Runx2 Overexpression in Bone Marrow Stromal Cells Accelerates Bone Formation in Critical-Sized Femoral Defects

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The repair of large nonunions in long bones remains a significant clinical problem due to high failure rates and limited tissue availability for auto- and allografts. Many cell-based strategies for healing bone defects deliver bone marrow stromal cells (BMSCs) to the defect site to take advantage of the inherent osteogenic capacity of this cell type. However, many factors, including donor age and *ex vivo* expansion of the cells, cause BMSCs to lose their differentiation ability. To overcome these limitations, we have genetically engineered BMSCs to constitutively overexpress the osteoblast-specific transcription factor Runx2. In the present study, we examined Runx2-modified BMSCs, delivered via polycaprolactone scaffolds loaded with type I collagen meshes, in critical-sized segmental defects in rats compared to unmodified cells, cell-free scaffolds, and empty defects. Runx2 expression in BMSCs accelerated healing of critical-sized defects compared to unmodified BMSCs and defects receiving cell-free treatments. These findings provide an accelerated method for healing large bone defects, which may reduce recovery time and the need for external fixation of critical-sized defects.

Introduction

THE REPAIR OF LARGE bone defects in humans remains a significant clinical problem affecting ~600,000 people in the United States per year and costing upward of 5 billion dollars annually.^{1,2} Despite successful bone healing with auto- and allografts, which are the most common clinical treatments for large nonunions, failure rates for these procedures remain unacceptably high. Autografts contain the appropriate cues for osteogenesis and elicit no immunogenicity, but donor-site morbidity and pain affect as many as 30% of patients who have undergone a bone harvest from the iliac crest.^{3,4} Allografts address some of these sourcing issues but are further complicated by an increased risk of infection, poor revascularization and remodeling, and a high rate of fracture.^{5–7} As an alternative approach for bone repair, cell-based regenerative medicine strategies are being developed to address the problems associated with current bone grafting procedures.^{8–11}

For successful repair and remodeling of large bone defects, a cell population capable of producing and remodeling bone must be present in the defect site. These cells can either be recruited from host tissues or delivered to the defect via surgical intervention. Bone marrow stromal cells (BMSCs)

offer an attractive solution to cell sourcing for bone tissue engineering because they represent a multipotential cell population that contributes to the early stages of fracture healing in bone.¹² Human BMSCs can be isolated from bone marrow and delivered directly to a defect site to induce healing.^{13,14} BMSCs have the capability of differentiating into osteoblasts, among other cell types of the mesodermal lineage, and they mineralize constructs both *in vitro* and *in vivo*.^{15–19} BMSCs also enhance bone healing in critical-sized orthotopic defects in rodents and large animals compared to treatment with a scaffold alone.^{20–23} Recently, Marcacci *et al.* have reported successful healing of large bone defects in humans treated with autologous BMSCs seeded on bioceramic scaffolds. These implantations were initially reported in 2001, and a 6–7-year follow-up of each patient demonstrated that complete bone implant integration was achieved in all patients as determined by radiography and computed tomography (CT) analysis.^{24,25}

The success of these cell-seeded constructs makes a compelling case for the use of autologous BMSCs in treating large bone defects in humans. However, multiple factors affect the differentiation of BMSCs into bone-producing osteoblasts. For example, *in vitro* expansion of BMSCs, a necessary step to obtain sufficient numbers for implantation, causes

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dedifferentiation and subsequent loss of mineralization capacity.^{26–28} Further, the mineralization potential and proliferation rate of BMSCs varies widely with individual donors and is significantly affected by donor age.^{29–31} These complications with BMSC function suggest that the treatment of large bone defects with autologous BMSCs may not produce effective results for all patients. A strategy for overcoming these difficulties with BMSCs would provide great benefit to clinical bone healing.

To address these limitations with BMSCs, extensive work has focused on bone morphogenetic protein (BMP) delivery to host cells for the upregulation of osteoblastic differentiation.^{32–36} However, low biostability and/or diffusion of these soluble factors away from a defect site makes the effective dosage for human BMP treatment very high, which in turn makes treatment with BMPs an expensive therapy.³⁷ Furthermore, as a soluble factor, diffusion of BMP away from the delivery site decreases the amount of bone formed in an implant and may lead to unregulated signaling in remote sites.³⁸ A recent retrospective study of complications due to spinal fusion in the United States demonstrated that BMP leads to 50% more complications in cervical spinal fusion compared to fusion without BMP.³⁹ As an alternative osteoinductive factor, we have focused on delivering the type 2 runt-related Cbfa1 gene *Runx2*, which encodes an osteoblast-specific transcription factor that works intracellularly to upregulate a host of bone-specific genes, including osteocalcin and collagen I.^{40–42} *Runx2* plays an important role in both bone development and bone remodeling/repair.^{43,44} Homozygous deletion of *Runx2* in mice causes the formation of a completely nonmineralized, cartilaginous skeleton and results in immediate postpartum death.^{45,46} Mice heterozygous for *Runx2* display a pathology similar to that observed in the skeletal disease cleidocranial dysplasia.^{46,47} In fact, the human disease, cleidocranial dysplasia, occurs as a result of genetic mutations in *Runx2*.^{47–49} The role of *Runx2* as an osteoblastic transcription factor in human BMSCs has been demonstrated *in vitro* via DNA binding assays.⁵⁰ Further, dominant negative expression of *Runx2* after osteoblast differentiation causes skeletal abnormalities,⁴⁴ while *ex vivo* overexpression of *Runx2* in both osteoblastic and nonosteoblastic cells promotes upregulation of bone-specific genes and subsequent mineralization.^{42,51–53} Finally, overexpression of *Runx2* in BMSCs accelerates osteoblastic differentiation and subsequent mineralization both *in vitro* and *in vivo* compared to unmodified BMSCs,^{54,55} making *Runx2* overexpression an attractive option for regulating BMSC function.

Recently, our group and others have reported significant healing of critical-sized defects in a calvarial defect model treated with BMSCs engineered to overexpress *Runx2*.^{40,56,57} Although these findings suggest the potential of *Runx2* to promote healing of large nonunions in long bones, results obtained from calvarial defect studies do not necessarily translate to healing in long bones due to the involvement of the dura mater in healing cranial defects.⁵⁸ In the present study, we examined the effects of constitutive overexpression of *Runx2* in BMSCs implanted in critical-sized segmental defects in rat femurs. This model provides a more rigorous test bed than a cranial defect by eliminating contributions to healing from the dura mater. This study is the first to investigate *Runx2*-modified BMSCs for the treatment of large nonunions in long bones, providing better insight

into the use of *Runx2* as a cell-based gene therapy strategy for future clinical bone defect healing.

Materials and Methods

Cell harvest and isolation

Rat BMSCs were isolated from the hind legs of 7-week-old male Lewis rats (Charles River Labs) by commonly used methods.^{59,60} For each independent *in vivo* experiment performed, a separate cell harvest was generated by pooling the femoral and tibial cells from 1 to 2 rats. To isolate the cells, rats were euthanized by CO₂ inhalation (Georgia Tech IACUC-approved protocol), and the hind limbs were removed, taking care to minimize bleeding and subsequent clotting. Surrounding soft tissue was removed from each bone, and the femurs and tibiae were soaked in a cell culture medium consisting of α Minimal Essential Medium (α MEM) (Invitrogen), 10% fetal bovine serum (FBS; HyClone), 1% penicillin/streptomycin (pen/strep; Invitrogen), and 0.3 μ g/mL fungizone. The distal end of each bone was removed and the marrow was flushed from the diaphysis via centrifugation (2 min, 500 g) into a sterile tube. The marrow pellet was then resuspended, and transferred to tissue-culture-grade polystyrene (Corning). Three days after harvest, plates were rinsed twice in phosphate-buffered saline (PBS) to remove nonadherent hematopoietic cells, and a fresh medium was added. The medium was changed every 3 days. When plates were 80%–90% confluent, passage 0 cells were cryopreserved in 10% dimethylsulfoxide in FBS at -80°C overnight, and then transferred to liquid nitrogen for long-term storage.

Scaffold fabrication

Medical-grade polycaprolactone (PCL) scaffolds were manufactured in 9-mm-thick sheets by fused deposition modeling (Osteopore International). Scaffolds were reproducibly cut from PCL sheets using dermal biopsy punches 4 mm in diameter (Miltex). MicroCT analysis was used to characterize the structural parameters of the scaffolds (Fig. 1A). For *in vivo* studies, scaffold porosity was 81%–85%, average pore size was 890 μ m, and average rod/strut thickness was 310 μ m.

For all studies, scaffolds were cleaned in 70% ethanol for 30 min, rinsed three times in sterile water to remove ethanol, and then soaked in PBS for 10–30 min before cell seeding. For *in vivo* studies, an additional step was added before scaffold sterilization for endotoxin removal. For this step, scaffolds were rinsed in 70% ethanol for 4 days on a shaker plate, and ethanol was replaced once daily. Endotoxin levels were below the U.S. Food and Drug Administration's recommended 0.5 EU/mL, as determined by the limulus amoebocyte lysate (LAL) chromogenic assay (Cambrex).

To increase cell retention in PCL scaffolds, collagen meshes were produced within the scaffold pores by lyophilizing a collagen solution as previously described.⁶¹ Briefly, a sterile solution of 1.5 mg/mL type I collagen (MP Biomedicals) was incubated within sterile PCL scaffolds at 37°C for 30 min. Constructs were then frozen for 1 h at -80°C and lyophilized overnight to produce a fibrous mesh of collagen within the scaffolds.

Retroviral transduction

The *Runx2* type II MASNSLF isoform was expressed via the pTJ66 vector as a single bicistronic mRNA sequence en-

FIG. 1. Scaffolds, Runx2 plasmid, and femoral fixation plate used for segmental defect surgeries. (A) Cross-sectional microCT image of a PCL scaffold. (B) Diagram of Runx2 plasmid showing IRES for eGFP expression. (C) An explanted femur showing the modular fixation plate attached to the bone via stainless steel screws. Notches in the polysulfone plate (marked with arrows) are spaced 8.0 mm apart ensuring each defect is created at the same size. CT, computed tomography; PCL, polycaprolactone; IRES, internal ribosomal entry site; eGFP, enhanced green fluorescent protein. Color images available online at www.liebertonline.com/ten.

coded by murine cDNA (Fig. 1B). An internal ribosomal entry site, located downstream from and adjacent to the Runx2 insert, allowed coexpression of a fusion protein of zeocin resistance and enhanced green fluorescent protein (eGFP). eGFP expression was quantified via flow cytometry and used as a measure of Runx2 transduction efficiency.^{52,53} Empty pTJ66 vector, which was missing the Runx2 insert but still encoded the fusion protein, was used as an empty vector control.

To package Runx2 retrovirus, Φ NX helper cells, stably transfected with the Runx2 plasmid, were grown to subconfluency in Dulbecco's modified Eagle's medium containing 10% FBS and 1% pen/strep. Twenty-four hours before the first viral harvest, the medium was changed and cells were transferred to a 32°C incubator to minimize heat-induced viral degradation. To harvest virus, medium supernatant was collected every 12 h, filtered at 0.45 μ m, snap frozen, and stored at -80°C.

To transduce primary BMSCs with Runx2 or empty-vector virus, BMSCs were expanded to passage 3, trypsinized, and seeded into T-75 flasks at a density of 5000 cells/cm². One day after seeding into flasks, the medium was replaced with retrovirus supplemented with 0.4 μ g/mL polybrene, as previously described.⁵² Briefly, flasks were incubated with a viral medium at 32°C for 15 min, and then centrifuged at 1200 g for 30 min. The viral medium was then replaced with fresh α MEM containing 10% FBS, 1% pen/strep, and 0.3 μ g/mL fungizone, and cells were incubated at 37°C. After 12 h, a second transduction was performed. Two days after transduction, cells were trypsinized from flasks and seeded onto scaffolds or analyzed for eGFP expression by flow cytometry.

Cell seeding on scaffolds

Immediately before cell seeding, PCL-collagen scaffolds were prewet in PBS, and wicked on KimwipesTM to remove excess fluid. Unmodified, Runx2-modified, and empty-vector-modified cells (passage 4) were trypsinized and seeded at 500,000 cells per scaffold in 50 μ L of the medium (25 μ L per side). Scaffolds were placed in scaffold holders inside 24-well plates and incubated at 37°C to allow cell attachment to the scaffolds. After 30 min of incubation, 2 mL of

the medium was added to each well for complete submersion of each scaffold. The medium was changed every 3 days.

To ensure that equal cell numbers were seeded onto all scaffolds, unmodified BMSCs were seeded onto PCL-collagen scaffolds, as described above, in three separate batches ($n = 4$ scaffolds for each batch). Three days after cell seeding, DNA content on each batch of scaffolds was analyzed via Picogreen staining (Quant-iTTM Picogreen[®] dsDNA Assay Kit; Molecular Probes) as previously described.^{62,63} Briefly, constructs were rinsed in PBS and then frozen at -80°C. Thawed samples were dried in a Savant DNA120 SpeedVac Concentrator (Thermo Electron Corporation), and digested in 700 μ g/mL Proteinase K (Promega) for 48 h in a 45°C water bath. Picogreen staining was used to quantify average DNA content/cell numbers on each batch of scaffolds. No differences in cell number were observed among groups (data not shown), confirming that cell seeding was reproducible across multiple batches.

Flow cytometry for transduction efficiency

For each *in vivo* study performed, a sample population of cells was harvested and analyzed for transduction efficiency. Briefly, 3 days after Runx2 or empty-vector transduction, cells were trypsinized (0.05% trypsin/ethylenediaminetetraacetic acid; Invitrogen) and resuspended in PBS with 10% FBS. Cell suspensions were centrifuged for 5 min at 200–300 g, resuspended in PBS, and then passed through a 40 μ m filter (BD Falcon). eGFP fluorescence was measured on a flow cytometer (Becton Dickinson; BD LSR II), and 10,000 events were measured for each sample. Data were analyzed using WinMDI v.2.8.

Live/Dead staining

Cell viability on PCL scaffolds was assessed using the Live/Dead kit (Invitrogen). Unmodified and Runx2-modified cells were seeded onto PCL scaffolds with and without collagen meshes. Three days after seeding, constructs were rinsed three times in PBS, and stained with 4 μ M calcein and

4 μ M ethidium homodimer in PBS for 45 min at room temperature. After staining, constructs were rinsed three times in PBS, and imaged on a confocal microscope (Zeiss LSM 510 NLO). LSM 5 Image Browser was used to stack groups of two-dimensional image slices.

Segmental defect surgery

Femoral defects were created bilaterally as previously described.⁶⁴ Briefly, 13- to 15-week-old female Lewis rats were anesthetized using isoflurane, and the hind limbs were shaved and swabbed with cycloheximide and alcohol to prepare the skin for incision. An anterior incision was made from the hip to the knee to allow blunt separation of the quadriceps muscles, exposing the femur. Before the defect was created, a modular fixation device was attached to the bone for mechanical support. The device consisted of two stainless steel plates affixed directly to the bone via screws and one polysulfone plate, which spanned the defect and was attached to the stainless steel plates (Fig. 1C). Use of this modular system was advantageous for postmortem mechanical testing because the polysulfone plate could be removed before defect testing without removing the screws or stainless steel plates from the bone, thus avoiding any incidental damage to the repair tissue before testing. Further, use of a polysulfone plate for support allowed noninvasive *in vivo* X-ray analysis of defects due to the low X-ray attenuation of polysulfone. After the fixation device was attached, an 8.0-mm segment of bone, amounting to $50.6 \pm 1.9 \text{ mm}^3$ (as measured by postmortem microCT analysis, $n=8$), was removed via bone saw. A scaffold was then press fit into the defect. Notches in the polysulfone plate, spaced 8.0 mm apart, ensured that each defect was consistently created the same length (Fig. 1C). Muscle was closed around the plate and defect using Vicryl sutures, and the skin was closed using sutures and wound clips.

For *in vivo* studies, five groups were tested: empty defect control (no scaffold or cells were placed in the defect) and PCL-scaffolds seeded with (i) unmodified BMSCs, (ii) Runx2-modified BMSCs, (iii) empty vector-modified BMSCs, or (iv) no cells (cell-free scaffolds). These groups are outlined in Table 1.

After surgery, animals were given three daily doses of buprenorphine at 0.03 mg/kg for two consecutive days and three doses of 0.01 mg/kg on the third day to control pain. Animals were monitored daily for signs of pain and distress, progress of wound closure, regular eating habits, and normal ambulation. A small percentage (<8%) of rats developed infections in or around the surgery site, or experienced mechanical failure of the fixation device. These animals were removed from the study and euthanized, and any data col-

lected from these animals were excluded. Two weeks post-surgery, when skin wounds had completely healed, animals were anesthetized with isoflurane and wound clips were removed. At 4, 8, and 12 weeks postsurgery, animals were anesthetized with isoflurane and the hind legs were scanned via radiography and microCT as described below. At 12 weeks postsurgery, animals were euthanized by CO₂ inhalation, and the femurs, along with surrounding muscle tissue, were harvested for postmortem microCT evaluation, histology, Fourier transform infrared (FTIR) analysis, and mechanical testing.

Radiography and microCT analyses

Every 4 weeks after surgery, two-dimensional X-ray images of each sample were noninvasively obtained using an MX-20 Specimen Radiography System (Faxitron X-ray Corporation) to make gross morphological observations of bone formation in each defect site. For X-ray analysis, animals were anesthetized in a gas chamber with 5% isoflurane and maintained under anesthesia using 2% isoflurane flow into a face mask. Each hind leg of anesthetized animals was scanned for 15 s with an X-ray beam energy of 23 kV. X-ray images were blindly assessed for extent of bridging and assigned a bridging score from 0 to 5, as previously described.⁶⁵

In addition to radiographic imaging, samples were noninvasively analyzed every 4 weeks postsurgery by microCT using a vivaCT 40 (Scanco Medical) to quantify bone volume in each defect site. Sample sizes for each group and time point were as follows: empty defect $n=5$, PCL $n=7$, empty vector $n=2$, BMSC $n=11$, Runx2 $n=11$ (4 weeks); empty defect $n=5$, PCL $n=7$, empty vector $n=2$, BMSC $n=10$, Runx2 $n=9$ (8 weeks); and empty defect $n=5$, PCL $n=6$, empty vector $n=2$, BMSC $n=9$, and Runx2 $n=9$ (12 weeks). For microCT, animals were anesthetized in the same manner as for radiography and placed in a rodent holder with one leg outstretched for scanning. The defect area in between the stainless steel plates of the fixation device was imaged with an X-ray beam energy of 55 kVp and intensity of 109 μ A, and the integration time was 200 ms. Scanning resolution was 38 μ m. After imaging was complete, noise was reduced from three-dimensional reconstructions of each scan by applying a Gaussian filter (sigma = 1.2, support = 2) using the Scanco Medical microCT Evaluation Program. Images were thresholded at 270 mg HA/ccm to isolate mature bone from soft tissue and the polymer scaffold and polysulfone plate. Bone volume was quantified using directly computed values.

Histological analysis

One sample from each cell group, with bone volume closest to the 12-week median bone volume for that group, was chosen as a representative sample for histological analysis. After euthanasia, these samples were immediately fixed in 10% neutral buffered formalin. One day after fixation, soft tissue was removed, and specimens were placed in fresh formalin. Before embedding, fixed tissues were scanned *ex vivo* in formalin via microCT as described above to allow matching of histological sections with microCT slices. After scanning, specimens were dehydrated in a series of alcohols, cleared in xylene, and embedded in methyl methacrylate. Ground sections, 50–80 μ m thick, were prepared by Wasatch

TABLE 1. OUTLINE OF EXPERIMENTAL GROUPS FOR *IN VIVO* SEGMENTAL DEFECTS

Group name	Defect contains
Empty defect	No scaffold and no cells
PCL	PCL scaffold (cell-free)
Empty Vector	PCL with empty vector BMSCs
BMSC	PCL with unmodified BMSCs
Runx2	PCL with Runx2-BMSCs

PCL, polycaprolactone; BMSC, bone marrow stromal cells.

Histo Consultants, Inc., and stained using Sanderson's Rapid Bone Stain™ and a van Gieson counterstain.⁶⁶ Stained histological sections were then matched to thresholded microCT scans to confirm that microCT analysis was representative of mature bone.

FTIR spectroscopy

Explanted samples for FTIR analysis were wrapped in PBS-soaked gauze and frozen at -20°C until use. One representative sample from each group underwent the analysis. Upon thawing, chips of mineralized tissue were removed from the defect area of each sample using a bone cutter. Care was taken to remove only newly formed tissue in the defect area and no native host bone. Mineral chips were fixed in ethanol, dried overnight at 50°C , ground with a mortar and pestle, pressed into KBr pellets, and read on a Nexus 470 FT-IR (Thermo Nicolet) using 64 scans at 4 cm^{-1} resolution. Native bone from age-matched Lewis rat femurs was used as a positive control for FTIR bone spectra. Mineral recovered from the defect region of empty defect samples was used as a negative control.

Mechanical testing

Samples for mechanical testing were explanted, wrapped in PBS-soaked gauze and frozen at -20°C until use. Torsional properties for intact age-matched femora from Lewis rats were also measured. Mechanical testing was performed as previously described.⁶⁴ Briefly, samples were thawed in room temperature PBS and most of the soft tissue was removed, taking care not to mechanically disrupt tissue in the defect site. The ends of each bone were potted in Wood's metal up to the polysulfone plate and secured with pins into potting blocks. Blocks were loaded onto an ElectroForce® mechanical testing machine (Elf 3200; Bose), and the polysulfone plate was removed just before testing. Samples were loaded in torsion at a displacement rate of $3^{\circ}/\text{s}$ up to 360° . Maximum torque before failure was recorded for each sample.

Statistical analysis

Data from two independent studies were pooled, and a mixed model analysis of variance was used to define experiment and individual animal as sources of error. This analysis was performed using the Hierarchical Linear Mixed Models function in Systat v12, which uses a Satterthwaite approximation to account for differences in sample sizes. For mechanical strength, the Tukey–Kramer method was used to determine differences between the pooled cell conditions (BMSC and Runx2) and empty defects, and the Pearson product-moment correlation coefficient was calculated for the correlation analysis. In addition, ridge regression using the Hoerl–Kennard model^{67,68} was used to assess the relationship between torsion, bone volume, and bridging score instead of the conventional linear multi-variate regression because bone volume and bridging score are not orthogonal. A p -value <0.05 was considered significant.

Results

Runx2 expression and cell viability

To measure transduction efficiency of the Runx2 and empty-vector retroviruses, eGFP expression was measured via flow cytometry. Unmodified cells were used as a control population, and transduction efficiency of retrovirus-transduced cells was determined using the 2% of background method.⁶⁹ Runx2-modified cells showed 40% transduction efficiency as measured by eGFP expression compared to control cells (Fig. 2A). Empty-vector-transduced cells showed 60% transduction efficiency (data not shown). These transduction efficiencies are in agreement with previous reports.⁶⁰

Cell-seeded PCL scaffolds with and without collagen meshes were analyzed to determine cell viability and retention on the scaffolds. Live/Dead staining of cells on scaffolds 3 days postseeding shows that unmodified and Runx2-modified BMSCs are viable on PCL scaffolds both with and without collagen meshes. Scaffolds with collagen

FIG. 2. Runx2-modified BMSCs show high eGFP expression at 3 days posttransduction and are viable on PCL scaffolds containing lyophilized collagen mesh. **(A)** Flow cytometric detection of eGFP expression in unmodified and Runx2-modified BMSCs. Transduction efficiency of Runx2-modified cells is 40% compared to unmodified controls. **(B)** Confocal live/dead images of unmodified and Runx2-modified BMSCs on PCL scaffolds with and without collagen meshes. Live cells are shown in green and dead cells in red. Scale bar is $200\text{ }\mu\text{m}$. Cells populate the pore volume of the PCL by adhering to collagen meshes lyophilized inside the scaffold. BMSCs, bone marrow stromal cells. Color images available online at www.liebertonline.com/ten.

meshes promote an even distribution of cells throughout the pore volume of the PCL, while scaffolds without meshes do not retain cells in the pore volume (Fig. 2B). PCL scaffolds with collagen meshes were subsequently used for all *in vivo* studies, and "PCL" hereon refers to PCL with collagen mesh.

Radiography and microCT analysis

Immediately after surgery, animals were monitored several times daily for signs of pain or stress, regular eating habits, and normal ambulation. Within 1 week after surgery, signs of stress were minimal, regular eating had returned, and normal ambulation using both hind limbs was restored.

To monitor bone formation in critical-sized defects, animals were anesthetized every 4 weeks and defects were scanned via X-ray and microCT. X-ray images show gross morphological changes in bone growth at the defect site over time. Whereas empty defects, PCL, and BMSC groups showed minimal bone formation, Runx2 showed substantial increases in bone growth over time (Fig. 3). Representative X-ray images from each group are shown in Figure 3A, and the corresponding three-dimensional microCT reconstructions at 12 weeks are shown in Figure 3B. No differences were observed between unmodified BMSC and empty vector groups (data not shown).

At 4, 8, and 12 weeks postsurgery, bone volume in all defects was quantified via microCT. Negligible bone formation occurred at the ends of host bone in empty defects and cell-free PCL-treated defects. However, bone formation in BMSC- and Runx2-BMSC-treated defects increased over time (Fig. 3C). At 4 and 8 weeks postsurgery, Runx2 defects contained significantly more bone than unmodified BMSC defects ($p < 0.05$, $n = 8$). However, at 12 weeks, bone volume in Runx2 defects was not significantly different compared to unmodified BMSC defects ($p = 0.059$) (Fig. 3C). On average, the amount of bone formed in Runx2-treated defects at 12 weeks was about 50% of the total bone volume removed from the femur during surgery. It should be noted that the PCL scaffold occupies volume within each defect, which may contribute to the observed lack of difference between Runx2 and BMSC groups at 12 weeks. On the whole, this differential time course of bone formation in BMSC- and Runx2-treated defects indicates that Runx2-modified BMSCs initially accelerate bone formation in critical-sized defects, but that the osteogenic capacity of unmodified BMSCs eventually produces a similar level of bone as Runx2-modified BMSCs.

Histological analysis

To more fully characterize areas of high attenuation imaged by microCT, a representative sample from each cell group was subjected to histological analysis at 12 weeks. Before embedding, samples were scanned via microCT and thresholded in the same manner described above. Samples were then stained with Sanderson's Rapid Bone Stain™, which distinguishes areas of mineralized bone from demineralized connective tissue and osteoid, revealing mineralized bone tissue in a red/pink color and demineralized osteoid in blue/green.⁷⁰ Because these samples underwent both Sanderson's stain and microCT scanning, matching slices from histology and microCT were compared. This analysis shows that areas of high attenuation that were

thresholded in microCT and used as a measure of bone volume in Figure 3 directly match areas of red/pink staining defined as mineralized bone tissue in the Sanderson's stain. Histology and CT images for Runx2 are shown in Figure 4. Histology and CT images for BMSC also demonstrated this correlation (data not shown).

FTIR spectroscopic analysis of repair tissue

To determine the chemical composition of bone formed in the defects, tissue taken from the defect area of a representative sample from each group was analyzed by FTIR spectroscopy (Fig. 5). Spectra from native bone (positive control) contain all peaks expected for biologic apatite, including amide peaks for protein at 1700 and 1550 cm^{-1} , a small carbonate peak at 900 cm^{-1} , a broad phosphate peak for stretching vibrations at 900–1200 cm^{-1} , and a phosphate doublet for bending vibrations at 525–625 cm^{-1} .^{60,71,72} Cell-loaded samples having either unmodified BMSCs or Runx2-modified BMSCs displayed all of these bands (Fig. 5), indicating that the mineralized tissue in these defects was a biological, carbonate-containing hydroxyapatite. Cell-free PCL scaffolds also displayed most of these bands; however, the CO_3 and PO_4 (bending) peaks were less prominent in these cell-free samples than native control bone or cell-loaded samples, and the PO_4 doublet indicative of crystalline apatite was not evident. Finally, empty defect negative controls showed amide peaks and some phosphate deposits; however, CO_3 peaks and prominent PO_4 (stretching) peaks were not present, indicating that the tissue deposited in these samples did not have the chemical composition of native bone.

Mechanical strength of repaired defects is dependent on bridging

To assess mechanical functionality of the repair tissue present in critical-sized defects, femurs were harvested 12 weeks postsurgery and subjected to postmortem torsional testing. A representative torque–displacement curve for Runx2-engineered cell-treated defects is shown in Figure 6A. Age-matched intact femora demonstrated a maximum torque of $279.5 \pm 31.0 \text{ N} \cdot \text{mm}$ ($n = 8$), while maximum torque sustained for BMSC- and Runx2-treated defects was 28.8 ± 18.8 and $28.3 \pm 17.5 \text{ N} \cdot \text{mm}$, respectively. Although microCT revealed differences in bone volume between BMSC and Runx2 defects at 4 and 8 weeks, maximum torque sustained at 12 weeks was not significantly different between these two groups (Fig. 6B). Stiffness and work to failure were calculated as the slope of the linear region (highlighted in Fig. 6A) and the area beneath the curve up to the maximum torque, respectively. No significant differences among experimental groups were observed for either of these parameters (data not shown). However, pooling of the data for cell-treated groups (Runx2 and BMSC) demonstrated a significant enhancement in maximum torque for cell-seeded scaffolds compared to empty defect negative controls (Fig. 6C).

We hypothesized that mechanical strength is dependent not only on bone volume but also on defect bridging. Without full attachment (i.e., complete bridging) samples that have large bone volumes are expected to sustain low torque loads. To demonstrate that mechanical strength of the defects is dependent not only on bone volume but also on defect bridging, X-ray images for all samples were blindly

FIG. 3. Runx2-modified BMSCs accelerate bone formation in critical-sized defects. **(A)** X-ray images of femoral defects showing representative images for each group. **(B)** MicroCT images showing the same samples from **(A)** at 12 weeks. **(C)** Bone volume is significantly greater in Runx2-treated defects than in BMSC-treated defects at 4 and 8 weeks ($*p < 0.05$). At 12 weeks, bone formation due to unmodified BMSCs is not significantly different from Runx2-modified cells ($p = 0.059$), indicating that Runx2-modified cells accelerate healing, but unmodified cells produce similar levels of bone at late time points. Error bars represent standard error of the mean.

FIG. 4. Histological analysis confirms that areas of high attenuation revealed by microCT are bone. (A) Sander-son's rapid bone stain for a Runx2-modified BMSC sample showing bone in red/orange and soft tissue in blue/green. (B) A corresponding two-dimensional microCT slice from the same sample, thresholded to isolate areas of high attenuation within the sample. MicroCT analysis shows high correlation with Sanderson's bone stain. Color images available online at www.liebertonline.com/ten.

assessed for their extent of bridging and assigned a bridging score from 0 to 5. Scoring criteria and representative X-ray images for each score are shown in Figure 7A, where 0 is no bone in the defect and 5 is a fully bridged defect. Whereas some samples contain a large amount of bone in the defect site, high mechanical strength is only present when this bone is firmly attached to both the proximal and distal ends of the host bone. A Pearson product-moment correlation analysis shows a positive correlation between bone volume and maximum torque ($r = 0.54$, $p = 0.027$), and the inclusion of bridging scores in the correlation plot shows that samples with higher bridging scores generally have greater mechanical strengths (Fig. 7B). In addition, a ridge regression analysis was performed with torsion as the dependent variable and bone volume and bridging score as independent variables. For this analysis, a Hoerl-Kennard ridge regression model was used instead of the conventional linear multivariate regression because bone volume and bridging score are not orthogonal.^{67,68} This analysis yielded regression fit parameters (constant = 9.88, coefficient for bone volume = 0.46, coefficient for bridging score = 3.22, ridge parameter =

0.6) and an adjusted R^2 value of 0.41. Test of significance indicated that this regression fit accurately describes the experimental data ($p < 0.020$). Although there are no significant differences in torsional strength between BMSC- and Runx2-treated defects, a distribution of bridging scores from each group shows that Runx2-modified defect scores are shifted toward fully bridged or nearly fully bridged (scores 4 and 5) compared to unmodified BMSC scores (Fig. 7C). A Kruskal-Wallis analysis of variance by ranks demonstrates that the bridging score distribution for Runx2-treated defects is statistically different compared to that for BMSC-treated defects ($p < 0.05$).

Discussion

This study examined the effects of Runx2 expression in BMSCs on the repair of critical-sized segmental defects in rat femurs. Compared to scaffolds containing unmodified BMSCs, scaffolds alone, or empty defects, bone repair was accelerated in defects treated with Runx2-engineered BMSCs. At both 4 and 8 weeks postsurgery, quantitative

FIG. 5. FTIR spectra demonstrate that the chemical composition of the repair tissue for cell-treated defects is similar to that of native bone. Bands characteristic of biologic hydroxyapatite, namely, a small carbonate peak at $855\text{--}890\text{ cm}^{-1}$, a broad phosphate peak at $900\text{--}1200\text{ cm}^{-1}$, and a phosphate doublet at $525\text{--}625\text{ cm}^{-1}$, are present in Runx2-modified and unmodified BMSC-treated defects, as well as native bone. Cell-free scaffold spectra contain peaks that are shifted compared to native bone, and empty defects do not contain characteristic peaks.

FIG. 6. Mechanical properties of repaired segmental defects. **(A)** Representative torque–displacement curve for Runx2-treated defects. The curve was plotted as the 20° moving average. **(B)** Despite differences in bone volume between Runx2-treated and BMSC-treated defects, maximum torque is not significantly different. (Empty $n=5$, PCL $n=6$, BMSC $n=9$, Runx2 $n=8$). **(C)** Pooled cell-treated groups (Runx2 and BMSC) have significantly greater strength than empty defects (*Different from empty defect, $p < 0.05$). Error bars represent standard error of the mean.

microCT analysis showed significantly more bone formation in defects treated with Runx2-modified BMSCs compared to unmodified BMSCs, indicating accelerated healing due to Runx2 treatment. At 12 weeks postsurgery, no differences in bone formation between unmodified and Runx2-modified BMSCs were present, probably due to the inherent osteogenic capabilities of this cell type. At all time points, cell-free and empty defects showed negligible bone formation. Taken together, this study demonstrates that Runx2 accelerates healing of large bone defects *in vivo*.

Runx2 has been described as a molecular switch for osteoblastic differentiation,⁴³ yet few studies have examined the potential use of Runx2 in healing critical-sized bone defects. Three previous critical-sized orthotopic analyses of Runx2-modified BMSCs have been performed, all focusing

on calvarial defects.^{40,56,57} Although the cranial defect model provides relevant information regarding the healing of craniofacial defects, conflicting evidence regarding the role of periosteum, dura mater, and surrounding healthy bone tissue in cranial defect healing makes separation of host response from treatment effect difficult in a cranial model.⁵⁸ In particular, dura mater has been shown to have a significant effect on cranial defect healing.⁷³ To our knowledge, no other study to date has examined the use of Runx2 as a gene therapy strategy for the healing of critical-sized segmental defects in long bones. The present study examined Runx2 treatment in a segmental defect model, where contributions from the dura mater are not present, making this a more rigorous test bed for the healing of large bone defects.

FIG. 7. Maximum torque is correlated with bone volume and extent of defect bridging. **(A)** Bridging score criteria and representative X-ray images of bridging scores. **(B)** Samples with higher bridging scores (shown in the legend) are shifted to the upper right of a bone volume versus maximum torque correlation graph. **(C)** The distribution of bridging scores for Runx2-treated defects is right shifted compared to that for BMSC-treated defects ($p < 0.05$). Color images available online at www.liebertonline.com/ten.

In addition to differences in anatomical location between this study and other orthotopic studies of Runx2, factors such as species, time points, and scaffold types also varied between the studies. Zheng *et al.* implanted adenovirally transduced Runx2-BMSCs on collagen sponges for 4 weeks in BALB/c mice. Using manual segmentation of radiographic images, they found significantly more bone in defects treated with Runx2-transduced cells over unmodified cells, cell-free scaffolds, and empty defects.⁵⁶ Zhao *et al.* used adenoviral transduction to deliver Runx2 and LacZ (control) to BMSCs. Cells were implanted on gelatin sponges for 7 weeks in C57BL/6 mice, and CT quantification showed more bone in defects treated with Runx2 over LacZ. However, no cell-free or empty defect controls were included in this study.⁵⁷ Our group has also previously reported on Runx2 treatment of cranial defects. We have investigated BMSCs retrovirally transduced with Runx2 and implanted on PCL scaffolds for 4 weeks in the rat calvarium. MicroCT analysis showed more bone in Runx2-treated defects, compared to unmodified controls and empty defects, after a 21-day pre-culture period. However, due to the open pore structure of our PCL scaffolds, cell-free scaffolds performed as well as

Runx2-engineered cell-loaded scaffolds in this study.⁴⁰ Given these differences, all three previous orthotopic studies comparing Runx2-transduced BMSCs to unmodified or LacZ-transduced BMSCs showed a significant increase in bone formation due to Runx2 treatment, in agreement with the present study.

Although Runx2-modified BMSCs accelerated bone repair in critical-sized defects in this study, unmodified BMSCs eventually produced equivalent amounts of bone, pointing to the inherent ability of BMSCs to mineralize bone defects. Many studies have demonstrated successful healing of bone defects using unmodified BMSCs in preclinical trials,^{20–22} and recently, a pilot study of the implantation of autologous human BMSCs for repair of large bone defects in humans was reported by Marcacci *et al.* This study included four patients with large bone diaphysis defects, for which previous treatment with conventional surgical therapies had failed. Each patient underwent a BMSC harvest from the iliac crest, and the isolated cells were then cultured and seeded onto hydroxyapatite scaffolds custom made to fit the size and shape of the defects. For patients 1, 2, and 4, complete consolidation between the implant and host bone was ra-

diographically evident between 5 and 7 months postsurgery, at which time external fixation devices were removed. For these patients, limb function was gradually regained within 8 months postsurgery. For patient 3, whose injury was more complex and involved the elbow joint, a custom-made cast was fitted over the defect after removal of the Ilizarov apparatus at 8 months postsurgery. This patient recovered limb function after 16–24 months postsurgery. For all patients, a 6–7-year follow-up revealed that stable bone–implant integration was maintained.²⁴ The success of this study makes treatment with autologous BMSCs an attractive option for patients with large bone defects. However, a reduction in the time needed for external fixation is desirable, making the accelerated healing strategy presented in the current study relevant for clinical application.

The success of unmodified BMSCs for healing critical-sized defects in other preclinical studies may largely depend on the scaffold type used to deliver the cells. For example, all of the aforementioned studies that demonstrate successful healing (i.e., complete bridging) of segmental defects when treated with unmodified BMSCs employed the use of a hydroxyapatite scaffold to deliver the cells to the defect site. The osteoconductive properties of hydroxyapatite have been reviewed elsewhere.⁷⁴ In brief, calcium phosphate ceramics demonstrate bioactivity and osteoconductivity leading to rapid and strong osseointegration with host bone tissue when implanted into an orthotopic site. In fact, implantation of HA alone (without cells) has been shown to heal osteotomy defects in human patients.⁷⁵ In the present study, the use of a polymer scaffold, which is not inherently osteoconductive, in combination with the rigorous 8-mm segmental defect test bed, may explain why defects treated with unmodified BMSCs were not fully healed after 12 weeks. This result is in agreement with other studies that also show nonunion of segmental defects treated with unmodified BMSCs when a nonceramic scaffold is the delivery vehicle.^{76,77} Recent evidence suggests that the modification of PCL scaffolds with hydroxyapatite nanoparticles increases the bone forming response of cells seeded on the scaffolds.⁷⁸ In the present study, the rationale behind including a collagen matrix within PCL scaffolds was to increase the therapeutic load of cells delivered the defect site. It is possible that incorporation of an osteoconductive material into our scaffolds would further enhance the cellular response.⁷⁹

A distinguishing aspect of this study over many reports of segmental defect healing is the use of mechanical testing to assess functionality of the defects. Many studies that report successful healing of bone defects rely solely on a combination of X-ray analysis and histological evaluation as a measure of defect healing. However, this approach does not provide functional information, which is necessary to fully evaluate the success of a given treatment strategy for bone healing.⁸⁰ Of the few groups that report mechanical testing analysis, the most common test method is torsion.^{36,64,81–84} The current study demonstrates the importance of including mechanical testing analysis to evaluate bone healing because; in this case, the significant differences in bone volume measured by microCT did not translate to significant differences in torsional strength between Runx2- and BMSC-treated defects at 12 weeks postimplantation. However, we did observe that samples with greater bridging scores generally

had greater strength than samples with lower bridging scores. Longer time points may be necessary to fully evaluate the effects of gene- and cell-based therapies for bone repair in this model. The present results argue that functional mechanical evaluation of bone defects should be used in conjunction with other methods for complete analysis of both bone mass and bone quality.

In summary, we have demonstrated accelerated bone healing in critical-sized femoral defects due to treatment with Runx2-modified BMSCs delivered on synthetic polymer scaffolds. Although Runx2 modification did not enhance defect bridging or biomechanical function after 12 weeks compared to unmodified BMSCs, this genetic modification strategy specifically upregulates osteoblastic differentiation, thereby offering an accelerated means of bone formation with the potential to shorten healing time for large bone defects in humans. With further development, the use of Runx2 as a gene therapy treatment for large bone defects could become a valuable strategy for the acceleration of bone healing, reducing recovery time and the need for external fixation.

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Disclosure Statement

No competing financial interests exist.

References

1. Bucholz, R.W. Nonallograft osteoconductive bone graft substitutes. *Clin Orthop Relat Res* **395**, 44, 2002.
2. Kretlow, J.D., and Mikos, A.G. Review: mineralization of synthetic polymer scaffolds for bone tissue engineering. *Tissue Eng* **13**, 927, 2007.
3. Rawashdeh, M.A., and Telfah, H. Secondary alveolar bone grafting: the dilemma of donor site selection and morbidity. *Br J Oral Maxillofac Surg* **46**, 665, 2008.
4. Gottfried, O.N., and Dailey, A.T. Mesenchymal stem cell and gene therapies for spinal fusion. *Neurosurgery* **63**, 380, 2008.
5. Mankin, H.J., Hornicek, F.J., and Raskin, K.A. Infection in massive bone allografts. *Clin Orthop Relat Res* **432**, 210, 2005.
6. Sorger, J.I., Hornicek, F.J., Zavatta, M., Menzner, J.P., Gebhardt, M.C., Tomford, W.W., and Mankin, H.J. Allograft fractures revisited. *Clin Orthop Relat Res* **382**, 66, 2001.
7. Ito, H., Koefoed, M., Tiyyapatanaputi, P., Gromov, K., Goater, J.J., Carmouche, J., Zhang, X., Rubery, P.T., Rabinowitz, J., Samulski, R.J., Nakamura, T., Soballe, K., O'Keefe, R.J., Boyce, B.F., and Schwarz, E.M. Remodeling of cortical bone allografts mediated by adherent rAAV-RANKL and VEGF gene therapy. *Nat Med* **11**, 291, 2005.

8. Cancedda, R., Giannoni, P., and Mastrogiacomo, M. A tissue engineering approach to bone repair in large animal models and in clinical practice. *Biomaterials* **28**, 4240, 2007.
9. Awad, H.A., Zhang, X., Reynolds, D.G., Guldberg, R.E., O'Keefe, R.J., and Schwarz, E.M. Recent advances in gene delivery for structural bone allografts. *Tissue Eng* **13**, 1973, 2007.
10. Kimelman, N., Pelled, G., Helm, G.A., Huard, J., Schwarz, E.M., and Gazit, D. Review: gene- and stem cell-based therapeutics for bone regeneration and repair. *Tissue Eng* **13**, 1135, 2007.
11. Hutmacher, D.W., and García, A.J. Scaffold-based bone engineering by using genetically modified cells. *Gene* **347**, 1, 2005.
12. Einhorn, T.A. The cell and molecular biology of fracture healing. *Clin Orthop Relat Res* (**355 Suppl**), S7, 1998.
13. Smiler, D., and Soltan, M. Bone marrow aspiration: technique, grafts, and reports. *Implant Dent* **15**, 229, 2006.
14. Muschler, G.F., Boehm, C., and Easley, K. Aspiration to obtain osteoblast progenitor cells from human bone marrow: the influence of aspiration volume. *J Bone Joint Surg Am* **79**, 1699, 1997.
15. Ishaug, S.L., Crane, G.M., Miller, M.J., Yasko, A.W., Yaszemski, M.J., and Mikos, A.G. Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. *J Biomed Mater Res* **36**, 17, 1997.
16. Cartmell, S., Huynh, K., Lin, A., Nagaraja, S., and Guldberg, R. Quantitative microcomputed tomography analysis of mineralization within three-dimensional scaffolds *in vitro*. *J Biomed Mater Res A* **69**, 97, 2004.
17. Goshima, J., Goldberg, V.M., and Caplan, A.I. Osteogenic potential of culture-expanded rat marrow cells as assayed *in vivo* with porous calcium phosphate ceramic. *Biomaterials* **12**, 253, 1991.
18. Krebsbach, P.H., Kuznetsov, S.A., Satomura, K., Emmons, R.V., Rowe, D.W., and Robey, P.G. Bone formation *in vivo*: comparison of osteogenesis by transplanted mouse and human marrow stromal fibroblasts. *Transplantation* **63**, 1059, 1997.
19. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143, 1999.
20. Bruder, S.P., Kraus, K.H., Goldberg, V.M., and Kadiyala, S. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. *J Bone Joint Surg Am* **80**, 985, 1998.
21. Kon, E., Muraglia, A., Corsi, A., Bianco, P., Marcacci, M., Martin, I., Boyde, A., Ruspanini, I., Chistolini, P., Rocca, M., Giardino, R., Cancedda, R., and Quarto, R. Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones. *J Biomed Mater Res* **49**, 328, 2000.
22. Petite, H., Viateau, V., Bensaid, W., Meunier, A., de Pollak, C., Bourguignon, M., Oudina, K., Sedel, L., and Guillemin, G. Tissue-engineered bone regeneration. *Nat Biotechnol* **18**, 959, 2000.
23. Werntz, J.R., Lane, J.M., Burstein, A.H., Justin, R., Klein, R., and Tomin, E. Qualitative and quantitative analysis of orthotopic bone regeneration by marrow. *J Orthop Res* **14**, 85, 1996.
24. Marcacci, M., Kon, E., Moukhachev, V., Lavroukov, A., Kutepov, S., Quarto, R., Mastrogiacomo, M., and Cancedda, R. Stem cells associated with macroporous bioceramics for long bone repair: 6- to 7-year outcome of a pilot clinical study. *Tissue Eng* **13**, 947, 2007.
25. Quarto, R., Mastrogiacomo, M., Cancedda, R., Kutepov, S., Mukhachev, V., Lavroukov, A., Kon, E., and Marcacci, M. Repair of large bone defects with the use of autologous bone marrow stromal cells. *N Engl J Med* **344**, 385, 2001.
26. Banfi, A., Muraglia, A., Dozin, B., Mastrogiacomo, M., Cancedda, R., and Quarto, R. Proliferation kinetics and differentiation potential of *ex vivo* expanded human bone marrow stromal cells: implications for their use in cell therapy. *Exp Hematol* **28**, 707, 2000.
27. Derubeis, A.R., and Cancedda, R. Bone marrow stromal cells (BMSCs) in bone engineering: limitations and recent advances. *Ann Biomed Eng* **32**, 160, 2004.
28. Yeon Lim, J., Jeun, S.S., Lee, K.J., Oh, J.H., Kim, S.M., Park, S.I., Jeong, C.H., and Kang, S.G. Multiple stem cell traits of expanded rat bone marrow stromal cells. *Exp Neurol* **199**, 416, 2006.
29. Mendes, S.C., Tibbe, J.M., Veenhof, M., Bakker, K., Both, S., Platenburg, P.P., Oner, F.C., de Bruijn, J.D., and van Blitterswijk, C.A. Bone tissue-engineered implants using human bone marrow stromal cells: effect of culture conditions and donor age. *Tissue Eng* **8**, 911, 2002.
30. Phinney, D.G., Kopen, G., Righter, W., Webster, S., Tremain, N., and Prockop, D.J. Donor variation in the growth properties and osteogenic potential of human marrow stromal cells. *J Cell Biochem* **75**, 424, 1999.
31. Kretlow, J.D., Jin, Y.Q., Liu, W., Zhang, W.J., Hong, T.H., Zhou, G., Baggett, L.S., Mikos, A.G., and Cao, Y. Donor age and cell passage affects differentiation potential of murine bone marrow-derived stem cells. *BMC Cell Biol* **9**, 60, 2008.
32. Hanada, K., Dennis, J.E., and Caplan, A.I. Stimulatory effects of basic fibroblast growth factor and bone morphogenetic protein-2 on osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells. *J Bone Miner Res* **12**, 1606, 1997.
33. Huang, Y.C., Kaigler, D., Rice, K.G., Krebsbach, P.H., and Mooney, D.J. Combined angiogenic and osteogenic factor delivery enhances bone marrow stromal cell-driven bone regeneration. *J Bone Miner Res* **20**, 848, 2005.
34. Gazit, D., Turgeman, G., Kelley, P., Wang, E., Jalenak, M., Zilberman, Y., and Moutsatsos, I. Engineered pluripotent mesenchymal cells integrate and differentiate in regenerating bone: a novel cell-mediated gene therapy. *J Gene Med* **1**, 121, 1999.
35. Edgar, C.M., Chakravarthy, V., Barnes, G., Kakar, S., Gerstenfeld, L.C., and Einhorn, T.A. Autogenous regulation of a network of bone morphogenetic proteins (BMPs) mediates the osteogenic differentiation in murine marrow stromal cells. *Bone* **40**, 1389, 2007.
36. Hsu, W.K., Sugiyama, O., Park, S.H., Conduah, A., Feeley, B.T., Liu, N.Q., Krenek, L., Virk, M.S., An, D.S., Chen, I.S., and Lieberman, J.R. Lentiviral-mediated BMP-2 gene transfer enhances healing of segmental femoral defects in rats. *Bone* **40**, 931, 2007.
37. Bishop, G.B., and Einhorn, T.A. Current and future clinical applications of bone morphogenetic proteins in orthopaedic trauma surgery. *Int Orthop* **31**, 721, 2007.
38. Uludag, H., D'Augusta, D., Golden, J., Li, J., Timony, G., Riedel, R., and Wozney, J.M. Implantation of recombinant human bone morphogenetic proteins with biomaterial carriers: a correlation between protein pharmacokinetics and osteoinduction in the rat ectopic model. *J Biomed Mater Res* **50**, 227, 2000.

39. Cahill, K.S., Chi, J.H., Day, A., and Claus, E.B. Prevalence, complications, and hospital charges associated with use of bone-morphogenetic proteins in spinal fusion procedures. *JAMA* **302**, 58, 2009.
40. Byers, B.A., Guldberg, R.E., Hutmacher, D.W., and García, A.J. Effects of Runx2 genetic engineering and *in vitro* maturation of tissue-engineered constructs on the repair of critical size bone defects. *J Biomed Mater Res A* **76**, 646, 2006.
41. Gersbach, C.A., Le Doux, J.M., Guldberg, R.E., and García, A.J. Inducible regulation of Runx2-stimulated osteogenesis. *Gene Ther* **13**, 873, 2006.
42. Phillips, J.E., Guldberg, R.E., and García, A.J. Dermal fibroblasts genetically modified to express Runx2/Cbfa1 as a mineralizing cell source for bone tissue engineering. *Tissue Eng* **13**, 2029, 2007.
43. Ducy, P. Cbfa1: a molecular switch in osteoblast biology. *Dev Dyn* **219**, 461, 2000.
44. Ducy, P., Starbuck, M., Priemel, M., Shen, J., Pinero, G., Geoffroy, V., Amling, M., and Karsenty, G. A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. *Genes Dev* **13**, 1025, 1999.
45. Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R.T., Gao, Y.H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S., and Kishimoto, T. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* **89**, 755, 1997.
46. Otto, F., Thornell, A.P., Crompton, T., Denzel, A., Gilmour, K.C., Rosewell, I.R., Stamp, G.W., Beddington, R.S., Mundlos, S., Olsen, B.R., Selby, P.B., and Owen, M.J. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* **89**, 765, 1997.
47. Mundlos, S., Otto, F., Mundlos, C., Mulliken, J.B., Aylsworth, A.S., Albright, S., Lindhout, D., Cole, W.G., Henn, W., Knoll, J.H., Owen, M.J., Mertelsmann, R., Zabel, B.U., and Olsen, B.R. Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. *Cell* **89**, 773, 1997.
48. Zhang, Y.W., Yasui, N., Kakazu, N., Abe, T., Takada, K., Imai, S., Sato, M., Nomura, S., Ochi, T., Okuzumi, S., Nogami, H., Nagai, T., Ohashi, H., and Ito, Y. PEBP2alphaA/CBFA1 mutations in Japanese cleidocranial dysplasia patients. *Gene* **244**, 21, 2000.
49. Lee, B., Thirunavukkarasu, K., Zhou, L., Pastore, L., Baldini, A., Hecht, J., Geoffroy, V., Ducy, P., and Karsenty, G. Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia. *Nat Genet* **16**, 307, 1997.
50. Shui, C., Spelsberg, T.C., Riggs, B.L., and Khosla, S. Changes in Runx2/Cbfa1 expression and activity during osteoblastic differentiation of human bone marrow stromal cells. *J Bone Miner Res* **18**, 213, 2003.
51. Xiao, Z.S., Hinson, T.K., and Quarles, L.D. Cbfa1 isoform overexpression upregulates osteocalcin gene expression in non-osteoblastic and pre-osteoblastic cells. *J Cell Biochem* **74**, 596, 1999.
52. Byers, B.A., Pavlath, G.K., Murphy, T.J., Karsenty, G., and García, A.J. Cell-type-dependent up-regulation of *in vitro* mineralization after overexpression of the osteoblast-specific transcription factor Runx2/Cbfa1. *J Bone Miner Res* **17**, 1931, 2002.
53. Gersbach, C.A., Byers, B.A., Pavlath, G.K., and García, A.J. Runx2/Cbfa1 stimulates transdifferentiation of primary skeletal myoblasts into a mineralizing osteoblastic phenotype. *Exp Cell Res* **300**, 406, 2004.
54. Byers, B., Guldberg, R., and García, A. Synergy between genetic and tissue engineering: Runx2 overexpression and *in vitro* construct development enhance *in vivo* mineralization. *Tissue Eng* **10**, 1757, 2004.
55. Zhao, Z., Zhao, M., Xiao, G., and Franceschi, R.T. Gene transfer of the Runx2 transcription factor enhances osteogenic activity of bone marrow stromal cells *in vitro* and *in vivo*. *Mol Ther* **12**, 247, 2005.
56. Zheng, H., Guo, Z., Ma, Q., Jia, H., and Dang, G. Cbfa1/osf2 transduced bone marrow stromal cells facilitate bone formation *in vitro* and *in vivo*. *Calcif Tissue Int* **74**, 194, 2004.
57. Zhao, Z., Wang, Z., Ge, C., Krebsbach, P., and Franceschi, R.T. Healing cranial defects with AdRunx2-transduced marrow stromal cells. *J Dent Res* **86**, 1207, 2007.
58. Aalami, O.O., Nacamuli, R.P., and Longaker, M.T. Roles of periosteum, dura, and adjacent bone on healing of cranial osteonecrosis—discussion. *J Craniofac Surg* **14**, 380, 2003.
59. Javazon, E.H., Colter, D.C., Schwarz, E.J., and Prockop, D.J. Rat marrow stromal cells are more sensitive to plating density and expand more rapidly from single-cell-derived colonies than human marrow stromal cells. *Stem Cells* **19**, 219, 2001.
60. Byers, B.A., and García, A.J. Exogenous Runx2 expression enhances *in vitro* osteoblastic differentiation and mineralization in primary bone marrow stromal cells. *Tissue Eng* **10**, 1623, 2004.
61. Porter, B.D., Lin, A.S., Peister, A., Hutmacher, D., and Guldberg, R.E. Noninvasive image analysis of 3D construct mineralization in a perfusion bioreactor. *Biomaterials* **28**, 2525, 2007.
62. Phillips, J.E., Hutmacher, D.W., Guldberg, R.E., and García, A.J. Mineralization capacity of Runx2/Cbfa1-genetically engineered fibroblasts is scaffold dependent. *Biomaterials* **27**, 5535, 2006.
63. Gersbach, C.A., Byers, B.A., Pavlath, G.K., Guldberg, R.E., and García, A.J. Runx2/Cbfa1-genetically engineered skeletal myoblasts mineralize collagen scaffolds *in vitro*. *Bio-technol Bioeng* **88**, 369, 2004.
64. Oest, M.E., Dupont, K.M., Kong, H.J., Mooney, D.J., and Guldberg, R.E. Quantitative assessment of scaffold and growth factor-mediated repair of critically sized bone defects. *J Orthop Res* **25**, 941, 2007.
65. Wojtowicz, A.M., Shekaran, A., Oest, M.E., Dupont, K.M., Templeman, K.L., Hutmacher, D.W., Guldberg, R.E., and García, A.J. Coating of biomaterial scaffolds with the collagen-mimetic peptide GFOGER for bone defect repair. *Biomaterials* **31**, 2574, 2010.
66. Reyes, C.D., Petrie, T.A., Burns, K.L., Schwartz, Z., and García, A.J. Biomolecular surface coating to enhance orthopaedic tissue healing and integration. *Biomaterials* **28**, 3228, 2007.
67. Hoerl, A., and Kennard, R. Ridge regression: applications to nonorthogonal problems. *Technometrics* **12**, 69, 1970.
68. Hoerl, A., and Kennard, R. Ridge regression: biased estimation for nonorthogonal problems. *Technometrics* **12**, 55, 1970.
69. Overton, W.R. Modified histogram subtraction technique for analysis of flow cytometry data. *Cytometry* **9**, 619, 1988.
70. Sanderson, C., and Bachus, K.N. Staining technique to differentiate mineralized and demineralized bone in ground sections. *J Histotechnol* **20**, 119, 1997.
71. Paschalis, E.P., Betts, F., DiCarlo, E., Mendelsohn, R., and Boskey, A.L. FTIR microspectroscopic analysis of normal human cortical and trabecular bone. *Calcif Tissue Int* **61**, 480, 1997.

72. Bonewald, L.F., Harris, S.E., Rosser, J., Dallas, M.R., Dallas, S.L., Camacho, N.P., Boyan, B., and Boskey, A. von Kossa staining alone is not sufficient to confirm that mineralization *in vitro* represents bone formation. *Calcif Tissue Int* **72**, 537, 2003.
73. Ozerdem, O.R., Anlatıcı, R., Bahar, T., Kayaselcuk, F., Barutcu, O., Tuncer, I., and Sen, O. Roles of periosteum, dura, and adjacent bone on healing of cranial osteonecrosis. *J Craniofac Surg* **14**, 371, 2003.
74. LeGeros, R.Z. Properties of osteoconductive biomaterials: calcium phosphates. *Clin Orthop Relat Res* **395**, 81, 2002.
75. Meyer, S., Floerkemeier, T., and Windhagen, H. Histological osseointegration of a calciumphosphate bone substitute material in patients. *Biomed Mater Eng* **17**, 347, 2007.
76. Fialkov, J.A., Holy, C.E., Shoichet, M.S., and Davies, J.E. *In vivo* bone engineering in a rabbit femur. *J Craniofac Surg* **14**, 324, 2003.
77. Turgeman, G., Pittman, D.D., Muller, R., Kurkalli, B.G., Zhou, S., Pelled, G., Peyser, A., Zilberman, Y., Moutsatsos, I.K., and Gazit, D. Engineered human mesenchymal stem cells: a novel platform for skeletal cell mediated gene therapy. *J Gene Med* **3**, 240, 2001.
78. Wutticharoenmongkol, P., Pavasant, P., and Supaphol, P. Osteoblastic phenotype expression of MC3T3-E1 cultured on electrospun polycaprolactone fiber mats filled with hydroxyapatite nanoparticles. *Biomacromolecules* **8**, 2602, 2007.
79. Hutmacher, D.W., Schantz, J.T., Lam, C.X., Tan, K.C., and Lim, T.C. State of the art and future directions of scaffold-based bone engineering from a biomaterials perspective. *J Tissue Eng Regen Med* **1**, 245, 2007.
80. Liebschner, M.A. Biomechanical considerations of animal models used in tissue engineering of bone. *Biomaterials* **25**, 1697, 2004.
81. Cook, S.D., Baffes, G.C., Wolfe, M.W., Sampath, T.K., and Rueger, D.C. Recombinant human bone morphogenetic protein-7 induces healing in a canine long-bone segmental defect model. *Clin Orthop Relat Res* **301**, 302, 1994.
82. Cook, S.D., Baffes, G.C., Wolfe, M.W., Sampath, T.K., Rueger, D.C., and Whitecloud, T.S., 3rd. The effect of recombinant human osteogenic protein-1 on healing of large segmental bone defects. *J Bone Joint Surg Am* **76**, 827, 1994.
83. Cook, S.D., Wolfe, M.W., Salkeld, S.L., and Rueger, D.C. Effect of recombinant human osteogenic protein-1 on healing of segmental defects in non-human primates. *J Bone Joint Surg Am* **77**, 734, 1995.
84. Rai, B., Oest, M.E., Dupont, K.M., Ho, K.H., Teoh, S.H., and Guldborg, R.E. Combination of platelet-rich plasma with polycaprolactone-tricalcium phosphate scaffolds for segmental bone defect repair. *J Biomed Mater Res A* **81**, 888, 2007.

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