Gene Therapy to Enhance Allograft Incorporation After Host Tissue Irradiation

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Abstract Structural bone allografts are used to reconstruct large skeletal defects after tumor surgery. Although allograft-related complications are declining, the use of perioperative radiation therapy is associated with a poorer outcome. Recently, BMP-2 levels in the host bed were reportedly diminished after exposure to radiation doses consistent with those used perioperatively to treat musculoskeletal sarcoma. Reintroduction of this osteogenic protein may circumvent the deleterious effects of

preoperative radiation on allograft incorporation. We introduced a novel polymeric BMP-2 gene delivery system into the host-allograft junctions at the time of transplantation in an ovine tibial defect model with or without preoperative exposure to 50 Gy radiation. After 4 months, we noted no radiographic or histologic improvements in allograft incorporation after preoperative radiation and BMP-2 reintroduction; however, 50 Gy radiation was associated with increased porosity in the interface regions and poorer radiographic healing. We identified no BMP2-expressing cells or protein in the interface at the study end point, suggesting the polymeric gene delivery system was unable to promote extended expression of the protein or induce a healing response. Although gene therapy may hold promise as a novel technique to improve allograft incorporation, our data do not support that contention with the current approach.

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Each author certifies that his or her institution has approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research.

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Introduction

Allograft transplantation is a mainstay for reconstruction of massive bone defects after tumor surgery. Multiple studies document allograft limb salvage for musculoskeletal sarcomas does not negatively influence survival compared with amputation [2, 14, 17, 33, 36, 40–42, 44, 45, 47–49, 53, 54]. The improved ability to perform small-margin resection with favorable local tumor control rates can be attributed to improvements in preoperative imaging and the use of neoadjuvant therapy such as radiation and chemotherapy [2, 36, 41, 43, 44, 48, 49, 52, 55]. However, several clinical studies demonstrate perioperative radiation therapy is associated with a higher incidence of allograft-related complications, including nonunion [15, 25] and fracture [24] and poorer overall outcome [22, 34]. Irradiation

decreases osteoclast and osteoblast numbers and also diminishes local vascularity within the exposed soft tissues and bone, which is believed to affect neoangiogenesis and vascular invasion during bone healing [7]. Furthermore, radiation reduces viable numbers of undifferentiated multipotential mesenchymal cells *in vitro* and, perhaps more importantly, also inhibits differentiation into osteoblast cell lineages in surviving mesenchymal cells [27].

We previously demonstrated preoperative radiation therapy administered in clinically relevant total doses and fractions histologically and mechanically delayed allograft incorporation [15]. Furthermore, preoperative irradiation diminished BMP-2 protein and mRNA expression at the graft-host junction. Because BMPs are important inducers of endochondral bone formation and enhance healing of critical-sized bone defects [1], we theorized diminished BMP-2 expression at the graft-host junction may be responsible for poor allograft incorporation and therapeutic interventions aimed at enhancing BMP-2 expression in host cells at the graft-host junction may overcome these negative effects.

Although exogenously delivered BMP-2 protein has beneficial effects in fracture healing studies [21], the protein has a short 20- to 30-minute half-life *in vivo* [51], thus requiring supraphysiological doses to successfully treat bone defects [23], and the purification process is arduous and expensive. Furthermore, sustained release (greater than 3 weeks) of the protein is difficult to achieve [10]. BMP-2 gene therapy to enhance bone healing has been previously successful [11, 12, 46]. Using an ovine osteoporosis model, Egermann and coworkers [11] demonstrated accelerated fracture healing mechanically and histologically after injection of adenoviral particles containing human BMP-2 DNA. An additional study [4] demonstrated direct intraosseous injection of adenoviral vectors encoding BMP-2 was able to heal critical size defects in rabbits as well as rats.

We hypothesized sustained local delivery of the rhBMP-2 gene would increase BMP-2 protein expression at the graft-host junction and enhance allograft incorporation (radiographically and histologically) despite the host's exposure to preoperative radiation.

Materials and Methods

We divided 24 sheep into two groups: 12 received preoperative irradiation to the right tibia and surrounding soft tissue and 12 received no preoperative irradiation. After acclimation, a 5-cm middiaphyseal tibial osteotomy was created in the right tibia in all sheep and reconstruction was performed using a size-matched frozen allograft and bone plate. At the time of surgery, six irradiated and six

nonirradiated sheep received 100 mg of polymeric microspheres containing a BMP-2-encoded plasmid implanted at the graft-host junctions, whereas six irradiated and six nonirradiated sheep received 100 mg of microspheres containing a plasmid encoded with a marker gene (GFP) but no BMP-2 sequence. Monthly radiographs were taken to monitor allograft healing over a 4-month timespan at which time animals were euthanized. Subsequently, the allograft-host bone constructs were harvested, sectioned, and prepared for histomorphometric and immunohistochemical analyses.

A power calculation was performed to determine the number of animals needed per group. To detect a 20% difference in percent new bone formation between treatment groups using an alpha value of 0.05 and a power of 0.8, and presuming allograft incorporation in the irradiated group of sheep that did not receive the active gene would be affected as previously observed [15], six animals were needed per group. Eighteen of the 24 sheep (75%) used for this study were included in the radiographic, immunohistochemical, and histologic analyses. Six sheep ($n = 1$, GFP-0Gy; $n = 2$, GFP-50Gy; $n = 1$, BMP2-0Gy; $n = 2$, BMP2-50Gy) sustained hind-limb fracture as a result of the surgical procedure and were euthanized before completion of the study.

Poly(lactic-co-glycolic acid) (PLGA) microspheres containing BMP-2 plasmid were prepared by a water-oil-water emulsion solvent evaporation method [29, 37]. Briefly, 1.8–2 mL of 30 mM Tris-HCl (pH 8.5) containing 10 mg of plasmid DNA complexed with a 1:1 mixture of the monocationic lipid 1,2, dioleoyl-3-trimethylammonium propane (DOTAP), and helper lipid dioleoyl phosphatidylethanolamine (DOPE) at a molar ratio of 1:7 was emulsified in 20 mL of dichloromethane containing 300 mg of poly(lactide) (PLA; Birmingham Polymers, Pelham, AL) using a homogenizer at 18,000 rpm. The resulting primary emulsion was added to 150 mL of 1% (w/v) polyvinyl alcohol (PVA; Aldrich, Milwaukee, WI) and homogenized at 8000 rpm to form a water-oil-water double emulsion. The resultant emulsion was added dropwise into 300 mL of 0.5% (w/v) PVA and stirred for 4 hours at room temperature, completely removing dichloromethane and causing precipitation of PLA microspheres. The microspheres were collected by ultracentrifugation at 12,000 rpm for 30 minutes at room temperature, washed three times with distilled water, and lyophilized. PLGA microspheres were stored at -20°C until use. PLGA microspheres encapsulating plasmid DNA encoding GFP were prepared by this procedure in which the aqueous phase was composed of 200 mg of plasmid 30 mM Tris buffer (pH 8.5).

Twelve skeletally mature adult Rambouillet x Columbia ewes (54–82 kg) were humanely euthanized using a rapid intravenous bolus of 20 mL of pentobarbital sodium

solution (400 mg/mL) and used as allograft donors. Allografts consisted of cortical bone 5 cm in length harvested from the midtibial diaphyses. Specimens were harvested under strict sterile conditions. Soft tissue and periosteum were mechanically stripped until clean and marrow elements were removed using sterile saline flushing before freezing. Allografts were wrapped in gauze sponges soaked in sterile saline, double-wrapped, and frozen at -70°C for at least 72 hours before implantation.

We used 24 skeletally mature adult Rambouillet x Columbia ewes as recipients. Humane care and use of sheep was maintained in strict accordance with National Institutes of Health guidelines. Before surgery, 12 sheep received 50 Gy of radiation to the right tibia and surrounding soft tissues through bilateral opposing portals. Radiation was administered in 2-Gy fractions on a Monday through Friday schedule using a linear accelerator teletherapy unit. Sheep were sedated for radiation therapy using a combination of intravenous butorphanol and diazepam. The 12 irradiated sheep were randomized to one of two groups, each containing six sheep. The BMP2-0 Gy group received 100 mg of microspheres containing the BMP-2 gene at the time of surgery. The GFP-50 Gy group received 100 mg of microspheres containing the green fluorescence protein (GFP) marker gene. Twelve sheep received no irradiation and were randomly assigned to one of two groups of six control sheep. The BMP-2-0 Gy group received 100 mg of microspheres containing the BMP-2 gene at the time of surgery. The GFP-0 Gy group received 100 mg microspheres containing the GFP gene.

Six days after the end of radiation therapy, preemptive analgesia began by placing transdermal fentanyl patches on the shaved forearm skin of each sheep and adding non-steroidal antiinflammatory drugs to the feed. Seven days after the end of radiation therapy, anesthesia was induced using intravenous diazepam and ketamine. We maintained anesthesia using isoflurane (1.5%–3%) in oxygen with intravenous fluid support maintained until recovery. All sheep received 2.2 mg/kg of cefazolin intravenously at induction and every 8 hours during surgery. The right hind

limb was aseptically prepared for surgery. We performed a standard medial approach to the tibia and created a 5-cm middiaphyseal complete tibial cortical defect (osteotomy) using an oscillating power saw cooled with saline irrigation. Tibial defects were reconstructed using size-matched ovine fresh-frozen cortical allografts that had been sterilely procured and stored at -70°C for at least 72 hours. The allografts were thawed in 0.9% saline solution immediately before implantation. Allografts were secured to the host bone with a 12-hole, 4.5-mm dynamic compression plate using ASIF/AO principles. We placed four cortical screws in each allograft and four in the proximal and distal host bone. Before closure, the 100 mg of microspheres containing the specified gene was mixed with saline into a thick paste and placed immediately adjacent to the proximal and distal host-graft junction using a spatula. Muscles and soft tissue were opposed around the graft and the subcutaneous tissue and skin closed routinely. We ensured proper graft apposition and implant positioning on immediate postoperative radiographs.

Sheep were permitted full weightbearing throughout the duration of the study. Calcein green fluorochrome label was intravenously administered (20 mg/kg) to all sheep 3 days and 14 days before euthanasia. Four months after surgery, sheep were humanely euthanized using intravenous pentobarbital sodium.

Lateral and anteroposterior radiographs of the tibia were obtained 60 days postsurgery and every 30 days thereafter under sedation. A veterinary board-certified radiologist (HPT) blinded to treatment group and familiar with radiographic assessment of allograft healing scored all radiographs in accordance with the system developed by the International Society of Limb Salvage (ISOLS) (Table 1) [19]. Prior research [39, 57] has demonstrated strong agreement between radiographic scores for allograft healing as measured with the ISOLS system and mechanical and histologic outcome measures in a canine intercalary allograft model.

After euthanasia, we harvested the right tibia and removed the plate and screws. The specimens were

Table 1. Radiographic evaluation grading criteria form based on the scoring system developed by the International Society of Limb Salvage

Score	Union	Resorption	Fracture	Graft shortening	Implant
Excellent (4)	Union; osteotomy line not visible	No resorption	No fracture	No shortening	No change
Good (3)	$\geq 75\%$; osteotomy line still visible	$< 25\%$; no fracture	Incomplete fracture	< 2 cm	Minor changes; bending $< 10^{\circ}$
Fair (2)	25% to 75%	25% to 50%; no fracture	Simple fracture without displacement	2 to 4 cm	Bending $> 10^{\circ}$; broken device not affecting graft
Poor (1)	$< 25\%$; no evidence of callus	$> 50\%$	Simple fracture with displacement or comminuted fracture	> 4 cm	Failure of device with damage to the graft

sectioned in the longitudinal, sagittal plane creating medial and lateral tibial halves. Medial sections were processed for undecalcified histology and lateral longitudinal sections were fixed in 10% neutral formalin, decalcified in a 12.5% HCl-EDTA solution, dehydrated in graded solutions of ETOH (75%–100%), and cleared with xylene. Lateral specimens were then processed using standard paraffin histology techniques and subjected to immunohistochemical staining for BMP-2 and GFP.

After fixation in 10% neutral formalin, the medial tibial sections were placed in fresh 70% ETOH for 1 week and then dehydrated in graded solutions of ETOH (70%, 95%, and 100%) and xylene over the course of 5 weeks. The samples were infiltrated with a series of solutions containing methylmethacrylate, dibutyl phthalate, and benzoyl peroxide. The final methylmethacrylate solution was polymerized into a hardened plastic block and kept in the dark until completion of dynamic histomorphometry. One 100- μ m section was taken from each specimen block in the sagittal plane using an Exakt diamond blade bone saw (Exakt Technologies, Oklahoma City, OK). All sections were ground to 20 μ m thickness using an Exakt microgrinder. The sections remained unstained until after dynamic histomorphometric analyses (Fig. 1), then were stained using Sanderson's rapid bone stain (Surgipath, Richmond, IL), and counterstained with acid fuchsin to provide color differentiation between tissue types for semiautomatic image analysis.

Fig. 1 Dynamic histomorphometry was performed to quantify the effect of endogenously delivered rh-BMP2 plasmid DNA in the presence or absence of an irradiated host tissue bed on mineral apposition rate (MAR) in 18 regions of interest per slide (proximal section shown here), including host, allograft, and interface (host/allograft junction) regions. Static histomorphometric parameters of interest included trabecular thickness in the interface region as well as porosity in the host, allograft, and interface.

High-resolution digital images were acquired for fluorescent and stained sections using an Image Pro Imaging system (Media Cybernetics, Silver Spring, MD). The fluorescent images were used to measure mineralizing surface (MS), mineral apposition rate (MAR), and bone formation rate (BFR) at the host/allograft interface region. Host/allograft interface porosity and periosteal and endosteal callus area were measured on stained sections.

The following antibodies were used for immunohistochemical staining on the serial sections using the avidin-biotin peroxidase complex method in accordance with Farhadieh and coworkers [16]: rabbit (ab14933; Abcam, Cambridge, MA) and goat (sc-6895; Santa Cruz Biotechnology, Santa Cruz, CA) polyclonal antibodies raised against BMP-2; rabbit (ab290; Abcam) and goat (sc-5385; Santa Cruz Biotechnology) polyclonal antibodies raised against GFP. The concentration for each antibody was optimized in preliminary studies.

In preparation for staining, 4- μ m serial sections of formalin-fixed, decalcified tissue were deparaffinized and hydrated. Antigen retrieval was accomplished by placing the slides into a citrate buffer (Vector Laboratories, Burlingame, CA) preheated to 100°C. Slides and buffer were allowed to cool for 30 minutes. Endogenous peroxidase activity was then quenched with methanol containing 3% hydrogen peroxide for 30 minutes at room temperature.

We inhibited nonspecific binding with SNIPER blocking solution (Biocare Medical, Concord, CA) for 10 minutes at room temperature. Immunostaining was subsequently carried out by applying the appropriate concentration of primary antibody to each sample and incubating for 1 hour at room temperature. Monoclonal mouse antibody against alkaline phosphatase (sc-57502; Santa Cruz Biotechnology) served as the positive control. We included negative controls, using phosphate-buffered saline (PBS) instead of primary antibodies, with each series of slides. After incubation, the samples were washed in PBS and incubated with the biotinylated secondary antibody solution (Dako-Cytomation, Carpinteria, CA) for 30 minutes at room temperature. The avidin-biotin peroxidase complex (Vector Laboratories) was then added for 10 minutes. We counterstained with hematoxylin. Positively stained cells in the interface as well as endosteal and periosteal callus regions of interest were evaluated with light microscopy.

Paraffin-embedded bone sections containing the host/allograft interface were also subject to Western blotting for BMP-2 and GFP. Briefly, we cut three 10- to 15- μ m thick sections from paraffin-embedded bone tissue and placed them into 1.5-mL microcentrifuge tubes. The tissue was then cleared with xylene and rehydrated. Protein obtained from pellets generated from repeated vortex in extraction buffer (Qiagen, Valencia, CA) was loaded onto a 12% Tris-HCl-ready gel and SDS PAGE was run at 100 V for

1 hour. After protein transfer to a nitrocellulose membrane, we incubated the membranes overnight in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% nonfat dried milk. The next day, the membrane was incubated in primary rabbit polyclonal antibodies for GFP and BMP-2 (Abcam) for 1 hour at room temperature. After repeated washing, secondary antirabbit IgG, horseradish peroxidase (HRP) conjugate was added and incubated for 1 hour. Chemiluminescence detection solution (GE Healthcare, Piscataway, NJ) was then added and incubated for 5 minutes. Subsequently, we exposed the membranes for 1 to 15 minutes on Kodak Biomax Light film and developed them in an automated developer. Using the same primary antibody, fresh and paraffin-embedded sheep liver and spleen served as the positive control.

Effect of endogenously delivered rhBMP-2 DNA in the presence or absence of preoperative 50 Gy host bed irradiation after 4 months of healing on MS, MAR, BFR, interface porosity, and total callus area was evaluated with a one-way analysis of variance followed by a Dunnett's multiple comparison procedure with the GFP-0 Gy treatment group serving as the control. We analyzed differences in radiographic scores between all treatment groups at the monthly time points using a Kruskal-Wallis test. When differences were detected at the $p < 0.05$ level, we performed multiple comparisons using Wilcoxon rank sum tests. All analyses were performed with SigmaStat (Systat Software, Inc, San Jose, CA).

Results

Scores for radiographic union at the proximal and distal host-allograft junctions were greater ($p = 0.016$) in the BMP2-0 Gy treatment group relative to the GFP-50 Gy treatment group after 4 months of healing (Fig. 2). Mean final radiographic scores for union in the GFP-50 Gy

treatment group tended to be lower than those of the BMP2-50 Gy or GFP-0 Gy treatment groups ($p = 0.156$ and $p = 0.063$, respectively). At the study end point, we noted no allograft resorption in the BMP2-0 Gy treatment group, whereas allografts in the GFP-50 Gy treatment groups were more ($p = 0.016$) resorbed. One nondisplaced allograft fracture was noted radiographically in the BMP2-0 Gy treatment group 2 months postoperatively but healed without complication by the study end point. One animal in the GFP-50 Gy treatment group sustained an allograft fracture 2 months postoperatively as evidenced radiographically by a vertical fissure along the medial cortical margin of the allograft. This fissure was still present at the study end point. Radiographic scores were similar ($p > 0.05$) with allograft fracture, shortening, and implant failure.

Porosity in the host-allograft interface was greater in the BMP2-50 Gy ($p = 0.020$) and GFP-50 Gy ($p = 0.023$) treatment groups relative to their nonirradiated controls after 4 months of healing (Fig. 3A). In the same region of interest (Fig. 3B), trabecular thickness was smaller in the irradiated groups relative to the GFP-0 Gy ($p = 0.005$) and BMP2-0 Gy ($p = 0.006$) groups. Total callus area was similar ($p > 0.05$) in all treatment groups. Presurgical irradiation or therapeutic delivery of BMP-2 did not influence ($p > 0.486$) MAR in the host-allograft interface between the four treatment groups after 4 months of healing (Table 2).

We identified neither BMP-2- nor GFP-expressing cells in the host-allograft interface or callus regions of interest after 4 months of healing. Several variations of the IHC protocol described here were also tried with no success. Our lack of positive findings using immunohistochemistry was confirmed with the additional attempt to isolate protein through Western blotting from these regions of interest, which failed to retrieve detectable amounts of BMP-2 and GFP from the decalcified tissue sections.

Fig. 2A–B Radiographic assessment of allograft healing was performed in accordance with the system developed by the International Society of Limb Salvage (ISOLS). (A) At the study end point, host graft unions in the BMP2-0 Gy treatment group proceeded to the highest degree of healing compared with all other treatment groups. (B) The cortical allografts in this group also underwent the least amount of resorption. *BMP2-0 Gy > GFP-50 Gy ($p < 0.05$).

Fig. 3A–B Static histomorphometric measurements indicated (A) host-allograft interface porosity was increased by presurgical irradiation. No effect of therapeutically delivered BMP-2 to this area of interest was noted either in the absence of presurgical irradiation ($p = 0.625$) or after exposure to 50 Gy of radiation before allograft

transplantations (ie, GFP-50 Gy versus BMP2-50 Gy, $p = 0.683$). (B) Trabeculae in the interface region were smaller in the GFP-50 Gy ($p = 0.005$) and BMP2-50 Gy ($p = 0.006$) treatment groups relative to their respective nonirradiated controls.

Table 2. Dynamic histomorphometric results from the interface (host/allograft) regions of interest

Treatment		Mineralizing surface (MS) MS/BP	Mineral apposition rate (MAR) ($\mu\text{m}/\text{d}$)	Bone formation rate MS*MAR
GFP-0 Gy	Proximal	0.5773 ± 0.3322	1.6040 ± 0.7691	1.1006 ± 0.7264
	Distal	0.7523 ± 0.1756	1.8234 ± 0.7212	1.4250 ± 0.5761
	Overall	0.6580 ± 0.2823	1.7053 ± 0.7459	1.2503 ± 0.6731
GFP-50 Gy	Proximal	0.5448 ± 0.3924	1.2012 ± 1.1269	0.9540 ± 0.9616
	Distal	0.7397 ± 0.3355	1.0745 ± 1.0930	0.9142 ± 0.9523
	Overall	0.6422 ± 0.3738	1.1379 ± 1.0983	0.9341 ± 0.9455
BMP2-0 Gy	Proximal	0.6559 ± 0.3669	1.5354 ± 0.8697	1.2310 ± 0.8741
	Distal	0.7304 ± 0.2563	1.5508 ± 0.6566	1.1899 ± 0.6120
	Overall	0.6954 ± 0.3122	1.5435 ± 0.7452	1.2092 ± 0.7706
BMP2-50 Gy	Proximal	0.6593 ± 0.3739	2.1739 ± 0.6310	1.4366 ± 0.9118
	Distal	0.4263 ± 0.5177	0.5929 ± 0.8966	0.6097 ± 0.9590
	Overall	0.6536 ± 0.4125	1.3834 ± 1.0432	1.2875 ± 0.9720

All values expressed as mean $\pm$ standard deviation; no significant differences ($p > 0.050$) in any dynamic interface properties (MS, MAR, BFR) as a result irradiation or BMP2 presence were detected by analysis of variance.

Discussion

Many processes, both biologic and nonbiologic, can affect the incorporation and functional capacity of structural bone allografts. Although the temporal sequence of cellular events believed directly responsible for allograft healing have been described [5, 20], the specific means by which perioperative irradiation affects these biologic processes are not fully characterized. Our previous *in vivo* study [15] suggested decreased expression of BMP-2 protein and mRNA at the host-allograft interface after exposure to clinically relevant doses of irradiation may, in part, explain the higher incidence of allograft-related complications such as nonunion [15, 25] and fracture [24]. Thus, the

overarching goal of the current study was to determine if sustained local delivery of the rhBMP-2 gene from a polymeric delivery system would enhance allograft incorporation as measured radiographically and histologically despite the host's exposure to preoperative radiation.

Our study is limited by the small sample size per treatment group. Although our *a priori* power analysis indicated a statistical power of 0.8 with six animals per treatment group, a total of six animals sustained hind limb fracture as a result of the surgical procedure and were not included for analysis. Thus, our findings should be interpreted cautiously. Second, BMP-2 protein expression at the host-allograft junction was only evaluated at single time point. Prior *in vitro* work with the PLGA delivery vector

demonstrated linear release of plasmid DNA for 16 weeks without a burst effect [38]. Our current experimental design precludes any temporal identification of expressed protein in the interface region of interest up to the 4-month time point. Thus, we are unable to definitively determine whether the released rhBMP-2 DNA was able to successfully transfect cells in the host-graft interface *in vivo*. However, our goal was not to characterize transfection efficacy using this gene therapy approach: rather, we asked whether sustained release of the naked BMP-2 plasmid in an area critical to affect union could promote healing in a large animal model. Finally, the radiographic evaluation of allograft healing was performed by a single board-certified radiologist and we therefore did not address interobserver variability in radiographic interpretation using the ISOLS scoring system.

Although used as a neoadjuvant therapy for the treatment of musculoskeletal sarcoma before en bloc resection of the tumor, preoperative radiation negatively affects the temporal cascade of healing events resulting in impaired and delayed allograft incorporation [15, 22, 24–26]. Our data confirmed the negative effects of 50 Gy irradiation administered presurgery to the host tissues on allograft incorporation both histologically and radiographically; however, we were unable to demonstrate any positive healing effect of the therapeutically delivered BMP-2 plasmid through a polymeric microcarrier in either the nonirradiated or irradiated tissue beds, thus disconfirming our initial hypothesis.

Because prior studies have demonstrated that exogenously delivered BMP-2 can overcome radiation-induced impairment of bone formation both *in vitro* and *in vivo* [26, 27, 56], our negative findings are confounding and perhaps illustrate that further investigation into our polymeric BMP-2 gene delivery system is warranted. Gene transfer by viral vectors represents a very ubiquitous approach in gene therapy studies, the most common vectors being adenoassociated viruses, retroviruses, and lentiviruses [32]. Much of the previous work using gene therapy to promote bone regeneration has used adenoassociated viral vectors because these vectors promote the highest levels of transgene expression and also promote transient gene expression [18], which presumably would be most beneficial to promote new bone development [32]. To date, BMP-2 gene transfer using adenoviral and adenoassociated viral vectors to promote bone regeneration after fracture [3], in critical-sized defects [4, 46], as well as in osteoporotic animal models [11–13], has been successful. Although viral-mediated intervention to upregulate endogenous BMP-2 expression in the aforementioned clinical settings has been well studied, very few studies to date have examined the effect of increased BMP presence in the host tissue bed after massive bone allograft transplantation. Because the

temporal sequence of events required to fully incorporate bone allografts requires more time than those required to promote fresh fracture healing [8, 20], if BMPs are to be used after transplantation, their half-life in the dynamic physiological environment must be extended so as to improve the clinical course of healing. The lack of improvement in allograft healing reported by Cullinane et al. [9] and Pluhar et al. [39] in canine femoral intercalary models using passive graft soaking and exogenous protein delivery to the host-allograft junctions through a biomaterial scaffold, respectively, supports the concept that BMP presence must be extended to promote healing.

Although viral vectors confer superior transfection efficiency over nonviral vectors, they are plagued with a myriad of adverse immunogenic responses and substantial safety concerns [31]. Nonviral approaches include delivery of naked DNA/plasmids by direct injection, liposome-mediated transfection, particle-mediated delivery (ie, gene gun), microseeding, electroporation, and polymer-DNA complex implantation, the latter of which was used in the current work. Because allograft infection and resorption remain very serious clinical problems [35], the use of a nonviral vector as a means to circumvent any additional and unnecessary immunogenicity in the host bed was deemed an appropriate means by which to deliver the BMP-2 gene and upregulate endogenous production of the osteogenic protein. Although no deep infections were noted in any of the animals, the BMP-2 plasmid-containing polymeric microspheres failed to promote enhanced allograft incorporation at the end point of this study, even in the absence of preoperative 50 Gy radiation therapy. The lack of a healing effect conferred by the microsphere delivery system may be multifactorial and future studies are warranted to determine whether this method of gene delivery holds promise as a therapeutic intervention to promote allograft incorporation. Although our preliminary *in vitro* work used the identical PLGA vector and plasmid [38], no prior *in vivo* work has been performed with this delivery system. It may be possible BMP-2 DNA was incorporated, transcribed, and translated by mesenchymal cells in the host-allograft junction into a functional osteogenic protein for a shorter period of time than noted *in vitro*. Resultantly, protein expression may have been limited to a timeframe far shorter than that needed to elicit any positive healing effect. In support of this, neither BMP-2 nor the marker protein was identified by immunohistochemical staining or protein sequester from the interface regions in any of the decalcified tissue samples. Future temporal studies in small animal models using this delivery system may further delineate the transfection efficacy and subsequent BMP-2 expression profile and thus the suitability of the delivery system for this particular clinical application.

Although the results presented here did not confirm the efficacy of a novel polymeric, BMP-2 gene delivery system, it is our contention that improved bone allograft incorporation may be achieved through the use of gene therapy and could have a substantial impact on the field of allograft transplantation science. In support of this, recent reports by Koefoed et al. [30] and Ito et al. [28] have demonstrated improved structural allograft incorporation in mouse models over a 6- and 4-week time period, respectively, using a recombinant adenoassociated viral vector (rAAV) to deliver various proteins believed responsible for promoting a favorable healing environment. Koefoed et al. [30] demonstrated lyophilizing 5×10^7 rAAV containing an activin receptor-like kinase-2 (caALK2) gene onto 4-mm murine femoral allografts induced endochondral bone formation directly on the cortical surface of the allograft as early as 2 weeks after transplantation. This early healing stage was followed by increased evidence of allograft remodeling throughout the remaining 2 weeks of the study as demonstrated histologically and through high-resolution three-dimensional microcomputed tomographic analysis. Using a unique combination of genes encoding for receptor activator of nuclear factor κ B ligand (RANKL) and vascular endothelial growth factor (VEGF), Ito et al. [28] reported that when these genes were delivered to the host bed adjacent to 4-mm murine femoral allografts, accelerated remodeling and revascularization of the grafted tissue was noted over the 4-week time period. Future studies may define whether gene therapy has any role in promoting structural allograft healing.

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