

## Dual growth factor delivery and controlled scaffold degradation enhance in vivo bone formation by transplanted bone marrow stromal cells

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### Abstract

Supraphysiological concentrations of exogenous growth factors are typically required to obtain bone regeneration, and it is unclear why lower levels are not effective. We hypothesized that delivery of bone progenitor cells along with appropriate combinations of growth factors and scaffold characteristics would allow physiological doses of proteins to be used for therapeutic bone regeneration. We tested this hypothesis by measuring bone formation by rat bone marrow stromal cells (BMSCs) transplanted ectopically in SCID mice using alginate hydrogels. The alginate was gamma-irradiated to vary the degradation rate and then covalently modified with RGD-containing peptides to control cell behavior. In the same delivery vehicle, we incorporated bone morphogenetic protein-2 (BMP2) and transforming growth factor- $\beta$ 3 (TGF- $\beta$ 3), either individually or in combination. Individual delivery of BMP2 or TGF- $\beta$ 3 resulted in negligible bone tissue formation up to 22 weeks, regardless of the implant degradation rate. In contrast, when growth factors were delivered together from readily degradable hydrogels, there was significant bone formation by the transplanted BMSCs as early as 6 weeks after implantation. Furthermore, bone formation, which appeared to occur by endochondral ossification, was achieved with the dual growth factor condition at protein concentrations that were more than an order of magnitude less than those reported previously to be necessary for bone formation. These data demonstrate that appropriate combinations of soluble and biomaterial-mediated regulatory signals in cell-based tissue engineering systems can result in both more efficient and more effective tissue regeneration.

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### Introduction

Many promising strategies for functional tissue engineering aim to replicate components of the natural cellular microenvironment by providing a synthetic extracellular matrix (ECM) [1] and by delivering growth factors [2]. These specific cues may allow one to regulate the phenotype of host or transplanted cells and guide tissue and organ regeneration. However, natural tissue regeneration is a

complex process in which responding cells are regulated by the coordinated action of several environmental cues. This suggests that appropriate presentation of multiple regulatory signals may be a prerequisite of effective tissue engineering strategies.

For bone tissue engineering, the strategy of providing multiple cues may be particularly important because bone formation and repair are regulated by many factors, including specific ECM properties and several growth factors [3]. Synthetic scaffolds with specific adhesion properties [4,5], degradation rates [6], and surface chemistries [7–9] have been shown to regulate in vivo bone formation. Natural bone development and regeneration are also regulated by a myriad of growth factors, among the most potent of which are members of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily [3]. The TGF- $\beta$  superfamily includes several

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isoforms of TGF- $\beta$ s and bone morphogenetic proteins (BMPs) that are expressed during bone formation and repair [10–13]. These growth factors have been delivered therapeutically to repair sites using a variety of methods, including bolus injection [14] or release from implant surfaces [15,16] and biodegradable polymers [17–20]. Delivery of TGFs and BMPs either alone or in conjunction with other growth factors has achieved mixed results, with good results in certain instances but minimal or even detrimental effects in other cases (reviewed in Refs. [21] and [22]). In cases where beneficial effects were observed, large doses of the growth factors were required (from 50  $\mu$ g to more than 60 mg of growth factor per gram of carrier). This raises concerns regarding the safety, cost, and effective delivery of these supraphysiological amounts of recombinant proteins [21].

The varying effects of previous efforts to deliver growth factors may be related to the delivery vehicle used, the concentrations and combinations of growth factors delivered, and the reliance on host cells for new bone tissue formation. We hypothesized that delivery of bone progenitor cells along with appropriate combinations of growth factors and scaffold characteristics would allow physiological doses of growth factors to be used to enhance bone regeneration. To test this hypothesis, we measured ectopic bone formation in alginate hydrogels covalently modified with RGD-containing peptides to control cell behavior [5]; this system has been used previously for osteoblast transplantation and bone tissue engineering [4–6]. In the current study, we delivered bone marrow stromal cells (BMSCs) rather than mature osteoblasts because BMSCs are a promising, clinically relevant autologous progenitor cell source [23]. We investigated the effects of physiological quantities of BMP2 and TGF- $\beta$ 3 on bone regeneration in the alginate system. The growth factors were delivered both individually and in combination, and we hypothesized that because BMP2 and TGF- $\beta$ 3 are both expressed during natural regeneration [3,11], we would observe enhanced bone formation with dual delivery. Finally, we tested the different growth factor conditions with both slowly and rapidly degrading scaffolds. The scaffold degradation rate is a critical design parameter for bone tissue regeneration because appropriate scaffold degradation provides new space for matrix deposition and coalescence, which may ultimately lead to improved quantity and quality of regenerated bone [6].

## Materials and methods

### *Preparation of peptide-modified alginate*

The degradation rate of the alginate hydrogels used in this study was controlled by gamma-irradiating the alginate to control its molecular weight and structure. Nonirradiated and irradiated peptide-modified alginate was prepared as described previously [6]. Briefly, MVG sodium alginate

powder (Pronova Biopolymers, Norway) was lyophilized and a portion was subjected to 5 Mrad gamma-irradiation (Phoenix Lab, University of Michigan). Nonirradiated and irradiated alginates were then modified with G<sub>4</sub>RGDSP peptides (Commonwealth Biotechnologies, Inc., Richmond, VA) by covalent coupling using standard carbodiimide chemistry [24] to achieve a peptide density of  $1.5 \times 10^5$  nmol/l when the alginate was reconstituted to a 2% w/w solution in  $\alpha$ -MEM.

### *Bone marrow stromal cell isolation*

Rat BMSCs were isolated as described previously [25], with slight modifications. Bone marrow from the femoral and tibial medullary cavities of 300 g Lewis rats was flushed using ice-cold  $\alpha$ -MEM supplemented with 20% fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25  $\mu$ g/ml Fungizone (all from Invitrogen, Carlsbad, CA). The flushed marrow cell suspension was passed repeatedly through a 22-gauge needle and filtered through a 100- $\mu$ m cell strainer before culturing. Marrow cells were plated in 225-cm<sup>2</sup> tissue culture flasks (marrow from one limb per flask) with  $\alpha$ -MEM supplemented with 20% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.25  $\mu$ g/ml Fungizone, and  $10^{-8}$  M dexamethasone (Sigma-Aldrich, St. Louis, MO). After 1 week of culture, adherent cells were rinsed thoroughly with phosphate-buffered saline and trypsinized for use in the implants.

### *Preparation and implantation of alginate implants*

Implants were prepared from nonirradiated alginate-G<sub>4</sub>RGDSP and from 5 Mrad treated alginate-G<sub>4</sub>RGDSP (both 2% w/w in  $\alpha$ -MEM). The aqueous alginate solutions were first combined with BMSCs to yield a density of approximately  $1 \times 10^6$  cells/implant. The alginate-BMSC mixtures were then combined with the growth factors. Four growth factor conditions were considered: (1) Blank (negative control), in which no growth factors were added to the implants; (2) BMP, in which 200 ng recombinant human BMP2 was added per implant; (3) TGF, in which 20 ng recombinant human TGF- $\beta$ 3 was added per implant; or (4) BMP + TGF, in which 200 ng BMP2 and 20 ng TGF- $\beta$ 3 were added per implant (Table 1). The growth factors, obtained lyophilized from R&D Systems (Minneapolis, MN), were reconstituted in 4 mM HCl and 1 mg/ml BSA, and were sterile filtered. Based on the implant volume (see below) and assuming the density of the implants was that of water, the final concentrations of total growth factors were 0.4, 4.0, and 4.4  $\mu$ g/g scaffold for TGF, BMP2, and BMP + TGF conditions, respectively. After addition of the BMSCs and growth factors, the alginates were cross-linked with calcium sulfate [5] and cast into 50 mm<sup>3</sup> rectangular implants (10  $\times$  5  $\times$  1 mm). Calcium sulfate is osteocompatible and has been used as a

Table 1  
Summary of treatments and implantation periods

	Weeks of implantation before retrieval			
	3	6	15	22
Nonirradiated alginate-G <sub>4</sub> RGDSP	–	–	–	Blank BMP TGF BMP + TGF
5 Mrad irradiated alginate-G <sub>4</sub> RGDSP	BMP + TGF	BMP + TGF	Blank BMP TGF BMP + TGF	–

bone graft substitute, primarily in a concentrated form [26,27], to treat bone defects. However, the effects of the very low concentration of calcium sulfate contained in the gels used in this study were most likely minimal compared to the effects of the peptide modification and growth factor delivery.

#### Implantation and histomorphometric analyses

University of Michigan and National Institutes of Health (NIH) animal care guidelines were followed in all procedures. The implants containing BMSCs and growth factors were implanted subcutaneously into the backs of male 4- to 5-week-old anesthetized CB-17 SCID mice. One implant was placed in each mouse and four to six mice were assigned to each condition. The nonirradiated implants were harvested after 22 weeks and the 5 Mrad irradiated implants were harvested after 15 weeks. Additional irradiated implants containing both BMP2 and TGF- $\beta$ 3 were harvested after 3 and 6 weeks. Harvested implants were fixed in 10% neutral-buffered formalin, paraffin embedded, serial sectioned, and stained with hematoxylin and eosin (H&E) or with aldehyde fuchsin, alcian blue, and eosin. Bone tissue was identified in the H&E sections, and the bone area (as a fraction of the total implant area) was determined using Adobe Photoshop (Adobe Systems, San Jose, CA) and ImageJ (NIH, <http://rsb.info.nih.gov/ij/>). Histomorphometric data were analyzed by ANOVA, with pairwise comparisons made using Student–Newman–Keuls (SNK) tests (ProStat 3.0, Poly Software International, Pearl River, NY). Data in graphs are presented as mean  $\pm$  standard error.

## Results

#### Bone formation with nonirradiated alginate

Bone formation was assessed in nonirradiated alginate-G<sub>4</sub>RGDSP implants containing BMSCs and growth factors 22 weeks after implantation. For all growth factor conditions, the nonirradiated implants were largely intact and retained their original shape (Fig. 1A). Histological exam-

ination revealed a dominance of residual alginate in all cases, with interspersed islands of cells (Figs. 1B–E). In all implants, there was little tissue formation, likely due in a large part to the continued presence of the alginate. In some cases, particularly for the BMP + TGF condition, there were round islands of bone tissue (Fig. 1E), but these made up a small fraction of the total implant area (Fig. 1F). While there was a trend for increased bone formation with the BMP + TGF condition, the differences between growth factor conditions were not statistically significant.

#### Bone formation with irradiated alginate

We hypothesized that the continued presence of residual alginate restricted tissue growth in the nonirradiated alginate implants. To investigate this further, we gamma-irradiated alginate to increase its degradation rate [6]. Implants produced with 5 Mrad irradiated alginate-G<sub>4</sub>RGDSP had sufficient mechanical integrity to allow their handling and implantation without damage to the construct. Bone formation was assessed 15 weeks after implantation. In contrast to the nonirradiated implants, the irradiated samples contained smaller islands of residual alginate surrounded by new tissue (Figs. 2A–D). This pattern of increased alginate degradation and new tissue growth was observed for all growth factor conditions. Similar to the nonirradiated implants, however, there was little bone tissue formation in the irradiated implants that contained no growth factor, BMP2 only, or TGF- $\beta$ 3 only (Figs. 2A–C). In contrast, when BMP2 and TGF- $\beta$ 3 were delivered together from the irradiated implants, there was a striking increase in bone formation with thick bony trabeculae surrounding the residual alginate (Fig. 2D). Histomorphometric analysis showed that the effect of dual growth factor delivery on bone formation was synergistic and significantly greater than any other condition tested (irradiated or nonirradiated) ( $P < 0.01$  by SNK; Fig. 2E).

#### Early bone and cartilage formation with dual growth factor delivery from irradiated alginate

The significant bone regeneration obtained with delivery of BMP2 and TGF- $\beta$ 3 from irradiated peptide-modified

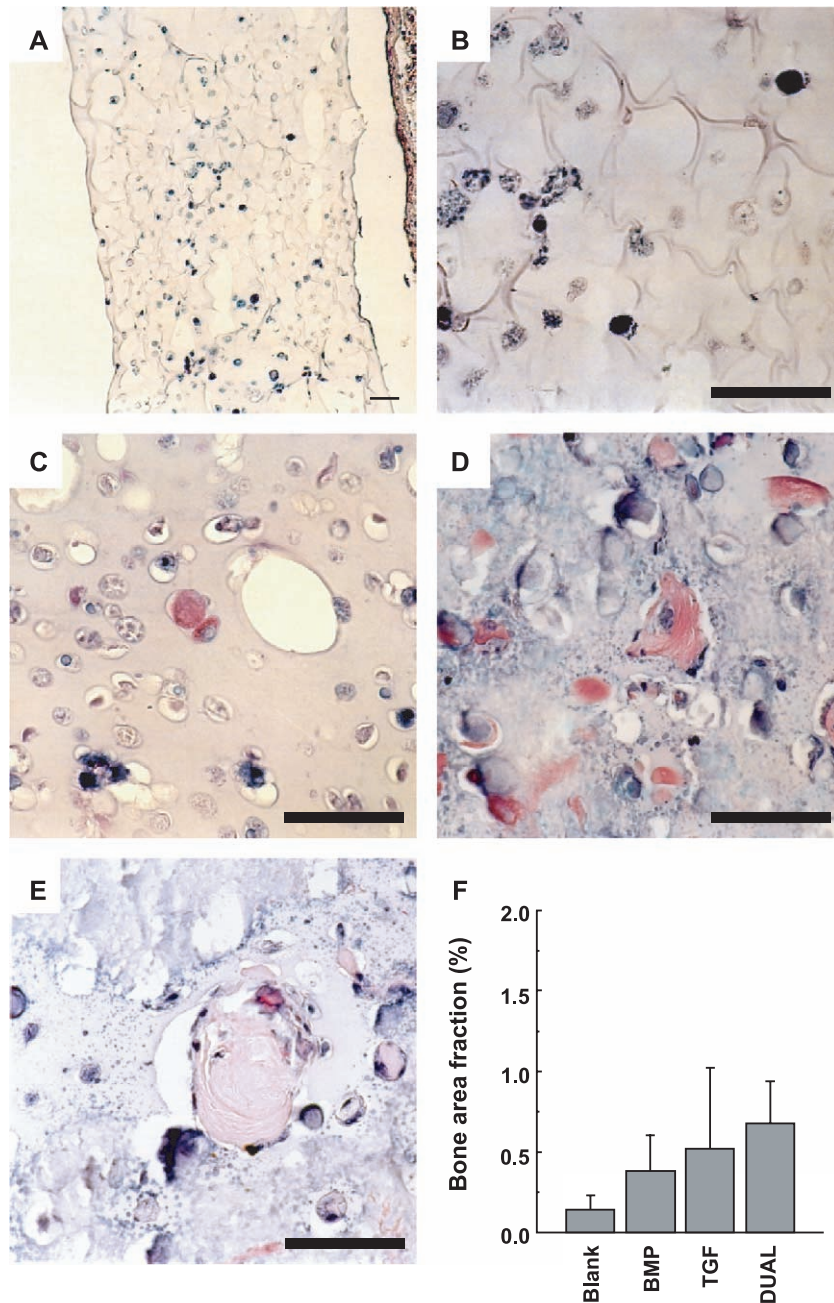


Fig. 1. Photomicrographs of H&E-stained sections from nonirradiated alginate implants after 22 weeks. (A) For all growth factor conditions, the nonirradiated implants were intact and retained their original shape. Bone formation (stained pink) was minimal in the (B) blank (no growth factor), (C) BMP2 only, (D) TGF-β3 only, and (E) BMP + TGF (dual growth factor) conditions. Where bone tissue formation did occur, it was confined to small islands within the residual alginate and made up <1% of the total implant area (F). Scale bar in photomicrographs represents 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

alginate led us to examine new tissue formation at earlier time points for this type of implant. Irradiated alginate-G<sub>4</sub>RGDSP implants containing BMSCs, BMP2, and TGF-β3 were harvested 3 and 6 weeks after implantation. Histological examination of these implants for bone and cartilage formation demonstrated that tissue regeneration was consistent with a pattern of endochondral ossification. Three weeks after implantation, there were large regions of cartilaginous tissue, particularly in the middle of the

implant volume, with interspersed thin strips of bone tissue (Fig. 3A). Six weeks after implantation, the regenerated tissue consisted of both cartilage and bone (Fig. 3B), with more bone than at the earlier time point. Fifteen weeks post-implantation, cartilaginous tissue was confined primarily to the periphery of the implant and the center regions of the implant (occupied by cartilage at earlier time points) contained bone tissue and marrow (Fig. 3C). Histomorphometric quantification confirmed that bone tis-

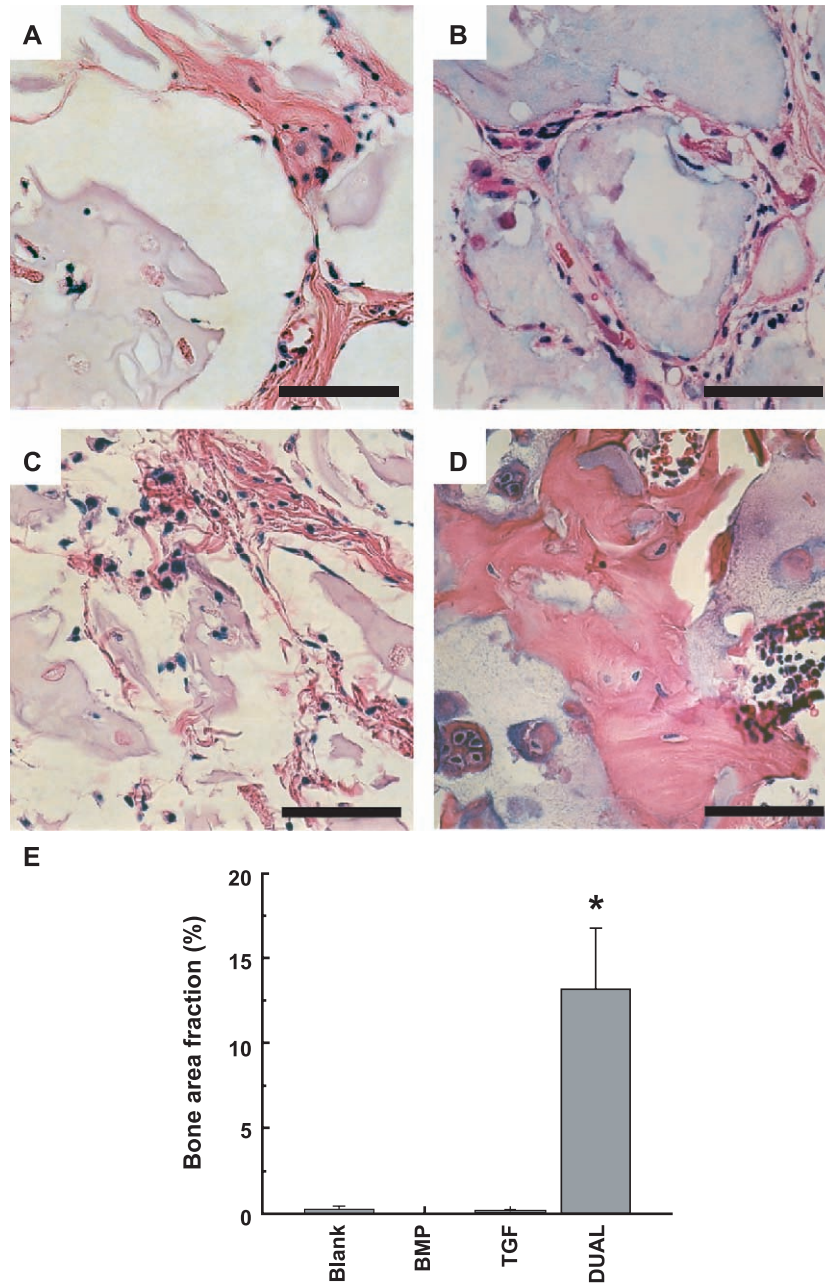


Fig. 2. Photomicrographs of H&E-stained sections from 5 Mrad irradiated alginate implants after 15 weeks. In contrast to the nonirradiated alginate implants, all of the irradiated implants contained small islands of residual alginate surrounded by new tissue. In the (A) blank, (B) BMP2 only, and (C) TGF-β3 only conditions, the new tissue was primarily fibrous, with little evidence of bone formation. However, in the implants with BMP + TGF (D), there was significant bone formation (pink). (E) Histomorphometric analysis confirmed the dual growth factor condition had significantly more bone tissue than any other condition analyzed (\* $P < 0.01$  by SNK). Scale bar in photomicrographs represents 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sue formation increased with time after implantation ( $P < 0.05$  by ANOVA; Fig. 3D).

## Discussion

Natural bone regeneration is a complex, coordinated process in which bone progenitor cells are regulated by several environment stimuli. In this study, we attempted to

replicate aspects of that environment using an alginate hydrogel system to regenerate bone in vivo. Our data indicate that BMSCs transplanted ectopically with this system produced significant bone tissue only in the presence of both BMP2 and TGF-β3, and then only when delivered from readily degradable scaffolds. This study demonstrates the critical roles of growth factors, scaffold characteristics, and appropriate combinations thereof in regulating therapeutic bone tissue regeneration.

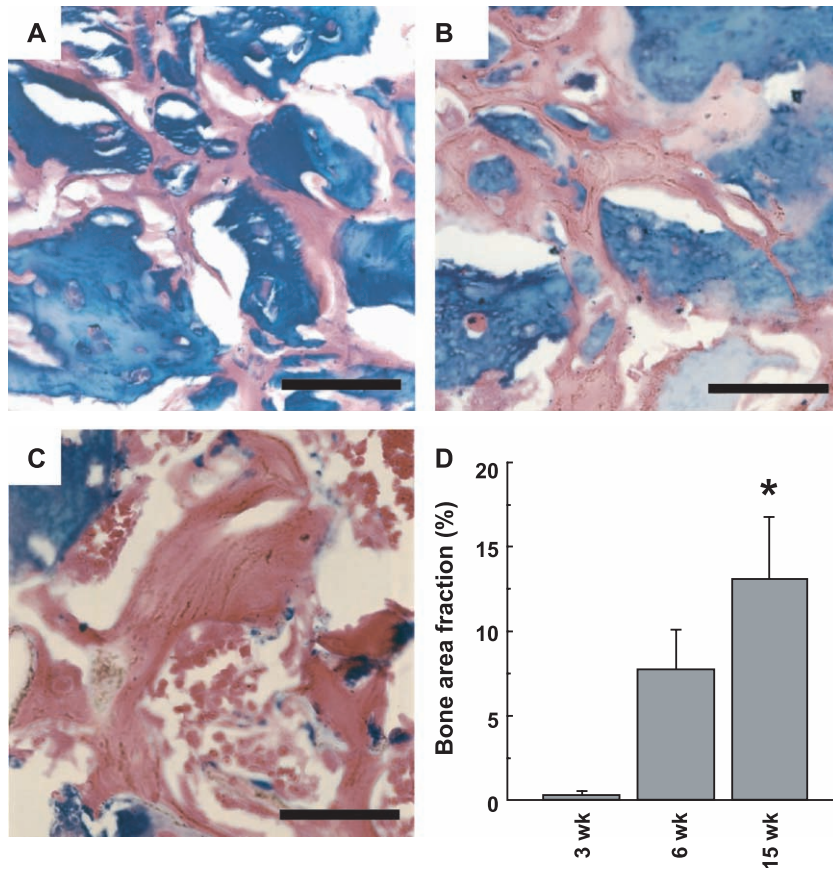


Fig. 3. Photomicrographs of sections from irradiated alginate implants with BMP2 and TGF- $\beta$ 3 after (A) 3, (B) 6, or (C) 15 weeks. Sections were stained with aldehyde fuchsin, alcian blue, and eosin to identify cartilage (deep blue), residual alginate (light blue), and bone (pink). (D) The amount of bone tissue in the implants with both growth factors increased with time after implantation, as the cartilage appeared to be replaced by bone ( $P < 0.05$  by ANOVA). \* $P < 0.05$  between the 3- and 15-week conditions by SNK. Scale bar in photomicrographs represents 50  $\mu$ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Previous studies have demonstrated bone regeneration with delivery of a single growth factor from polymeric carriers [17–20], but only at protein concentrations much greater than physiological levels, which are estimated to be about 1  $\mu$ g protein/g bone tissue for BMP [22]. Delivery of supraphysiological amounts of recombinant protein is costly and often impractical, raising questions as to the clinical utility of protein delivery for bone regeneration [21]. In the current study, we loaded the alginates with growth factors at physiological concentrations (approximately 0.4–4.4  $\mu$ g protein/g scaffold), and consistent with previous studies, little bone formation occurred when a single protein was delivered. However, when BMP and TGF- $\beta$ 3 were delivered together at physiologic concentrations, there was rapid and extensive bone formation, supporting our hypothesis that dual delivery of BMP2 and TGF- $\beta$ 3 enhances bone formation. These growth factors were selected because they are both expressed during natural bone regeneration, but appear to play distinct roles. During fracture healing, repair initiates with an inflammatory stage during which BMP2 messenger RNA (mRNA) is maximally expressed, followed by an intermediate chondrogenic phase during which TGF-

$\beta$ 2 and TGF- $\beta$ 3 mRNA show maximal expression, and a final osteogenic phase, in which BMPs 2, 3a, 4, 7, 8 mRNA are highly expressed [11]. Thus, BMP2 or TGF- $\beta$ 3 is involved in each phase of natural fracture repair, and the results we obtained with the irradiated alginates suggest a similar endochondral ossification process occurred in the implants only when both BMP2 and TGF- $\beta$ 3 were present. The striking response we observed with minimal amounts of protein may also be due in part to the co-delivery of cells and growth factors. Whereas previous efforts to deliver growth factors have relied on host cells in the surrounding tissue for bone formation, the present study is the first we are aware of in which progenitor cells and proteins are delivered together for bone regeneration. Our data suggest that encapsulation of progenitor cells with the growth factors in a single delivery vehicle may provide a more potent microenvironment for regeneration.

Bone tissue formation was regulated not only by the growth factor conditions, but also by the degradation rate of the scaffold. Minimal tissue formation occurred in the slowly degrading, nonirradiated implants apparently because the residual alginate limited the extent of matrix

deposition. Consistent with previous studies [6], the extent of tissue formation increased significantly in the more rapidly degrading gels, demonstrating the importance of tuning the scaffold degradation rate with the rate of new tissue formation. The scaffold degradation rate also likely influences the rate at which growth factors diffuse from the gel. Bone formation occurred primarily in the center of the implant volumes, suggesting the growth factors were largely retained in these regions, even in the more rapidly degrading gels. In contrast, bone formation was minimal in the peripheral regions from which growth factors could readily diffuse into the surrounding tissue. While impressive regeneration was achieved with the conditions used in this study, more extensive bone formation may be possible by manipulating the growth factor delivery and scaffold characteristics. For example, the doses, rates of delivery, and specific combinations of growth factors could be optimized. Furthermore, polymeric scaffolds that allow for delivery of multiple growth factors with distinct kinetics [28,29] could be employed to better replicate the temporal sequence of growth factor expression that occurs during natural bone repair [3]. The calcium cross-linked alginate hydrogels used in this study have nanometer size pores that likely do not permit extensive cell movement or tissue infiltration [30] before gel degradation. While the optimal pore diameter for bone ingrowth is likely in the micrometer range, the optimum for systems in which transplanted cells are used to regenerate bone has not been definitively established [31]. It is possible that increasing the pore size in alginate gels may lead to a more effective bone regeneration. Further, the effects of the gel degradation rate on bone formation in this study may be related to changes in gel porosity during the degradation process. Efforts have been made to generate macropores in alginate gels through techniques such as freeze-drying [32] or the creation and subsequent removal of gas pockets [30], and those types of alginate gels may be useful in bone regeneration.

We used alginate covalently modified with RGD-containing peptides in this study because this adhesion ligand significantly enhanced *in vivo* bone formation by rat calvarial osteoblasts in previous studies [4,5]. However, in the current study, there was little bone formation by BMSCs under similar no growth factor conditions. This suggests that the RGD-mediated signal alone was insufficient to induce large-scale osteogenic differentiation of the bone progenitor cells. Whether the RGD ligand modulated the BMSC response to the growth factors or played a critical role during later stages of endochondral ossification, as it does in systems with co-transplanted chondrocytes and osteoblasts [4], is uncertain from this study. However, matrix characteristics are known to regulate bone progenitor function [9,33–36], and further investigation of the effects of specific ligands on bone formation by BMSCs is required to better define design criteria for bone tissue engineering scaffolds.

In summary, we showed that delivery of BMP-2 or TGF- $\beta$ 3 individually from alginate hydrogels containing trans-

planted BMSCs resulted in negligible ectopic bone formation. In contrast, when the growth factors were delivered together from degradable hydrogels, there was significant bone formation by the transplanted BMSCs. Furthermore, bone formation was achieved with growth factor concentrations that were much less than those previously reported to be necessary for bone regeneration. Thus, appropriate delivery of multiple proteins might reduce the amount of protein required to achieve a desired effect, in essence increasing the potency of the growth factors in some cases. The incorporation of multiple regulatory signals into cell-based tissue engineering systems therefore may be a promising approach for more efficient and more effective tissue regeneration.

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