP-4)

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Gene therapy approaches to bone tissue engineering have been widely explored. While localized delivery of plasmid DNA encoding for osteogenic factors is attractive for promoting bone regeneration, the low transfection efficiency inherent with plasmid delivery may limit this approach. We hypothesized that this limitation could be overcome by condensing plasmid DNA with nonviral vectors such as poly(ethylenimine) (PEI), and delivering the plasmid DNA in a sustained and localized manner from poly(lactic-co-glycolic acid) (PLGA) scaffolds. To address this possibility, scaffolds delivering plasmid DNA encoding for bone morphogenetic protein-4 (BMP-4) were implanted into a cranial critical-sized defect for time periods up to 15 weeks. The control conditions included no scaffold (defect left empty), blank scaffolds (no delivered DNA), and scaffolds encapsulating plasmid DNA (non-condensed). Histological and microcomputed tomography analysis of the defect sites over time demonstrated that bone regeneration was significant at the defect edges and within the defect site when scaffolds

encapsulating condensed DNA were placed in the defect. In contrast, bone formation was mainly confined to the defect edges within scaffolds encapsulating plasmid DNA, and when blank scaffolds were used to fill the defect. Histomorphometric analysis revealed a significant increase in total bone formation (at least 4.5-fold) within scaffolds incorporating condensed DNA, relative to blank scaffolds and scaffolds incorporating uncondensed DNA at each time point. In addition, there was a significant increase both in osteoid and mineralized tissue density within scaffolds incorporating condensed DNA, when compared with blank scaffolds and scaffolds incorporating uncondensed DNA, suggesting that delivery of condensed DNA led to more complete mineralized tissue regeneration within the defect area. This study demonstrated that the scaffold delivery system encapsulating PEI-condensed DNA encoding for BMP-4 was capable of enhancing bone formation and may find applications in other tissue types.

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Introduction

Treatment of serious bone defects associated with nonunion fractures, and bone morbidity as a result of trauma or cancer poses a significant challenge in orthopedics, dentistry and plastic surgery.^{1–3} Various therapies have been employed to treat bone tissue loss, including autografts, allografts, and artificial materials such as metal alloys.^{4–6} The major drawbacks to autografts are an insufficient supply and surgical side effects, including donor site pain and infection. Allografts trigger concerns related to host response and disease transmission, and can be associated with infection and inflammation.^{7,8} Insertion of artificial materials (eg metal alloys) requires the removal of a significant amount of adjacent bone, and problems can arise at the prosthetic

material/bone interface.⁹ Although external fixation devices are capable of stabilizing fractures, the absence of viable bone at the defect site can lead to structural instability, infection, and bony erosion.¹⁰ The limitations of current therapies have driven the emergence of tissue engineering approaches for bone regeneration. These approaches are generally centered on the delivery of osteoinductive growth factors, using direct protein delivery or gene therapy approaches,^{11–14} implantation of osteogenic cells,^{5,15,16} and combining these approaches with osteoconductive scaffolds to promote bone regeneration.^{17,18} Direct protein delivery can suffer from protein instability, and inadequate post-translational modifications of recombinant proteins may limit their bioactivity.¹⁰ Alternatively, gene therapy approaches utilizing DNA encoding for osteogenic growth factors, such as bone morphogenetic proteins (BMPs), promises to be an effective approach for the treatment of osseous defects and diseases.^{19–23}

BMPs are a member of the transforming growth factor- β (TGF- β) superfamily, and have been implicated in the development of various tissues, most notably bone.^{24–26} A variety of BMPs have been isolated and cloned,

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including BMP-4. BMPs are responsible for initiating cartilage and bone progenitor cell differentiation,^{16–20} and sequencing new bone formation via endochondral ossification.²⁷ In the context of bone regeneration, BMPs act as chemotactic agents by initiating the recruitment of osteoprogenitors and mesenchymal stem cells towards bone defect sites.²⁴ In addition, BMPs serve as growth factors by stimulating angiogenesis (ie the formation of new capillary vessels from existing host vessels)²⁸ and proliferation of stem cells from surrounding mesenchymal tissues.^{29–31}

Gene therapy approaches to deliver BMPs to bone defect sites include *ex vivo* genetic modification of cells that are subsequently transplanted,^{20,22,23,26,32,33} injection of viral vectors containing BMP encoding sequences,²¹ and plasmid DNA-based approaches.¹⁰ The amount of BMP produced from *ex vivo* modified cells may be limited by their survival rate after transplantation, which may be especially low in larger defects, and this approach is complex and involves multisteps. Direct delivery of viral vectors is less complex, and these vectors generally display high gene transfer efficiency. However, introduction of these vectors poses concerns related to immunogenicity and uncontrollable expression, and disturbance of the host genome.^{34,35} Delivery of plasmid DNA has been pursued as an alternative approach to circumvent these issues. Plasmid DNA is economical and relatively simple to manufacture, and has a stable, flexible chemistry that lends itself for polymer-based drug delivery systems.¹⁴

The successful application of nonviral gene therapy approaches in bone regeneration relies on an appropriate delivery system and the safe and efficient expression of osteogenic growth factors. Biomaterials, including naturally derived materials such as collagen and demineralized bone matrix,³⁶ inorganic materials such as hydroxyapatite,³⁷ and synthetic materials such as poly (lactic-*co*-glycolic acid) (PLGA)³⁸ have been investigated as osteoconductive materials as well as growth factor delivery vehicles in bone tissue engineering. Collagen offers cell interaction through cell adhesion sequences inherent to this material, and a previous study using collagen as the delivery vehicle for plasmid BMP-4 demonstrated enhanced bone regeneration.¹⁰ However, collagen may be immunogenic and properties may vary from batch to batch. Demineralized bone matrix possesses osteoconductivity, but has limited availability and processing flexibility. Hydroxyapatite is biocompatible but not degradable.³⁹ Alternatively, PLGA has a history of clinical use and has proven to be an effective vehicle for osteogenic growth factor delivery¹⁷ and plasmid DNA in several applications.^{14,40,41} Although local gene therapy with plasmid DNA has been shown to be feasible, the low transfection efficiency and the high dose of plasmid DNA required with this approach pose obstacles for this approach. However, condensation of plasmid DNA using cationic macromolecules (eg poly (ethylenimine) (PEI))⁴² can greatly enhance the efficiency of cellular uptake and gene expression level. We have recently demonstrated that PEI-condensed DNA can be efficiently immobilized within porous PLGA scaffolds and this led to transfection of cells in contact with the scaffold *in vitro*.⁴³

This report investigates the effects of delivery of PEI-condensed plasmid DNA encoding for BMP-4 from

macroporous PLGA scaffolds in bone regeneration. The utility of this system for bone regeneration was evaluated utilizing a cranial critical-sized defect model,^{13,44} as a critical-sized defect allows one to clearly identify the effect of the intervention on bone regeneration. This system provides a platform that potentially allows osteoprogenitors or other cell types (eg, fibroblasts) surrounding the defect area to migrate into the defect site, and be transfected by the PEI-condensed plasmid DNA associated with the scaffold. The BMP-4 secreted from these cells may both drive the differentiation of osteoprogenitors into osteoblasts involved in bone regeneration, and possibly also increase recruitment of more host cells into the scaffolds. We hypothesized that the increased transfection as a result of the delivery of condensed DNA encoding for BMP-4, as compared to delivery of uncondensed plasmid DNA, would increase bone regeneration.

Results

Scaffolds incorporating condensed DNA, plasmid DNA and blank scaffolds were implanted into rat cranium critical-sized defects to examine orthotopic bone regeneration for time periods ranging from 3 to 15 weeks. Gross examination at the defect edge after retrieval displayed a seamless integration of implanted scaffold and host bone, both from the side view and top view. To confirm that the 9 mm defect was critical-sized, and thus would not heal without intervention, a control condition including no implantation of scaffold (defect left empty) was also included and analyzed at 15 weeks. The empty defect displayed only growth of a thin fibrous tissue layer and no evidence of bone formation and was not further analyzed (Figure 1a). This was consistent with previous studies where no spontaneous bridging of 8 mm cranial defects was observed.⁴⁵ Defects implanted with PLGA scaffolds were bridged with tissues and interspersed remnants of the polymer matrix (Figure 1b). Histological sections stained with hematoxylin and eosin (H&E) further confirmed the gross appearance of an integration between the host cortical bone and the newly formed cortical bone within the scaffold. This is characteristic of all samples at the defect edge where the scaffolds are in contact with the host bone, demonstrating the biocompatibility and osteoconductivity of this scaffold delivery system.

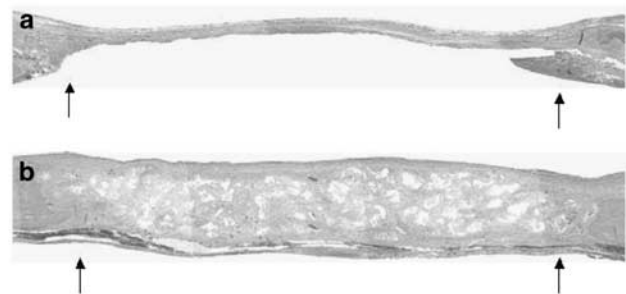


Figure 1 Implanted scaffolds support tissue ingrowth. H&E staining of sections of a critical-sized defect (a) without an implanted scaffold and (b) a defect 15 weeks after implantation of a PLGA scaffold. Arrows indicate the defect edges.

The extent of bone regeneration within scaffolds from each condition were compared by examining histological tissue sections at each time point. All scaffolds demonstrated extensive cellular infiltration. Cellular infiltration was prominent mainly at the defect edges at the 3- and 8-week time points for the blank scaffolds and the scaffolds encapsulating plasmid DNA, with decreasing density to the center of the defect. At 15 weeks, cellular infiltration was evenly observed throughout the scaffolds, possibly due to host cell infiltration from the dural region. For scaffolds encapsulating condensed DNA, cellular infiltration was more evident at the defect edges than at the center of the defect at the 3-week time point. At the 8- and 15-week time points, cellular infiltration was evenly distributed throughout the scaffold volume. In terms of bone formation, at 3 weeks, fibrous tissue prevailed within the defects implanted with blank scaffolds, and scaffolds delivering uncondensed plasmid DNA displayed small bony regions. Both conditions led to minimal bone formation at the defect edges only. In contrast, scaffolds incorporating condensed plasmid DNA revealed larger amount of bony nodules, both in number and size, although still mainly seen at the defect edges. At 8 weeks, defects with blank scaffolds were still filled with fibrous tissue, and the scaffolds delivering uncondensed plasmid DNA displayed small bony spicules near the defect edges. In contrast, the defects implanted with scaffolds delivering condensed plasmid DNA displayed bony trabeculae, and a greater amount of new bone than observed at 3 weeks. At 15 weeks, defects implanted with blank scaffolds displayed more

fibrous tissue than at previous time points, and minimal bone-like regions were observed near the defect edge along the dural region (Figure 2a). Scaffolds incorporating uncondensed plasmid DNA led to the formation of thin strips of bony tissue at this time (Figure 2b). Scaffolds delivering condensed plasmid DNA led to islands of interconnected bony trabeculae, with an even larger amount of bone than at the 8-week time point. Developing marrow could also be identified in this condition (Figure 2c). Higher magnification of the histological sections of these latter samples revealed that new bone formation was characterized by immature woven bone, with osteocytes embedded in the lacuna and osteoblasts lining the outer edge of the bone tissue (Figure 2d). In certain areas, bone formation consisted of both evolving immature woven bone, and mature bone, as characterized by a lamellar structure with interconnected bony trabeculae (Figure 2e). This finding demonstrated the active development of new bone tissue in this condition. There was no significant difference in inflammatory response between experimental conditions, and at each time point.

The composition of the ingrown tissue was next analyzed using bone-specific stains to confirm the observations from H&E-stained sections. Tissue sections from 15-week samples were stained with Goldner's trichrome to demonstrate the presence of osteoid matrix, as indicated by dark red staining. Histological analysis indicated that there were small regions of osteoid matrix within the defects treated with blank scaffolds (Figure 3a) and uncondensed DNA scaffolds (Figure 3b),

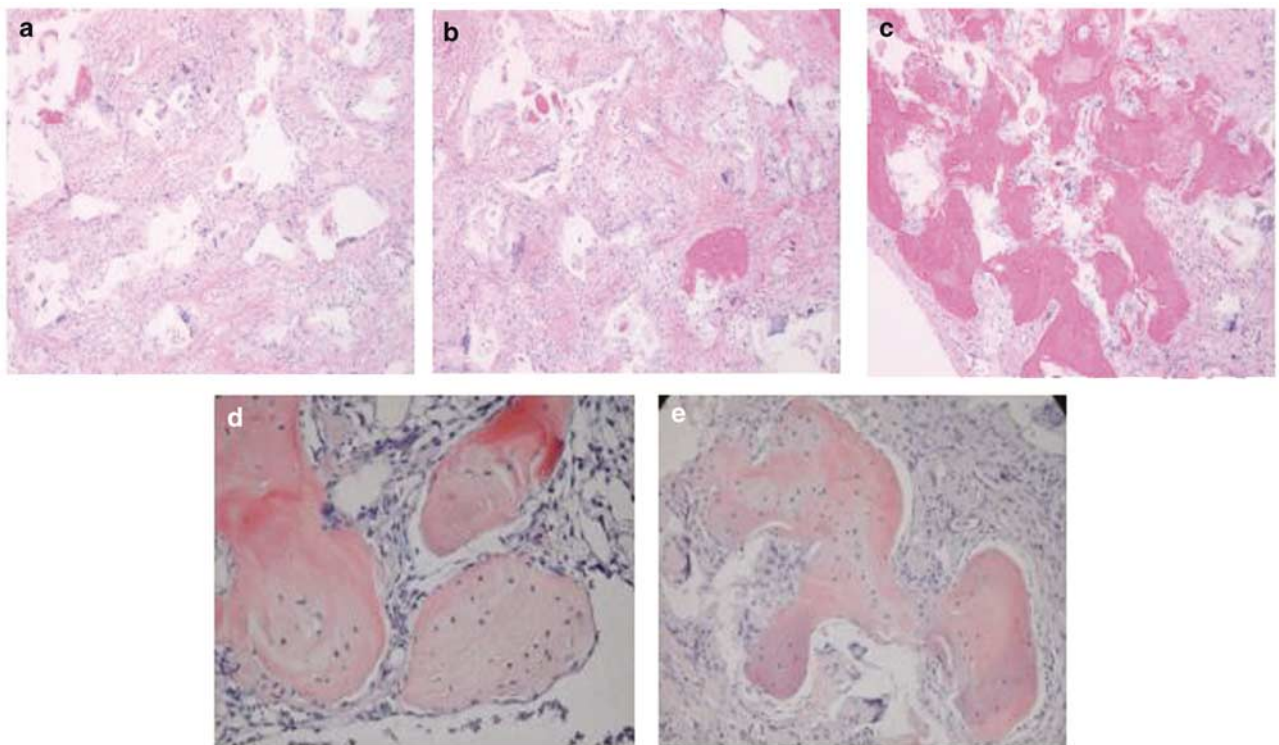


Figure 2 Photomicrographs of histological sections stained with H&E at 15 weeks. (a) Blank scaffold, (b) scaffold encapsulating plasmid DNA, (c) scaffold encapsulating condensed plasmid DNA. Analysis of a section from scaffolds encapsulating condensed DNA at a higher magnification (d and e). (d) Immature woven bone (w) with osteoblasts lining the edges, and developing marrow space (m) can be observed, as can (e) immature woven bone (w) consolidating into mature lamellar bone (l). Photomicrographs of a, b, and c were taken at $100\times$, and photomicrographs of b and c were taken at $400\times$.

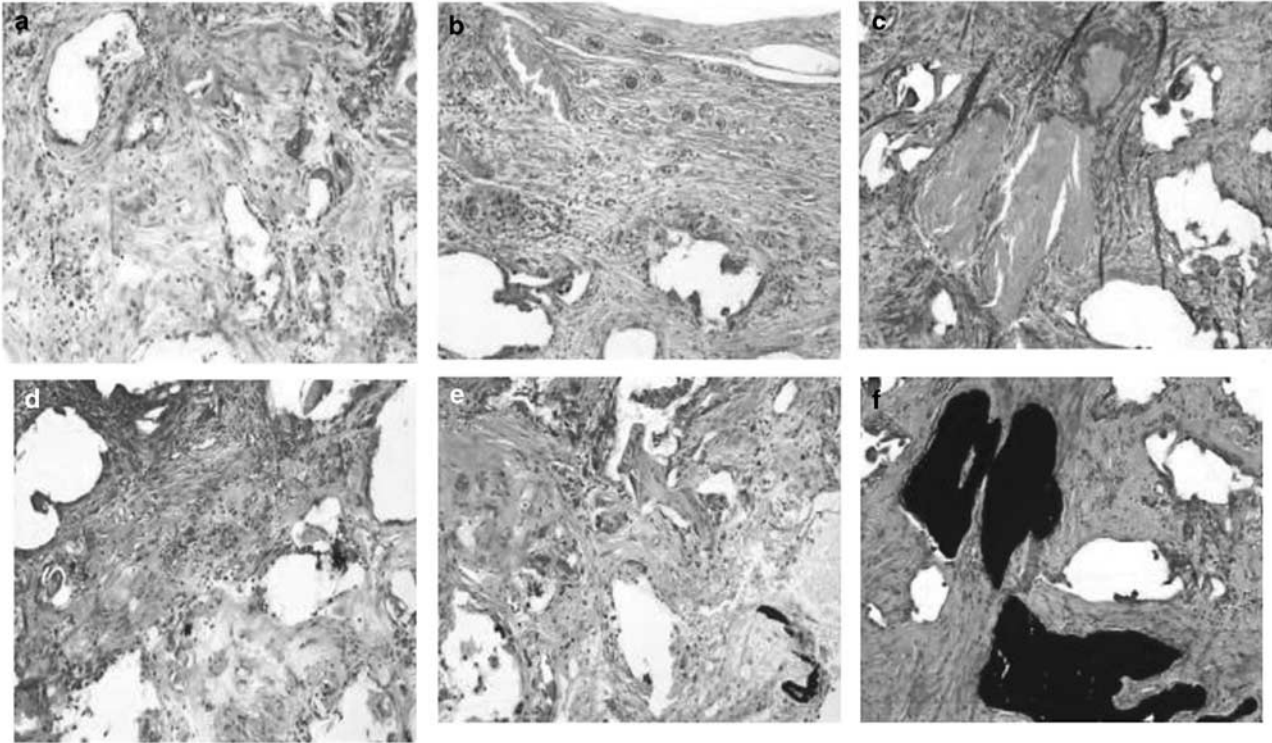


Figure 3 Photomicrographs of sections stained with Goldner trichrome (a–c) and von Kossa (d–f) at 15 weeks. (a, d) blank scaffolds, (b, e) scaffolds encapsulating plasmid DNA, and (c, f) scaffolds encapsulating condensed plasmid DNA. All photomicrographs were taken at 200 \times .

although a higher density of osteoid was observed in the latter. These regions were mostly observed near the defect edges. In contrast, scaffolds incorporating condensed DNA displayed a greater density of osteoid deposition than the blank scaffolds and scaffolds incorporating uncondensed DNA. The *de nova* bone formation was characterized by osteoblasts lining the edges and around the calcified region indicated by the positive staining (Figure 3c). The 15-week sections were also stained with von Kossa, which stains mineralized tissue dark purple to black. For blank scaffolds and scaffolds incorporating uncondensed DNA, only minimal mineralized tissues could be observed near the defect edges (Figure 3d and e). In contrast, there was a large amount of mineralized tissue regenerated within the scaffolds incorporating condensed DNA (Figure 3f), relative to blank scaffolds and scaffolds incorporating uncondensed DNA.

Immunostaining was also performed to confirm bone tissue formation and BMP-4 expression. The organic matrix of bone consists mostly (~90%) of type I collagen, and therefore most markers of bone tissues generally rely on the presence of specific noncollagenous proteins. To verify that Goldner's-positive staining (red staining) indicated the presence of bone matrix, tissue sections were also immunostained for bone sialoprotein (BSP), a glycoprotein that composes 15% of the noncollagenous protein content in bone matrix. The positive dark brown staining can be identified in localized regions at bone-forming sites (Figure 4a and b). BSP-positive regions were typically cell-free, yet surrounded by an organized monolayer of cells, as expected during bone matrix synthesis regulated by osteoblasts. Qualitative analysis

of BSP-positive regions also indicated the same trends indicated by the osteoid staining. At the 3- and 8-week time points, minimal BSP-positive stained regions can be observed in blank scaffolds and scaffolds encapsulating plasmid DNA, and they were mostly present near the defect edges, with no noticeable staining towards the center of the defect. Scaffolds encapsulating condensed DNA displayed more BSP-positive stained regions at the defect edges, and scattered positive stainings near the center of the defect. At 15 weeks, blank scaffolds and scaffolds encapsulating plasmid DNA demonstrated more positive staining than at previous time points and scarce positive staining in the middle of the scaffold. Scaffolds encapsulating condensed DNA exhibited more positive staining throughout the scaffold volumes, although these regions were more evident in areas approximately one-third of the distance from the defect edges. Positive BMP-4 expression by host cells for a 15-week time period was confirmed by performing immunostaining for BMP-4 on decalcified sections retrieved from an ectopic site (data not shown).

Histomorphometric analysis was performed to quantitatively evaluate bone formation. Analysis of H&E-stained sections demonstrated a significantly higher bone formation (at least 4.5-fold increase) within scaffolds incorporating condensed DNA, relative to blank scaffolds and scaffolds incorporating uncondensed DNA. Bone formation increased over time in each condition, and scaffolds incorporating condensed DNA displayed a statistically significant increase in bone formation relative to both the blank scaffolds and scaffolds incorporating uncondensed DNA at each time point (Figure 5). Quantification of osteoid area from

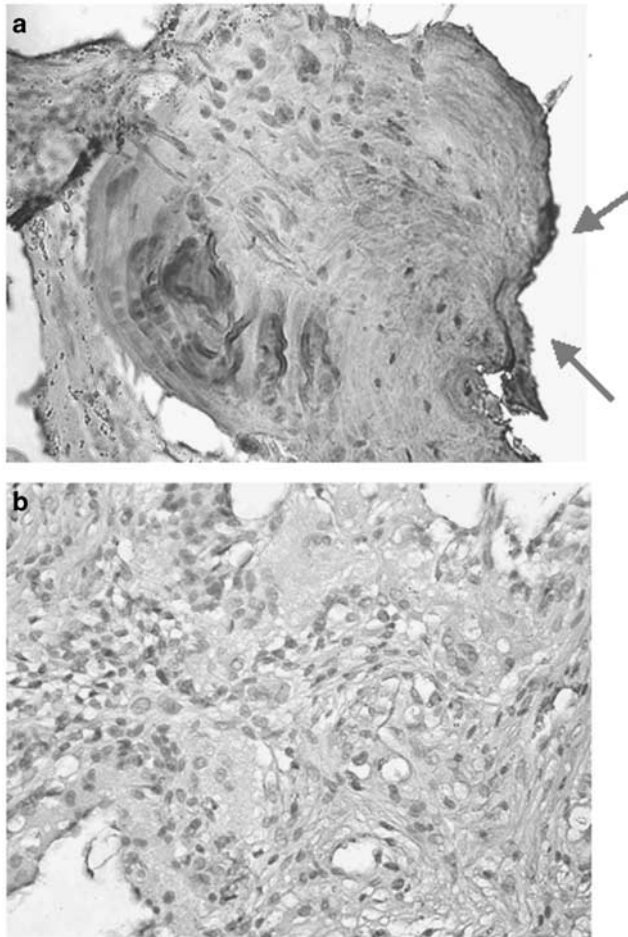


Figure 4 Photomicrographs of histological sections from 15 week samples immunostained for BSP. (a) Scaffold encapsulating condensed DNA, (b) Blank scaffold. The BSP-stained region in (a) contains localized regions of matrix deposition (dark brown staining) characteristic of these samples. Positive staining is indicated by the arrows. Photomicrographs were taken at 400 ×.

Goldner trichrome-stained sections also demonstrated a significantly greater amount of osteoid tissue within scaffolds incorporating condensed DNA, when compared to blank scaffolds and scaffolds incorporating uncondensed DNA (Figure 6a). Quantification of mineralized tissue area from sections stained with von Kossa similarly displayed a significant increase within scaffolds incorporating condensed DNA, compared with blank scaffolds and scaffolds incorporating uncondensed DNA (Figure 6b).

Three-dimensional microcomputed tomography (μ CT) images were taken to examine the microarchitecture and distribution of mineralization within scaffolds (Figure 7, circles superimposed on the images indicate the cranial defect edges). Blank scaffolds exhibited minimal mineralized regions, and the mineralized regions were confined mostly to the defect edge (Figure 7a). Scaffolds incorporating uncondensed DNA displayed small patches of mineralized tissue (Figure 7b). In contrast, scaffolds incorporating condensed DNA demonstrated multiple layers and islets of dispersed irregularly shaped mineralized regions within the implant region (Figure 7c). In addition, the scaffolds incorporating condensed

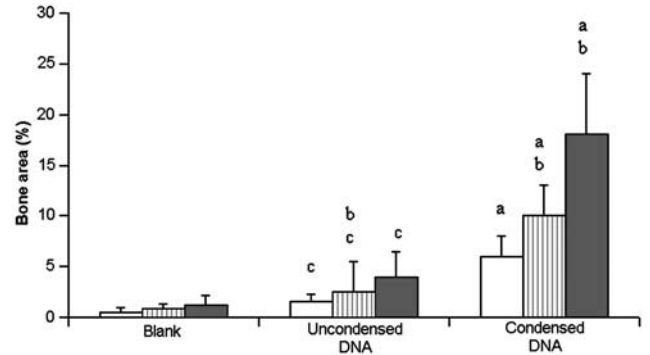


Figure 5 Histomorphometric quantification, based on H&E staining, of bone regeneration within implants at 3 weeks (blank bars), 8 weeks (striped bars), and 15 weeks (solid bars). Experimental values are reported as mean ($N=6$) \pm s.d. 'a' indicates statistical significance relative to blank scaffolds and uncondensed scaffolds at the specific time point, 'b' indicates statistical significance relative to the previous time point within each condition, 'c' indicates statistical significance relative to blank scaffolds ($P < 0.05$).

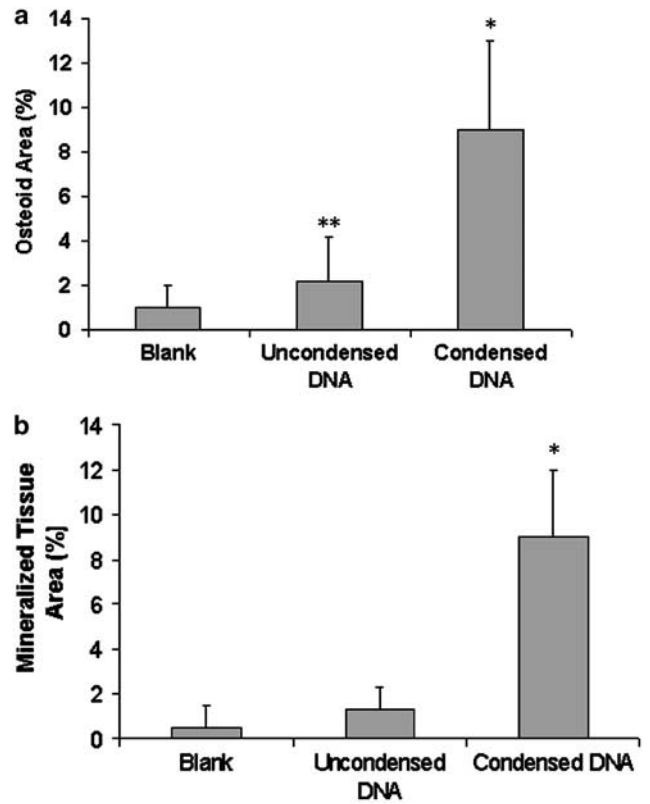


Figure 6 (a) Histomorphometric quantification of osteoid matrix area within the total scaffold area for each condition at 15 weeks. Experimental values are reported as mean ($N=6$) \pm s.d. (*) indicates statistical significance relative to the blank scaffold and uncondensed DNA scaffold, and (**) indicates statistical significance relative to the blank scaffold ($P < 0.05$). (b) Histomorphometric quantification of mineralized tissue area within the scaffold area for each condition at 15 weeks. Experimental values are reported as mean ($N=6$) \pm s.d. (*) indicates statistical significance relative to the blank scaffold and uncondensed DNA scaffold ($P < 0.05$).

DNA also appeared to have had more ingrowth of mineralized tissues from the defect edge, relative to the blank scaffolds and scaffolds incorporating uncondensed

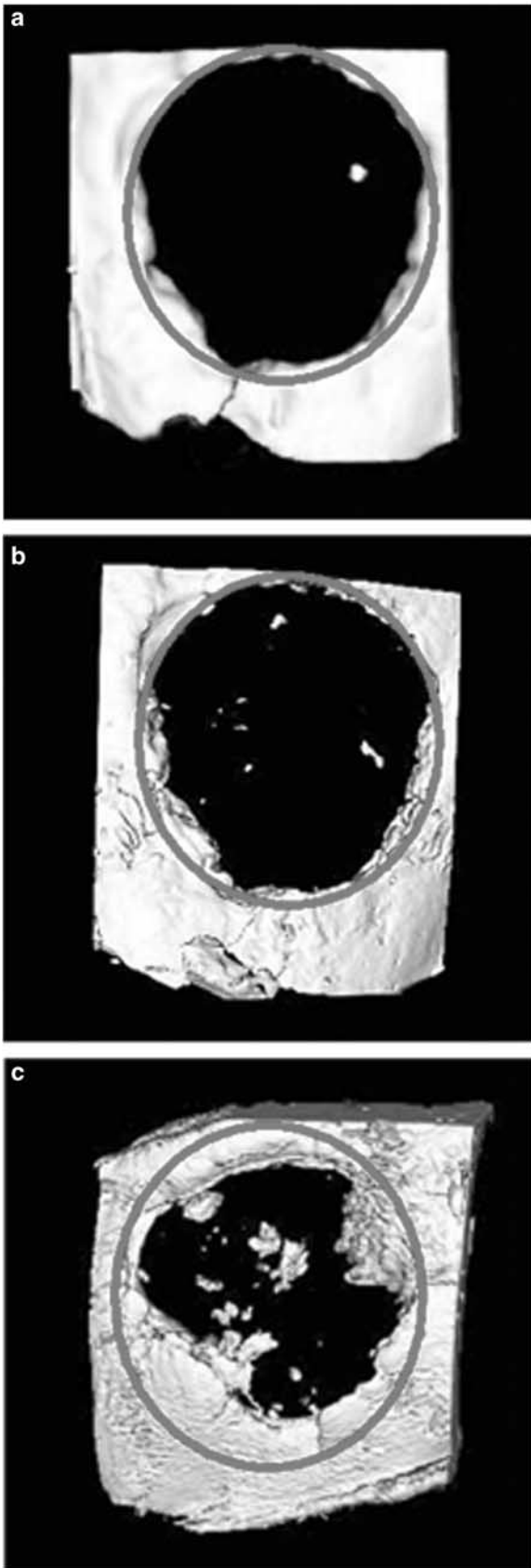


Figure 7 Three-dimensional μ CT images of (a) blank scaffold, (b) scaffold encapsulating plasmid DNA, (c) Scaffold encapsulating condensed plasmid DNA at 15 weeks. Image reconstructions were performed at a resolution of 18 μ m. The circles indicate the cranial defect edges.

DNA. The distribution of mineralization observed with this analysis was consistent with histological examinations.

Discussion

The potential of a polymeric delivery system encapsulating condensed DNA to promote bone regeneration was examined in this study. We have previously demonstrated that PLGA scaffolds encapsulating condensed DNA increased transfection *in vitro*⁴³ and *in vivo* (unpublished data), in studies using marker genes. The utility of this system was investigated in this study by incorporating condensed DNA encoding for BMP-4 within the scaffolds and examining the ability of the scaffolds to regenerate bone. In the present study, scaffolds containing condensed DNA enhanced bone regeneration within a cranial critical-sized defect compared to that of scaffolds containing uncondensed plasmid DNA and blank scaffolds during the 15-week experimental period. Furthermore, scaffolds containing condensed DNA displayed a significant increase in both osteoid and mineralized tissue area, when compared to the other two conditions.

Bone formation within scaffolds incorporating condensed DNA was qualitatively and quantitatively superior to that obtained with scaffolds containing uncondensed DNA scaffolds or blank scaffolds. This is likely a result of the enhanced transfection mediated by condensed plasmid DNA, as compared to uncondensed plasmid DNA.⁴⁶ The bone regeneration data suggest BMP-4 produced in the transfected cells was active and its expression was sustained. Previous studies using either protein or gene therapy usually reported enhanced bone formation over short periods of time,^{33,38} while a long-term effect on bone regeneration was achieved in this study. Histological examination of sections from scaffolds incorporating condensed DNA showed active *de novo* bone formation (woven) structure even at 15 weeks. This was evidenced by the presence of thick osteoid and plump osteoblasts surrounding and lining the seam. The evolving new bone consolidated with the interconnected mature lamellar bone during new bone development, and the increased mineralization in the condensed DNA versus plasmid DNA condition presumably indicated that enhanced production of BMP-4 facilitated the differentiation and maturation of infiltrating osteoblast precursor cells and osteoblasts. Scaffolds containing uncondensed plasmid DNA did exhibit a statistically significant increase in osteoid density, compared to blank scaffolds, at 15 weeks, but the magnitude of bone tissue formation was not high. This may indicate that the amount of BMP-4 produced with plasmid DNA delivery may be sufficient to initiate bone matrix synthesis, but was not enough to differentiate cells sufficiently to drive mineralization. Histological examination showed no evidence of cartilage formation within the defect area, indicating new bone likely developed in a mechanism directed by intramembranous ossification, which is the mechanism of bone formation generally considered to occur in cranial bone development.⁴⁷

The results of this study support the role of BMP-4 in promoting osteogenesis. The secreted BMP-4 may have acted as either or both a chemoattractant to recruit

precursor cells into the defect, and a differentiation factor for cells to drive bone formation.⁴⁸ These possibilities are supported by the finding that not only was bone formation increased within the scaffolds incorporating condensed DNA, but it was also increased at the defect edges when compared to blank scaffolds and scaffolds incorporating uncondensed DNA. There are several possible mechanisms that could contribute to this observation, including (1) continuous transfection of cells leading to active generation of BMP-4, and (2) increased recruitment of cells involved in forming immature new bone due to the secreted BMP-4, coupled with infiltrating cells responding to the secreted BMP-4. Comparison of the spatial and temporal changes in scaffold cellularity and expression of bone markers in scaffolds carrying condensed DNA suggested a correlation between the increased cellularity and enhanced expression of bone-specific markers near the defect edges. This may suggest that increased cellular infiltration from the host bone, due to the secreted BMP-4, contributes to the enhanced bone formation observed in this condition.

The plasmid delivery system utilized in this study bypasses some of the issues associated with other approaches and systems used to regenerate bone. This PLG system for plasmid DNA delivery has been previously used to enhance granulation tissue formation.¹⁴ However, the low transfection efficiency with this system led to a requirement for very high doses of plasmid DNA. We attempted to improve on the low transfection efficiency associated with plasmid DNA by condensing the plasmid DNA with PEI prior to incorporation into the scaffold. The delivery of condensed DNA reduced the dose necessary to elicit a biological response from the milligram dose range¹¹ to the microgram range. Bone regeneration in this approach was driven by the transfection of cells infiltrating into the scaffold from the surrounding host tissues, followed by the production of BMP-4 involved in bone formation. In contrast, cell transplantation approaches to bone regeneration typically require proper induction *in vitro*, and/or are often genetically modified prior to implantation.^{13,20,21} The therapeutic effect in either situation largely depends on the survival of the cells following delivery. The system described in this report bypassed the need for implanting external cell sources. Furthermore, implant success in this approach is not initially dependent on adequate vascularization, as opposed to a cellular transplantation approach, which requires rapid vascularization of the implanted cell mass to prevent ischemic necrosis.²⁷

Increased bone formation was achieved with this localized gene delivery system with a set of parameters for condensate formulation taken from previous *in vitro* results,⁴³ but further improvements in bone formation may result from modifying these parameters. Previous studies clearly indicated that *in vitro* and *in vivo* results may not necessarily correlate.⁴⁹ Further, the incorporation of growth factors into scaffolds that would facilitate the increased recruitment of cells may further enhance bone formation at early time points in this system. It has been previously speculated that fibroblasts were the cells transfected in this type of system.¹⁰ However, it is still necessary to identify what specific cell types were transfected, as this may allow specific targeting of these

cell types to further enhance transfection efficiency. The fate of cells post-transfection may also be important to trace, in order to determine whether transfected cells migrate out of the scaffold and participate in bone formation outside the scaffold. In addition, although this delivery system has been shown to be successful in promoting bone regeneration in a rat cranial defect model, the utility of this delivery system in other bone defect models (eg ectopic bone formation) or in animals more similar to human (eg primates) must be further assessed to know its true utility.

In conclusion, we have demonstrated that scaffolds incorporating condensed DNA encoding BMP-4 enhance bone regeneration within a cranial critical-sized defect model, compared to that of blank scaffolds and scaffolds incorporating uncondensed DNA. The results have implications for bone regeneration, in that this system allows for controlled and sustained delivery of osteogenic growth factors for directing bone formation. In addition, the use of a non-viral vector for delivering plasmid DNA, and the use of PLGA as an osteoconductive scaffold, as described in this study, may be more practical than the use of viral based vectors. Further consideration and inclusion of osteogenic components involved in bone development are warranted to extend the utility of this system in bone tissue engineering.

Materials and methods

Chemicals and materials

Branched PEI (MW 25 kDa) was purchased from Sigma/Aldrich (St Louis, MO, USA). Sucrose was purchased from Sigma (St Louis, MO, USA). Poly(D,L-lactic-co-glycolic acid) (PLGA) 85:15 was purchased from Alkermes, Inc. (Cincinnati, OH, USA). Reagents for von Kossa, toluidine blue, H&E and Goldner's staining were purchased from Fisher Scientific (Springfield, NJ, USA) or Sigma Chemical Company (St Louis, MO, USA). Antibodies for BMP-4 were purchased from Chemicon (Temecula, CA, USA). Staining for BSP was accomplished by using a primary antibody rabbit anti-human BSP (Chemicon, AB1854), and a secondary antibody biotinylated goat anti-rabbit IgG (BioCare Medical, GU600 H, universal link). Plasmid DNA encoding the bone morphogenetic protein-4 (pcDNA3-BMP-4) was produced by Fermentas (Hanover, MD, USA) and the gene encoding for BMP-4 was under the control of the cytomegalovirus promoter. The vector pcDNA-3 used for the construction of the plasmids was obtained from Invitrogen (Carlsbad, CA, USA). In brief, the plasmids were harvested in *Escherichia coli* strain DH5- α and purified followed by ethanol precipitation. The plasmids were then resuspended in TE buffer and quantified by absorbance at 260 nm, with the purity demonstrated by a 260/280 absorbance ratio ranging from 1.80 to 1.98.

Preparation of PLGA sponges

PEI DNA condensates were prepared by combining 200 μ g of plasmid in 4 ml HEPES buffer (5 mM, pH 7.4) with 4 ml of PEI (10 mM). Sucrose (1 wt/vol%) was added and condensates were rapidly frozen using dry ice in ethanol and lyophilized for 72 h. Freeze-dried PEI DNA condensates were combined with milled sucrose (250–425 μ m) and 85:15 polylactide:glycolide copolymer

(106–250 μm) to fabricate scaffolds as previously described.⁴³ Sponges were prepared at 91 wt% (Porogen: PLGA) by combining 728 mg of sucrose and PEI DNA condensate with 72 mg of PLGA. Sponges were fabricated by compression molding 800 mg of the dry mixture at 1500 psi for 1 min using a 13-mm die set from Pike Technologies (Madison, WI, USA) and a Carver Model 'C' hydraulic press (Pike Technologies, Madison, WI, USA). The compressed pellet (2 \times 13 mm) was then foamed into a scaffold via a gas foaming process⁵⁰ in a custom-designed stainless-steel high-pressure vessel using dry CO₂ gas at 800 psi for 24 h. A rapid reduction in pressure causes the polymer particles to expand and fuse into an interconnected structure. Sucrose was leached from the sponge by immersion in phosphate-buffered saline (PBS). Sponges containing naked DNA were also prepared by mixing lyophilized plasmid DNA (200 μg), sucrose and PLGA followed by the gas foaming and particulate leaching process.

In vivo bone regeneration in cranial critical-sized defect

The ability of condensed plasmid DNA (pcDNA3-BMP-4) to enhance bone regeneration *in vivo* was assessed by implantation of the PLGA scaffolds into a critical-sized defect created in the crania of Lewis rats (250–300 g). The conditions utilized were blank scaffolds, scaffolds encapsulating plasmid DNA, and scaffolds encapsulating condensed plasmid DNA.

Experimental parameters (charge ratio, dose of DNA) were derived from a previous *in vitro* optimization study.⁴³ Six samples were used for each condition at each time point. Treatment of experimental animals was in accordance with University of Michigan animal care guidelines, and all National Institutes of Health (NIH) animal handling procedures were observed. Animals were anesthetized by intraperitoneal injection of anesthetics (80 mg/kg ketamine and 10 mg/kg xylazine). A mid-longitudinal, 15 mm skin incision was made on the dorsal surface of the cranium, and care was taken to ensure that the periosteum was completely cleared from the surface of the cranial bone by scraping. A trephine bur was used to create a circular 9 mm diameter defect in the rat cranium (rat cranium critical defect size = 8 mm). The full thickness (~1.5–2 mm) of the cranial bone was removed and scaffolds were immediately placed in the defect. After 3, 8 and 15 weeks, the animals were euthanized with carbon monoxide, and the implants embedded in the surrounding native bone were retrieved. The implants were fixed for 24 h at 4°C in 10% zinc-buffered formalin. Following fixation, 3-, 8- and 15-week implants were decalcified via incubation in 10% EDTA, paraffin embedded and sectioned (Histology core facility, University of Michigan Dental School). The 15-week implants were also plastic embedded and sectioned (Pathology Associates, Inc.). Immunostaining for BSP and BMP-4 were accomplished on decalcified tissue sections. Goldner's staining and BSP and BMP-4 immunostaining were accompanied by a hematoxylin nuclear counterstain.

Analysis of bone regeneration

The 3-, 8-, and 15-week decalcified tissue sections were stained with H&E for histological examination and quantification of bone regeneration. The 15-week, non-decalcified tissue sections were stained with Goldner's

trichrome to identify osteoid or stained with von Kossa to identify mineralized tissue and imaged digitally at 100 \times magnification. Osteoid, indicated by red Goldner's staining, and fully mineralized tissue, indicated by black von Kossa staining, were identified using a Nikon Eclipse E800 light microscope. The osteoid area and mineralized tissue area, normalized to total tissue area, were computed using Image Pro Plus Software. Bridging of the defects with tissue was examined for all conditions via light microscopic analysis of standard H&E-stained sections 15 weeks postimplantation. To confirm that the 9 mm defect was critical-sized, we also created a defect without implanting a scaffold, and examined this condition at 15 weeks only. To verify that Goldner's-positive staining (red staining) indicated the presence of bone matrix, selected sections were also immunostained for BSP. In addition, one specimen from each condition at 15 weeks was imaged at a resolution of 9 μm using a three-dimensional μCT system, and reconstructed at a resolution of 18 μm in order to visualize the volume of new bone formation, and qualitatively examine the microarchitecture of the regenerated bone tissue.

Statistical analysis

Six scaffolds were prepared, implanted, and analyzed per condition at each time point, and statistical analysis was performed using InStat software. Statistically significant differences in histomorphometric analysis were determined using one-tailed Student's *t*-test ($P < 0.05$).

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