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ORIGINAL ARTICLE

Regional Gene Therapy with Transduced Human Cells: The Influence of "Cell Dose" on Bone Repair

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Regional gene therapy using a lentiviral vector containing the *BMP-2* complementary DNA (cDNA) has been shown to heal critical-sized bone defects in rodent models. An appropriate ''cellular dose'' needs to be defined for eventual translation into human trials. The purpose of this study was to evaluate bone defect healing potential and quality using three different doses of transduced human bone marrow cells (HBMCs). HBMCs were transduced with a lentiviral vector containing either *BMP-2* or green fluorescent protein (GFP). All cells were loaded onto compression-resistant matrices and implanted in the bone defect of athymic rats. Treatment groups included femoral defects that were treated with a low-dose (1×10^6 cells), standard-dose (5×10^6 cells), and high-dose (1.5×10^7 cells) HBMCs transduced with lentiviral vector containing *BMP-2* cDNA. The three control groups were bone defects treated with HBMCs that were either nontransduced or transduced with vector containing GFP. All animals were sacrificed at 12 weeks. The bone formed in each defect was evaluated with plain radiographs, microcomputed tomography (microCT), histomorphometric analysis, and biomechanical testing. Bone defects treated with higher doses of BMP-2-producing cells were more likely to have healed (6/14 of the low-dose group; 12/14 of the standard-dose group; 14/14 of the high-dose group; $\chi^2(2)=15.501$, $p<0.001$). None of the bone defects in the control groups had healed. Bone defects treated with high dose and standard dose of BMP-2-producing cells consistently outperformed those treated with a low dose in terms of bone formation, as assessed by microCT and histomorphometry, and biomechanical parameters. However, statistical significance was only seen between defects treated with high dose and low dose. Larger doses of BMP-2-producing cells were associated with a higher likelihood of forming heterotopic ossification. Femurs treated with a standard- and high-dose BMP-2-producing cells demonstrated similar healing and biomechanical properties. Increased doses of BMP-2 delivered through higher cell doses have the potential to heal large bone defects. Adapting regional gene therapy for use in humans will require a balance between promoting bone repair and limiting heterotopic ossification.

Keywords: gene therapy, nonunion, critical bone defect, lentiviral vector, BMP-2

Impact Statement

Critical bone loss may result from complex traumatic bone injury (i.e., open fracture or blast injury), revision total joint arthroplasty, and spine pseudoarthrosis. This is a challenging clinical problem to treat and regional gene therapy is an innovative means of addressing it. This study provides information regarding the quantity of cells or ''cell dose'' of transduced cells needed to treat a critical-sized bone defect in a rat model. This information may be extrapolated for use in humans in future trials.

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Introduction

COMPLEX TRAUMATIC BONE injury (i.e., open fracture or blast injury), revision total joint arthroplasty, and spine pseudoarthrosis are among the most difficult bone loss scenarios for which there is no consistently satisfactory clinical solution.[1] Autologous bone graft is considered the gold standard for graft material in such scenarios because it is osteoconductive, osteoinductive, osteogenic, and has minimal risk for an immunologic reaction.[1–3] However, bone graft has critical limitations, which include donor-site morbidity and there is only a limited quantity available.[2,4]

Recombinant human bone morphogenetic proteins (rhBMPs) have been one of the most studied osteobiologic alternatives to autograft. rhBMP-2 is FDA approved for lumbar spine fusion, open tibial fractures, and maxillofacial augmentation.[5–7] While early clinical results were very promising, further studies this past decade have brought concern over rhBMP-2's potential adverse effects, including heterotopic ossification, soft tissue swelling, and seroma formation.[8–10] It has been hypothesized that these side effects may be secondary to the supraphysiologic doses of rhBMP-2 required to induce adequate bone formation and the rapid release of the protein from the collagen carrier.[10–13] There is also evidence to suggest that this rapid release of the growth factor has a negative impact on bone repair since studies have demonstrated that a more prolonged release of growth factor is desirable and improves bone formation.[14,15]

Ex-vivo regional gene therapy with a lentiviral vector may provide a more effective delivery system for BMP-2 by not only avoiding the donor-site morbidity and quantity limitations of autologous grafts but also through a prolonged and consistent release of growth factors.[16] In a clinical scenario, autologous human bone marrow would be harvested from the iliac crest and expanded in tissue culture. The cultured cells would be transduced with lentiviral vector containing the complementary DNA (cDNA) for *BMP-2*.[16–18] We have demonstrated in a prior study that *ex-vivo* lentiviral gene therapy in a murine model can produce BMP-2 for 3 months.[19] However, duration of protein release alone may not be the only important factor as there is evidence to support the existence of a critical threshold of BMP-2 production for healing to occur.[18] We previously demonstrated that implanting 1×10^6 transduced rat bone marrow stromal cells (RBMSCs) into a rat femoral defect resulted in poorer defect-site healing than those implanted with 5×10^6 transduced RBMSCs.[18] The number of cells implanted also correlated with the amount of *in-vitro* BMP-2 production. The defects treated with more cells produced more BMP-2 and led to better healing. Consequently, mounting an adequate osteoinductive response is influenced by the number of cells implanted at the defect site and the amount of BMP-2 produced by those cells.

The ultimate goal of our *ex vivo* gene therapy strategy is to implant transduced human cells into humans to treat large bone defects. We already demonstrated that *ex-vivo* gene therapy with human bone marrow cells (HBMCs) is viable in an athymic rat model.[17] However, the size of a bony defect in humans will be much larger than the one in our rat model and will necessitate a much larger "cell dose." These larger doses may be associated with similar adverse events that were seen with supraphysiologic doses of rhBMP-2 as described above.

The purpose of the present study was to compare the bone formation in an athymic rat model with implantation of three different human cell doses: 1×10^6, 5×10^6, and 1.5×10^7 HBMCs. The largest cell dose was chosen to determine if there would be a proportionate increase in bone formation and quality, and to see if there would be an increased rate of heterotopic ossification from such a large dose. We hypothesized that with implantation of larger cellular doses of BMP-2-producing cells, more bone formation and better-quality bone repair would be seen.

Materials and Methods

Isolation and culture of HBMCs

HBMCs were harvested in a sterile fashion from the intramedullary femoral canal of healthy patients undergoing primary total hip arthroplasty (THA) for osteoarthritis of the hip at our institution. Institutional Review Board approval was obtained before collection of the deidentified bone marrow samples. Twenty-six healthy patients (14 male, 12 female) with an average age 58.6 ± 1.4 years were included. Exclusion criteria included patients with significant comorbidities, known history of human immunodeficiency viruses infection, hepatitis B or hepatitis C, or taking immunosuppressive or disease-modifying agents. The research team members were blinded to patient information other than sex and age.

HBMCs were harvested and cultured as outlined previously.[17] Briefly, bone marrow was transferred to sterile 50-cc tubes along with phosphate-buffered saline (PBS) in a 1:1 ratio. This suspension was layered on top of 20 mL of Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) and centrifuged at room temperature for 30 min at 400 *g*. The band of cells between the histopaque and plasma/PBS interface was aspirated to acquire the mononuclear cell fraction. These cells were treated with ACK lysing buffer (Lonza, Morristown, NJ), then washed twice and resuspended in Dulbecco's modified Eagle's medium (DMEM; Corning Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Gibco, Mexico). The isolated mononuclear cells were counted with an automated cell counter (Bio-Rad, Hercules, CA) using trypan blue. The freshly isolated mononuclear cells were plated at 40×10^6 cells/10 cm plate and expanded in tissue culture for five passages before transduction for use in the "two-step" approach according to previously established protocols.[20]

Viral transduction

Our previously established two-step transcriptional amplification (TSTA) lentiviral system overexpressing *BMP-2* (LV-TSTA-BMP-2) or green fluorescent protein (GFP; LV-TSTA-GFP) was used in this study.[21–23] Briefly, this TSTA system comprises two separate lentiviral vectors: the GAL4-VP16 transactivator vector (LV-RhMLV-GAL4-VP16) and the transgene expression vector encoding the gene of interest, in this case *BMP-2* or *eGFP* (LV-G5-BMP-2 or LV-G5-GFP). All lentiviral vectors were generated by transfecting 293T cells (American Type Culture Collection, Manassas, VA), as described in prior protocols.[21,24] The LV titers were determined by quantifying p24 protein in vector supernatant

using enzyme-linked immunosorbent assay (ELISA; Quantikine, R&D Systems, Minneapolis, MN). Only vectors with titers >0.50 TU/mL were used in the study.

HBMCs at passage 5 were plated in DMEM/10% FBS and transduced overnight with LV-RhMLV-GAL4-VP16 and LV-G5-BMP-2 at multiplicity of infection of 25/25 in the presence of 8 μg/mL polybrene. Following transduction, the cells were washed with PBS three times to remove any extracellular virus. Twenty-four hours after washing, the cells were harvested and, within 3 h, the cell pellet was implanted into the femoral defect rat model.

In vitro *BMP-2 production*

To determine BMP-2 production of our transduced cells, one plate of cells was set aside for *in vitro* testing. These cells were also transduced with LV-TSTA-BMP-2 overnight, washed with PBS and media, and then incubated for another 24-h period in fresh media. The cell culture supernatant was harvested 48 h after transduction. *In vitro* BMP-2 production was measured by ELISA in triplicate (Quantikine; R&D Systems, Minneapolis, MN). Results were then standardized by cell number and reported as nanograms of BMP-2 per 1×10^6 cells in a 24-h period. Nontransduced HBMCs were used as a negative control.

Animal model

To avoid an immune response to the human cells, nude rats were selected as the model. The study protocol was reviewed and approved by the university's Institutional Animal Care and Use Committee. A total of 57, 12–14-week-old male nude rats were used in this study. The animals were anesthetized and received buprenorphine SR immediately before incision. An anterolateral incision was made in the thigh, splitting the vastus lateralis and biceps femoris to expose the femur. Muscles were dissected off the femur and the periosteum was incised along the length of the diaphysis. A $23\times4\times4$ mm polyethylene four-hole plate (Findlay's Machine Shop, Los Angeles, CA) was securely placed on the femur using four 0.90-mm K-wires and further secured with two surgical steel cerclage wires proximally and distally. The critical-sized 6-mm mid-diaphyseal femoral defect was then created. A compression-resistant matrix consisting of collagen type I, hydroxyapatite, and β-tricalcium phosphate (Mastergraft strip; Medtronic, Minneapolis, MN) was then cut to size and loaded with HBMCs. A micropipette was used to load the HBMCs directly on top of the compression matrix. The matrix was allowed to soak up the cells for 3 min. The carrier and cells were implanted in the defect, and the wound was then closed with absorbable sutures. Antibiotics were added to the drinking water for 7 days postoperatively. After the surgery, animals were allowed to bear weight as tolerated.

Experimental groups

Athymic rats were randomly assigned to one of six groups, including: low-dose (1×10^6 cells) HBMCs transduced with LV-TSTA-BMP-2 (group I; $n=14$); standard-dose (5×10^6 cells) HBMCs transduced with LV-TSTA-BMP-2 (group II; $n=14$); high-dose (1.5×10^7 cells) HBMCs transduced with LV-TSTA-BMP-2 (group III; $n=14$); standard-dose (5×10^6 cells) HBMCs transduced with LV-TSTA-GFP (group IV; $n=5$); high-dose (1.5×10^7 cells) HBMCs transduced with LV-TSTA-GFP (group V; $n=5$); and high-dose (1.5×10^7 cells) HBMCs that were not transduced (group VI; $n=5$) (Table 1).

The standard dose of 5.0×10^6 cells was chosen because reliable bone defect-site healing was demonstrated in prior experiments with rat bone marrow cells (RBMCs) and in a pilot study using HBMCs (data not shown).[18] The low dose of 1.0×10^6 cells was selected because a low radiographic healing rate was seen in a prior study using the same dose of RBMCs.[18] The high dose of 1.5×10^7 cells was determined due to results from a pilot study, which demonstrated that this was the highest dose at which the cells completely saturated the matrix and no fluid leakage from the matrix was seen. Animals were euthanized at the 12-week postoperative time point. We then assessed bone healing with plain radiographs, microcomputed tomography (microCT), biomechanical testing, histologic (Hematoxylin and Eosin [H&E], Masson's Trichrome, and tartrate-resistant acid phosphatase [TRAP]) analysis, and histomorphometric analysis postmortem as described below. In the three treatment groups (groups I–III), the animals were randomized to undergo either histology/histomorphometry (five animals/group) or biomechanical testing (nine animals/group). In addition to routine husbandry care, the animals were examined regularly by laboratory staff for any wound dehiscence, pain, and soft tissue swelling throughout the 12-week postoperative period.

Radiographic evaluation

Plain radiographs were used to assess fixation, graft position, and bone healing at 4, 8, and 12 weeks postoperatively using an Ultrafocus 60 X-ray device (Faxitron Bioptics, Tucson, AZ). Using a previously established grading system, the 12-week radiographs were scored by three blinded observers (score of 0: no healing, score of 1: 0–25% healing, score of 2: 25–50% healing, score of 3: 50–75% healing, score of 4: 75–99% healing, and score of 5:

Table 1. The Six Study Groups Are Presented

Group	n	*Treatment*
I	14	Low-dose (1×10^6) HBMC/LV-TSTA-BMP2
II	14	Standard-dose (5×10^6) HBMC/LV-TSTA-BMP2
III	14	High-dose (1.5×10^7) HBMC/LV-TSTA-BMP2
IV	5	Standard-dose (5×10^6) HBMC/LV-TSTA-GFP
V	5	High-dose (1.5×10^7) HBMC/LV-TSTA-GFP
VI	5	High-dose (1.5×10^7) HBMC nontransduced

Experimental groups: group I defects were treated with a low dose of HBMCs transduced with a BMP2-encoding lentiviral vector, group II defects were treated with a standard dose of HBMCs transduced with a BMP2-encoding lentiviral vector, group III defects were treated with a high dose of HBMCs transduced with a BMP2-encoding lentiviral vector. Control groups: group IV defects were treated with a standard dose of HBMCs transduced with GFP-encoding lentiviral vector, group V defects were treated with a high dose of HBMCS transduced with GFP-encoding lentiviral vector, and group VI defects were treated with a high dose of HBMCs that were not transduced.

BMP2, bone morphogenetic protein-2; GFP, green fluorescent protein; HBMC, human bone marrow cell; LV, lentivirus; TSTA, two-step transcriptional amplification system.

100% healing).[17,25] A femoral defect was defined as healed when a bony bridge across both cortices of the defect was formed, restoring osseous continuity of the femur.

Microcomputed tomography

After reaching the 12-week postoperative timepoint, animals were euthanized. The experimental femurs were then harvested by careful removal of overlying soft tissue. Animals designated for biomechanical testing also had contralateral femurs harvested. The hardware used to fixate the experimental femur was removed in groups I–III but kept in place for groups IV–VI since the defects had not healed in these control groups. A microCT scan (μCT40; Scanco Medical, Bassersdorf, Switzerland) was completed on all experimental femurs to assess bone volume (BV) formation at the defect site.[21] Newly formed BV was separately calculated from the scaffold by applying hydroxyapatite-equivalent density thresholds of 375 mg/cm^3 for bone, and 750 mg/cm^3 for the scaffold. BV and total volume (TV) within the defect area were then calculated using Scanco Medical Bone Density Analysis software.

Histologic and histomorphometric analyses

After microCT imaging, five femurs in each group were processed for histologic and histomorphometric analyses. These specimens were fixed in 10% formalin, decalcified in 10% EDTA, then embedded in paraffin. Blocks were cut in the transverse and longitudinal planes as previously described.[21,26] These sections were stained with Masson's Trichrome, H&E and TRAP. Each section was assessed for bone formation and any signs of infection or inflammatory process. Bone formation within the femoral defect was evaluated with Bioquant analysis software (Bioquant Image Analysis, Nashville, TN) using a proximal and distal cut of Masson's Trichrome-stained sections. New bone within the region of interest, the defect, scaffold, and newly formed bone, was quantified, and bone area/tissue area (BA/TA) ratio was calculated for each end of the specimen, and then averaged for use in statistical analysis.

Biomechanical testing

Femora in groups I–III that were not processed for histological analysis were prepared for biomechanical testing using an established protocol.[17,18,27] Femora harvested from groups IV to VI did not undergo biomechanical testing because they failed to heal. The experimental and contralateral femurs were harvested from each rat. The specimens were wrapped in saline-soaked gauze and frozen at −20°C. Specimens were thawed on the morning of biomechanical testing. The proximal and distal ends of the femoral specimens were embedded in polymethylmethacrylate, where the longitudinal bone axis was centered with the axis of torsion. Torsional testing was performed by mounting each femur onto attachments on a rotating device (Mini Bionix; MTS, Minneapolis, MN). Each femur was then externally rotated, with the distal end rotating around the proximal end of the femur at 15° per minute until failure. Corresponding contralateral, intact femurs were used as controls. Torque-rotation curves were used to calculate peak torque and displacement, energy to failure, and torsional stiffness.

Statistical analysis

Statistical analysis was performed with IBM SPSS 23 software. All quantitative data are expressed as mean and standard error of the mean. *A priori* power analysis designed to detect differences in torsional failure between the two experimental groups required a sample size of nine animals for each group to achieve a power of 80% and a level of significance of 5%. Five animals per group were added for histologic and histomorphometric analyses to detect between-group differences with regard to BA/TA as previously described.[16,18] Fourteen animals were allocated to each experimental group (groups I–III) and 5 to each control group (groups IV–VI).

Shapiro–Wilk test was used to test for normality. Kruskal–Wallis test with Dunn *post hoc* analysis for pairwise comparisons were used to compare radiographic scores, microCT bone formation, histomorphometric bone formation, and biomechanical results across all groups. The distribution of every parameter was assessed by visual inspection of boxplots. One-way analysis of variance with Tukey's *post-hoc* test was used to compare *in vitro* BMP-2 production of cultured HBMC samples. Student's *t*-test and Mann-Whitney *U* test was used to compare the biomechanical results between the experimental femurs and corresponding contralateral, unoperated femurs. Significance level was set at 0.05.

Results

In vitro *BMP-2 production*

After HBMCs were harvested for *in vivo* implantation, HBMCs from groups I, II, and III (groups that were transduced with LV-TSTA-BMP-2 vector) were kept in culture for another 48 h. The supernatant was collected and the BMP-2 production was quantified through ELISA. Mean *in-vitro* BMP-2 production for groups I, II, and III were 83.0 ± 4.0, 86.7 ± 3.5, and 83.3 ± 4.4 ng/24 h/million cells, respectively. There was no significant difference in BMP-2 production by the transduced cells that were used in the different groups. A subgroup analysis of group I animals was completed to determine if there was a difference in *in-vitro* BMP-2 production by cells that were implanted between animals that went onto heal versus those that did not. There was a trend toward more BMP-2 production in those that healed but there was no significant difference between the corresponding *in-vitro* BMP-2 production of healed versus nonhealed animals (89.9 ± 5.9 vs. 77.8 ± 4.9 ng/24 h/million cells, respectively; $p = 0.14$).

Radiographic findings

Representative 8- and 12-week radiographs for each group are presented in Figure 1. Radiographic assessment of postoperative week 8 femurs demonstrated that none of the control groups (groups IV–VI) had healed while healed defects were noted in group I (5/14; 35.7%), group II (11/14; 78.6%), and group III (14/14; 100%). At 12 weeks, one additional femur had healed radiographically in group I (6/14; 42.9%) and one additional femur had healed radiographically in group II (12/14; 85.7%). Higher cell dosages of BMP-2-producing cells were significantly associated with an increased rate of healing ($\chi^2(2) = 15.501$, $p < 0.001$). A Kruskal–Wallis test was run to determine if there were

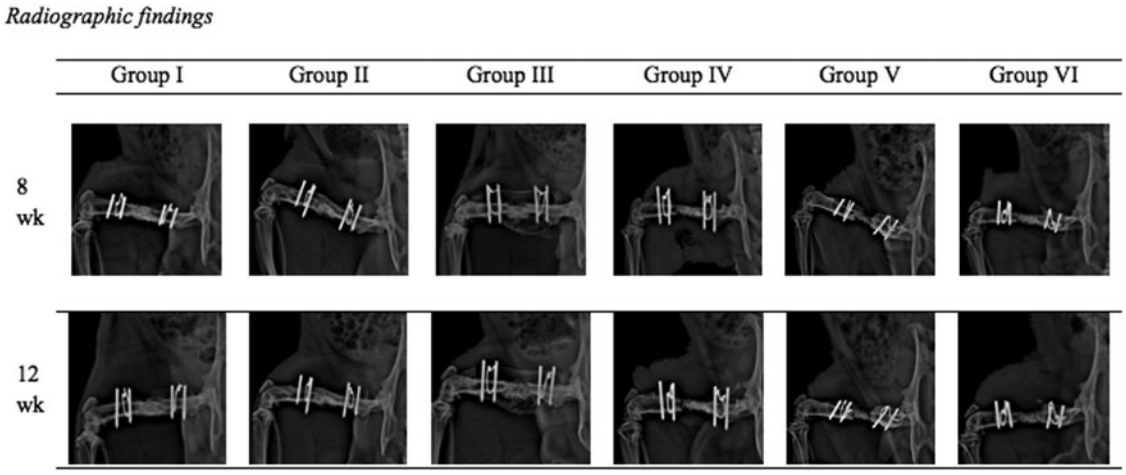

FIG. 1. Representative radiographic imaging of all six groups at 8 and 12 weeks. With increasing cell dosage, there was a corresponding increase in bone formation size at the defect sites. As expected, the control groups (IV, V, and VI) demonstrated minimal bone formation at the defect sites.

differences in radiographic scores between the six groups. *Post hoc* analysis revealed statistically significant differences in radiographic score mean ranks between group III and group I (43.82 vs. 23.68, respectively; $p = 0.013$). Group II radiographic score mean rank was not significantly different from that of either group I ($p = 0.48$) or group III ($p = 1.00$) (Fig. 2).

The presence of heterotopic ossification was also evaluated on the 12-week radiographs. Two distinct patterns of heterotopic ossification were observed based on location and amount of bone formation (Fig. 3). The first pattern involved thin wisps of bone that formed around the level of the defect site (pattern A). The second pattern involved small islands of bone proximal to the defect site (pattern B). Heterotopic ossification was seen in 2/14 (14.3%) group I animals; 7/14 (50%) of group II animals; 12/14 (85.7%) of group III animals; 0/5 (0%) of group IV animals; 1/5 (20%) of group V animals; and 1/4 (25%) of group VI animals. A chi square test for association was conducted between experimental group and heterotopic ossification. There was a statistically significant association between experimental group and presence of heterotopic ossification ($\chi^2(5) = 23.96$, $p < 0.001$). Higher doses of cells were associated with a higher rate of heterotopic ossification. Additionally, the pattern by which these formed appeared to be dependent on the dosage of cells. All of the animals from groups I, V, and VI demonstrated a pattern consistent with pattern A. Among group II animals that had heterotopic ossification, 2/7 demonstrated a pattern consistent with pattern B, whereas the remainder followed pattern A. Pattern B was seen more often in Group III animals (6/12). There was no statistically significant association between experimental group and pattern of heterotopic ossification ($\chi^2(4) = 4.71$, $p = 0.318$).

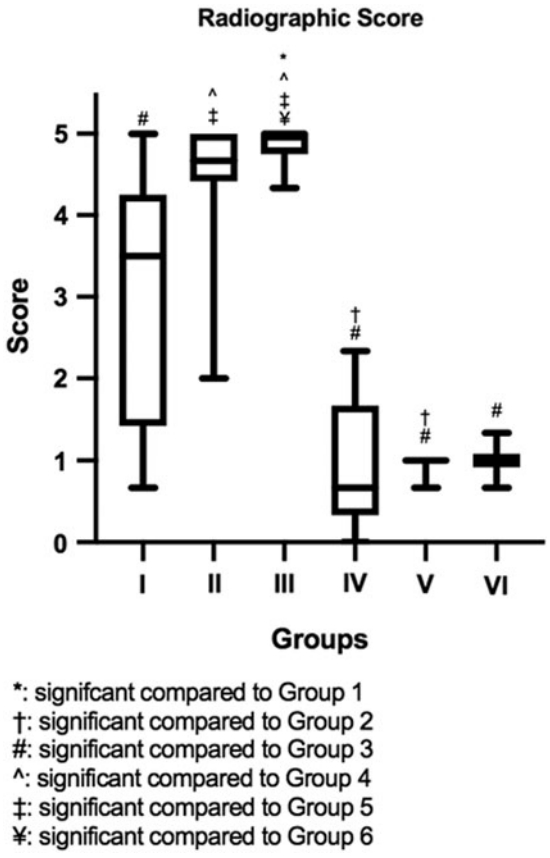

FIG. 2. Box plots of the six groups are presented. Medians are represented by horizontal lines within the box and the ends of the box represent the 25th and 75th percentiles or interquartile range. The *whiskers* represent the minimum and maximum values. Radiographic score by mean ranks increased from the control groups (IV: 17.55, V: 18.69, and VI: 17.15) to the treatment groups in increasing order of dosage: group I (27.20), II (44.70), and III (49.55). The mean rank of radiographic scores were statistically significantly different between groups ($\chi^2(5) = 36.77$, $p < 0.001$).

Bone formation on microCT

As seen with radiographic assessment, the 12-week microCT imaging demonstrated a higher rate of bone defect-site healing in animals treated with larger doses of BMP-2-producing cells (Fig. 4). One defect site in the low-dose group

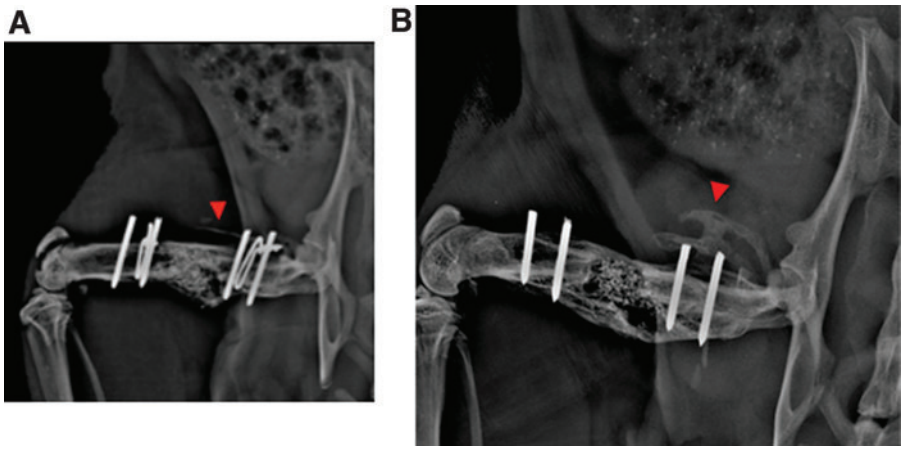

FIG. 3. Two distinct patterns of heterotopic ossification (*red arrow heads*) were observed. **(A)** Representative radiograph demonstrating the first pattern: thin wisps of bone around the area of the defect site. **(B)** Representative radiographic of the second pattern: small bone island proximal to the defect site. Color images are available online.

(group I) that was determined to be healed by radiographic assessment was deemed not healed by microCT. There was no other discrepancy between radiographic and microCT healing assessments. Group VI only had microCT data for three specimens because the data for one was lost during computed tomography (CT) scanner maintenance and another animal was euthanized before reaching the 12-week time point due to evidence of fixation failure at 8 weeks. The BV and bone volume fraction (BVF = Bone Volume [BV]/Total Volume [TV]) was calculated for each specimen. The average BV progressively increased with larger doses of BMP-2-producing cells. On average, group II formed approximately twice as much bone as group I (53.27 ± 5.35 vs. $26.71 \pm 4.53\,mm^3$, respectively). Group III formed approximately three times as much bone as group I (73.23 ± 5.67 vs. $26.71 \pm 4.53\,mm^3$, respectively). Mean BV for groups IV, V, and VI were 11.76 ± 4.60, 6.80 ± 1.47, and $11.12 \pm 3.35\,mm^3$, respectively. Similarly, there was a progressive increase in average BVF with larger doses of BMP-2-producing cells (Table 2). A Kruskal–Wallis test was conducted to determine if there were differences in BV and BVF between groups. The BV and BVF mean ranks of group III was significantly greater than those of group I. There were no significant differences in the mean rank for group II from those of both groups I and III.

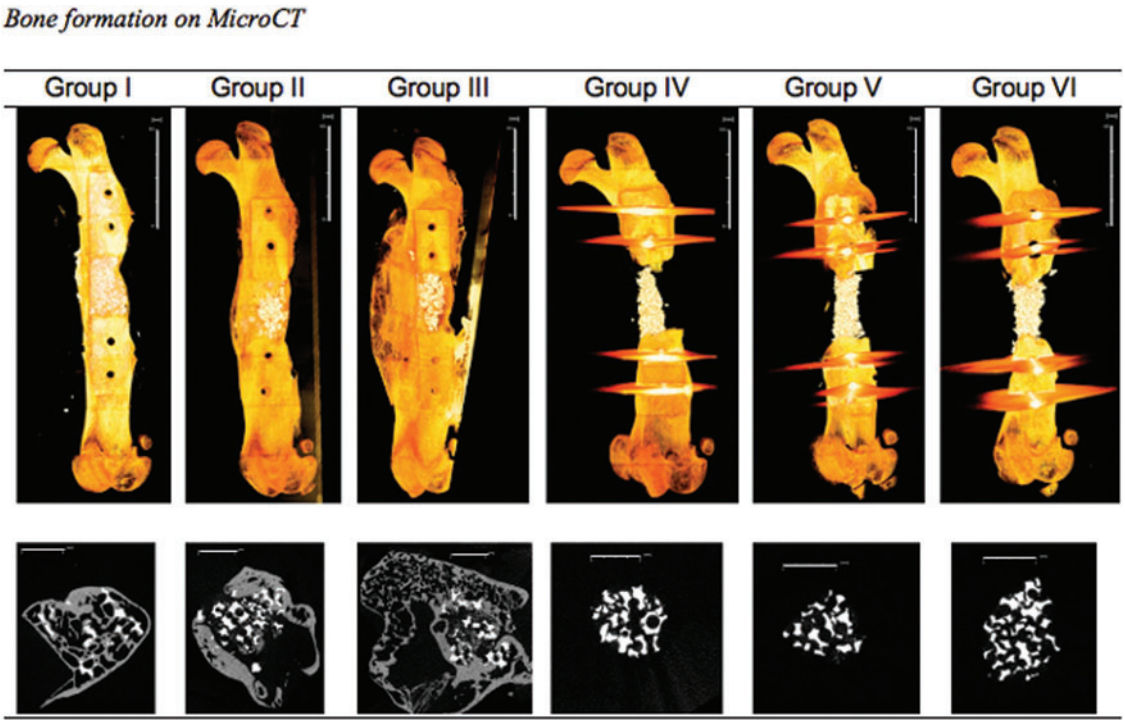

FIG. 4. Representative microCT scans with three-dimensional reconstructions and the corresponding axial images. The fixation pins led to metal artifact in the imaging studies, but these had to be left in place for the negative control groups (groups II–IV) since removal would have disrupted the alignment of the construct. The scale bars correspond to 10 mm. The axial images demonstrate bone formation in the treatment groups (I, II, and III) and no bone formation in the control groups (IV, V, and VI). The scale bars in the axial images correspond to 2 mm. microCT, microcomputed tomography. Color images are available online.

Table 2. Mean Bone Volume and Bone Volume Fraction as Measured on Microcomputed Tomography

microCT results

Group	n	*BV (mean ± SEM)*	*Mean rank*	*BVF (mean ± SEM)*	*Mean rank*
I: Low-dose HBMC/LV-TSTA-BMP2	14	$26.71 \pm 4.53\ m^3$	$21.21^{\#}$	$8.10\% \pm 1.36\%$	$21.71^{\#}$
II: Standard-dose HBMC/LV-TSTA-BMP2	14	$53.27 \pm 5.35\ mm^3$	$35.07^{\ddagger}$	$16.27\% \pm 2.59\%$	$35.57^{\ddagger}$
III: High-dose HBMC/LV-TSTA-BMP2	14	$73.23 \pm 5.67\ mm^3$	$44.86^{*,\wedge,\ddagger,¥}$	$19.42\% \pm 2.73\%$	$41.21^{*,\wedge,\ddagger}$
IV: Standard-dose HBMC/LV-TSTA-GFP	5	$11.76 \pm 4.60\ mm^3$	$11.00^{\#}$	$5.35\% \pm 2.09\%$	14.00
V: High-dose HBMC/LV-TSTA-GFP	5	$6.80 \pm 1.47 mm^3$	$6.20^{\dagger,\#}$	$3.41\% \pm 0.86\%$	$8.60^{\dagger,\#}$
VI: High-dose HBMC/nontransduced	3	$11.12 \pm 3.35\ mm^3$	$12.67^{\#}$	$5.58\% \pm 2.03\%$	16.00

The mean rank of each group is presented in separate columns. A Kruskal–Wallis test was conducted and the results of *posthoc* analysis between the six groups is also presented. BV and BVF of group III femurs were significantly greater compared with group I femurs. There was no significant difference between group II and group III femurs.

BV by mean ranks was statistically significantly different between groups, $\chi^2(5) = 38.38$, $p < 0.001$.

BVF by mean ranks was statistically significantly different between groups, $\chi^2(5) = 27.64$, $p < 0.001$.

*Significant compared with group I.

†Significant compared with group II.

#Significant compared with group III.

^Significant compared with group IV.

‡Significant compared with group V.

¥Significant compared with group VI.

BV, bone volume; BVF, bone volume fraction; microCT, microcomputed tomography.

Histologic and histomorphometric findings

Bony union was seen across the defect site on longitudinal section for all femurs that had demonstrated radiographic healing in groups I, II, and III (Fig. 5). Bony trabeculae and reconstitution of the medullary canal were noted. These femurs were also noted to have new bone formation on transverse sections that was incorporated within and around the compression matrices. Nearly all femurs in groups IV, V, and VI demonstrated minimal bone formation with bony ''caps'' over the distal and proximal defect sites and interposed fibrous tissue. One femur in group IV demonstrated new bone formation at the proximal and distal aspects of the defect site; however, the newly formed bone in this femur did not unite across the defect site as was seen in the treatment groups. There was no evidence of infection or inflammation noted on H&E and TRAP staining in any of the groups.

Bone area (BA) formation was quantified by histomorphometric analysis of transverse sections of each defect. BA and BA/TA comparisons across groups demonstrated a similar relationship as was seen with radiographic scores and microCT (Table 3). A Kruskal–Wallis test was conducted to determine if there were differences in BA and BA/TA between groups. While the treatment groups (groups I–III) had BA and BA/TA mean rank values that were greater than those of the control groups (groups IV–VI), only groups II and III had BA and BA/TA mean rank values that were statistically greater than those of the control groups. Among treatment groups, groups II and III had greater histomorphometric mean rank values than group I, but only group III's BA mean rank was found to be statistically greater compared with group I.

Biomechanical testing

There were nine designated femurs in groups I, II, and III for biomechanical testing. Mean biomechanical results were compared across the treated femurs. Only five of the group I femurs had enough stability to undergo torsional testing. The four specimens that could not be tested were given a ''0'' for all parameters. In contrast, all 18 femurs in groups II and III were stable enough to undergo torsional testing. Femora from groups IV, V, and VI were not tested since none healed across the defect site and were not stable enough for mounting. Femora treated with higher doses of BMP-2-producing cells were associated with higher stiffness, peak torque, and total energy (Table 4). A Kruskal–Wallis test was conducted to determine if there were

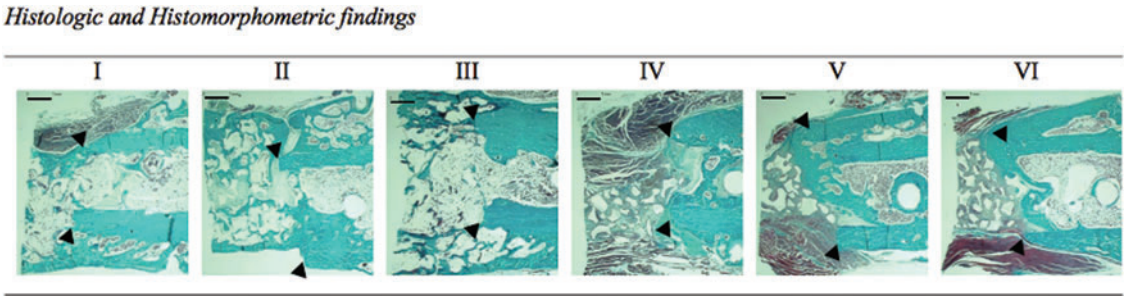

FIG. 5. Representative Masson Trichrome-stained longitudinal sections of the defect site interface from all groups. Defect site and bone formation interfaces are noted by *arrowheads*. As cell dosage increased, the bone area seen on histomorphometry also increased. The control groups were noted to have fibrous tissue in the defect site with bony caps formed at the defect site interface. The scale bars correspond to 1 mm. Color images are available online.

Table 3. Mean Bone Area and Bone Area/Tissue Area as Measured on Histomorphometry Are Presented

Group	N	*BA (mean ± SEM)*	*Mean rank*	*BA/TA (mean ± SEM)*	*Mean rank*
I: Low-dose HBMC/LV-TSTA-BMP2	14	$0.10 \pm 0.05\ mm^2$	$27.20^{\#}$	$8.60\% \pm 3.86\%$	28.95
II: Standard-dose HBMC/LV-TSTA-BMP2	14	$0.46 \pm 0.14\ mm^2$	$44.70^{\wedge,\ddagger,¥}$	$22.20\% \pm 5.61\%$	$44.25^{\wedge,\ddagger,¥}$
III: High-dose HBMC/LV-TSTA-BMP2	14	$0.54 \pm 0.05\ mm^2$	$49.55^{*,\wedge,\ddagger,¥}$	$21.80\% \pm 2.64\%$	$44.85^{\wedge,\ddagger,¥}$
IV: Standard-dose HBMC/LV-TSTA-GFP	5	$0.07 \pm 0.07\ mm^2$	$17.55^{\dagger,\#}$	$5.60\% \pm 5.48\%$	$19.50^{\dagger,\#}$
V: High-dose HBMC/LV-TSTA-GFP	5	$0.02 \pm 0.01\ mm^2$	$18.69^{\dagger,\#}$	$2.10\% \pm 1.31\%$	$19.13^{\dagger,\#}$
VI: High-dose HBMC/nontransduced	4	$0.04 \pm 0.03\ mm^2$	$17.15^{\dagger,\#}$	$4.13\% \pm 3.32\%$	$18.25^{\dagger,\#}$

The mean rank of each group is presented in separate columns. A Kruskal–Wallis test was conducted and the results of posthoc analysis between the six groups is also presented. BA of group III femurs was significantly greater compared with group I femurs. There was no significant difference between group II and group III femurs. There was no significant difference between any of the experimental groups (groups I, II, and III) with regard to BA/TA.

BA by mean ranks was statistically significantly different between groups, $\chi^2(5) = 37.35$, $p < 0.001$.

BA/TA by mean ranks was statistically significantly different between groups, $\chi^2(5) = 27.86$, $p < 0.001$.

*Significant compared with group I.

†Significant compared with group II.

#Significant compared with group III.

^Significant compared with group IV.

‡Significant compared with group V.

¥Significant compared with group VI.

BA, bone area; TA, tissue area.

between-group differences for stiffness, peak torque, peak displacement, and total energy to failure. Peak displacement did not significantly differ between all groups. *Post hoc* analysis revealed statistically significant differences between femora of group III and I for stiffness, peak torque, and total energy to failure. Although not significantly different, group II femora outperformed group I femora in stiffness (0.05 ± 0.01 vs. 0.02 ± 0.01 Nm/deg, respectively; $p = 0.144$), peak torque (0.34 ± 0.05 vs. 0.11 ± 0.05 Nm, respectively; $p = 0.063$), and total energy to failure (1.82 ± 0.29 vs. 0.47 ± 0.01 Nm·deg, respectively; $p = 0.05$).

The contralateral intact femurs, all underwent biomechanical testing, and served as the internal control within groups. The contralateral femurs generally outperformed each experimental femur. However, the femora of groups II and III were most biomechanically similar to intact femora. The experimental femurs for these two groups had statistically similar stiffness values to their contralateral counterparts. In addition, the mean peak torque for Group III femurs was not significantly different from that of the contralateral, intact femurs ($p = 0.50$).

Adverse events

No obvious signs of soft tissue swelling were noted in any animal over the 12-week postoperative period. One animal in group VI had to be euthanized at the 8-week time point due to radiographic evidence of fixation failure. Four total animals in groups I and III, two in each group, died before reaching the 4-week postsurgical time point. These animals were replaced with four additional animals. The average postsurgical time to death was ~3 weeks for the four animals making anesthesia complications unlikely. On postmortem autopsy, one of these animals was noted to have suffered from fixation failure with concern for possible infection. This was supported by the large aggregation of TRAP-stained cells seen on histologic analysis.

Discussion

Gene therapy treatment regimens are now being implemented to treat a variety of diseases. Lentiviral gene therapy, to treat β-thalassemia, was approved by the European Medicines Agency (EMA) in 2019 with ongoing

Table 4. Mean Biomechanical Results Are Presented

Biomechanical results (mean ± SEM)

Group	N	*Stiffness (Nm/deg)*	*Peak torque (Nm)*	*Peak displacement (deg)*	*Total energy to failure (Nm·deg)*
I: Low-dose HBMC/LV-TSTA-BMP2	9	$0.02 \pm 0.01^{\#}$	$0.11 \pm 0.05^{\#}$	5.94 ± 2.01	$0.47 \pm 0.25^{\#}$
(internal control) Contralateral group I	9	0.06 ± 0.00	0.58 ± 0.03	12.81 ± 0.36	7.53 ± 1.71
II: Standard-dose HBMC/LV-TSTA-BMP2	9	0.05 ± 0.01	0.34 ± 0.05	9.73 ± 0.55	1.82 ± 0.29
(internal control) Contralateral group II	9	0.06 ± 0.01	0.54 ± 0.05	12.19 ± 0.66	7.02 ± 1.64
III: High-dose HBMC/LV-TSTA-BMP2	9	0.09 ± 0.02*	0.51 ± 0.11*	8.95 ± 0.65	2.72 ± 0.64*
(internal control) Contralateral group III	9	0.07 ± 0.00	0.59 ± 0.04	11.93 ± 0.87	6.89 ± 1.40

A Kruskal–Wallis test was conducted and the results of *posthoc* analysis between the three experimental groups is also presented. Stiffness, peak torque, and total energy to failure of group III femurs were significantly greater than that of group I femurs. There was no significant difference between group II and group III femurs.

Median stiffness was statistically significantly different between groups, $\chi^2(2) = 11.80$, $p = 0.003$.

Median peak torque was statistically significantly different between groups, $\chi^2(2) = 10.82$, $p = 0.004$.

Median total energy to failure was statistically significantly different between groups, $\chi^2(2) = 8.71$, $p = 0.01$.

*Significant compared with group I.

#Significant compared with group III.

clinical trials for several other conditions.[28,29] An *ex vivo* regional gene therapy strategy to treat osteoarthritis is also being assessed in clinical trials. In this therapy (coined ''TissueGene-C''), allograft chondrocytes that have been modified using a retroviral vector containing *TGF-β1* cDNA are injected into osteoarthritic knees.[30–32] Early phase II results have been promising with significant improvement in function and decreased pain noted with use of ''TissueGene-C'' compared with a placebo.[32]

Ex vivo regional gene therapy is also a promising therapeutic option to treat bone loss situations, such as complex posttraumatic fractures, revision arthroplasty, and pseudoarthrosis of the spine. This therapy has the potential to provide the same advantages of autologous bone grafts while avoiding their donor-site morbidity and quantity limitations. Our gene therapy strategy provides osteogenic cells an osteoinductive signal (BMP-2), and is delivered on an osteoconductive scaffold directly into the appropriate anatomic site. A purported advantage of gene therapy over its recombinant protein counterpart is the sustained release of BMP-2 by transduced cells over time versus the rapid release that has been reported with rhBMP-2.[19] This sustained release can promote healing while potentially avoiding the adverse events associated with supraphysiologic doses of rhBMP-2.[10,11,14,15]

The primary goal of this study was to assess the influence of the dosage of transduced cells on bony healing of a critical-sized femoral defect. The high-dose group was specifically chosen to observe the effects of implanting a dosage of cells several times larger than what has been generally used in prior studies (5×0^6 BMP-2 transduced cells).[17,18,27] Treatment with a high dose of transduced cells (group III) clearly enhanced bone healing, especially compared with the results achieved with a low dose (group I). For example, femora from group III formed approximately three times more bone on microCT than femora from group I. In addition, biomechanical testing demonstrated that the experimental femurs had similar stiffness and mean torque values as the contralateral, intact femurs. The enhanced bone repair seen with the high-dose group suggests that this strategy can be adapted for treatment of the larger defects seen in humans.

The ultimate goal for regional gene therapy is to implant transduced cells in humans in a clinical setting. One step in that direction would be to determine an ideal number of cells required per defect unit size. We assessed three doses so it is not possible to formulate a dose–response curve to draw any definitive conclusions regarding the optimal cell dose. Prior work confirmed consistent healing with a dose of 5×10^6 BMP-2-transduced cells.[18] The findings of the present study confirm those results. Although group III femora demonstrated greater radiographic healing, bone formation, and biomechanical parameters than group II femora, all comparisons between group III and group II results were not statistically significant. The overall results suggest that treating with more than 5×10^6 cells may not be necessary to heal a 6 mm femoral defect in rats, but better-quality bone repair can occur by increasing the dose of cells. The lack of statistical significance between the two groups is likely due to the limitations of the rat femoral defect model. In addition, the study was only powered to detect torsional differences.

Tsuchida *et al.* demonstrated the potential of using allogeneic mesenchymal stem cells transduced with an adenovirus vector containing BMP-2 cDNA to treat critical-sized bone defects when an immunosuppressive reagent (FK 506) was coadministered.[33] Using a similar 6 mm femoral defect model, transduced RBMCs obtained from male Fisher 344 rats were implanted in both Fisher 344 and Brown Norway rats. A total of 8×10^6 transduced cells were implanted per defect site and 93% of the defects in their two treatment groups went on to completely heal by 8 weeks. This study did not comment on the rate of heterotopic ossification. While the vector, scaffold, and several other aspects of their methods differ from our model, their results with a cell dosage of 8×10^6 is in line with our findings that suggest that a high dose of 1.5×10^7 cells is not necessary to heal a 6 mm femoral defect.

The healing rate and biomechanical parameters achieved with 5×10^6 BMP-2-producing cells were comparable to that achieved with a high dose of 1.5×10^7 BMP-2-producing cells. We cannot comment on an optimal dosage to heal a 6 mm femoral defect in rats given the limitations of this study; however, the results of the present study demonstrate that 5×10^6 BMP-2-producing cells may be the threshold needed to achieve consistent results. Extrapolating this dose provides an idea of the number of cells that would be required to heal the larger bone defects seen in humans. The BV and BVF of a 6 mm segment of bone from the mid-femoral diaphysis in a 12-week-old rat is 31.21 mm^3 and 36%, respectively. These figures were obtained from a microCT scan of an intact, native rat femur. Consequently, 5×10^6 BMP-2-producing cells are necessary to heal a 31.21 mm^3 bone defect. This indicates that the required number of BMP-2-producing cells needed to heal a 1 mm^3 bone defect is 160,205 cells (5×10^6 cells/31.21 mm^3). When treating patients, a CT scan can be obtained to determine the volume of the bone defect and the 160,000 cells/1 mm^3 could be used to estimate the cells needed to treat a larger bone defect. This cell number may only be applicable to the treatment of rat bone, but, at the very least, these data provide a starting point for determining a cellular dose for humans since we used transduced human cells.

The transduced cells used to treat groups I, II, and III femora produced similar amounts of BMP-2 per million cells in the *in vitro* analysis. Since group I animals had the lowest healing rates, this suggests that the cell dose can influence healing. There is probably a threshold amount of BMP-2 production necessary to heal a defect. If the *in vitro* BMP-2 production per million cells was similar in all groups, then group II and group III femora received 5 times and 15 times the BMP-2 than the femora in group I, respectively. However, we are unable to determine the actual *in vivo* BMP-2 production. In addition, another critical element that influences bone healing may be the number of surviving cells in the defect. We did not measure this parameter in this study.

When assessing the healing of group I femurs, 5 out of 14 of the low-dose group healed the defect. Based on our prior rat data using the same cell dose, we had expected approximately two femurs to heal. In our subgroup analysis, the average *in vitro* BMP-2 production in healed femurs was 89.9 ± 5.9 ng/24 h/million cells, which was not significantly greater than the 77.8 ± 4.9 ng/24 h/million cells produced by

cells implanted in femurs that did not heal. This difference could be significant if more animals had been assessed. Data suggest that a certain *in vivo* BMP-2 threshold is necessary and therefore careful handling of the cells and cell survival in the defect have a critical impact on the bone repair.

Prolonged BMP-2 production is not without risk. Heterotopic ossification was seen at significantly increasing rates with higher level of cell dosages. In group III, this was most likely related to the oversaturation of the compression-resistant carrier; there was leakage of cells noted while implanting the group III carriers into the bone defect. While no formal functional assessment was performed, no obvious functional deficit was noted during examinations by husbandry and laboratory staff that occurred on a regular basis during the postoperative period. Furthermore, no soft tissue swelling was noted throughout the duration of the postoperative period. These results suggest that oversaturation of the cell carrier or scaffold should be avoided when using this strategy in humans to limit the leakage of cells from the defect site.

This study has a number of limitations. One limitation was that since just three different cell doses were studied, we were not able to generate a dose–response curve. Therefore, it is not possible to determine the healing response or heterotopic ossification rates with other cell doses. Another limitation is that the cell donor may be a potential confounding variable in the present study. Given the limitations of donor availability and the unknown impact of pooling human cells from multiple donors, we were unable to create a pool of HBMCs from multiple donors for implantation and instead assigned cells from specific donors to specific animals. However, the average BMP-2 produced by different donors included in each treatment group was similar as noted in the results above, so we believe that using different donors across animals likely had little, if any, significant effect on bone formation and healing. We also did not formally assess for an association between heterotopic ossification and functional status. Finally, we used HBMCs from femoral canals and not from the iliac crest, which would be the normal site of bone marrow harvest for use as an adjuvant in bone loss treatment.

There were four animals in both low-dose (group I) and high-dose (group III) groups that died early in the postoperative time period. While one likely died secondary to an infection, the causes of the other deaths are unknown. Athymic, nude rats have an impaired reactivity to bacterial disease due to a lack of thymus-dependent immunity.[34] This susceptibility necessitates sterile housing and care procedures for nude rats. It is impossible to say whether the deaths were a direct result of implantation of transduced cells or due to other reasons such as the rats' poor immune systems. The deaths were evenly distributed among both groups I (low-dose HBMC LV-TSTA-BMP-2) and III (high-dose HBMC LV-TSTA-BMP-2), which indicates the deaths were due to something other than the amount of cells being implanted.

Conclusion

Ex vivo regional gene therapy is a promising treatment option for the management of large bone loss clinical scenarios. The findings of this study demonstrate that treatment with larger cell doses of HBMCs transduced with lentiviral vectors containing BMP-2 cDNA is associated with higher bone formation and healing rates, but is also associated with higher rates of heterotopic ossification. When adapting the cellular dose for use in humans, these factors will need to be taken into consideration.

IRB Approval

This study required the use of HBMCs cultured from tissue specimen obtained from patients undergoing THA. This qualified for an expedited IRB review and did not require patient consent as the tissue being used would have been discarded. Please find a copy of the letter of approval from the IRB board at the University of Southern California attached among the uploads for this submission.

Authors' Contributions

Each named author has substantially contributed to conducting the underlying research and drafting this article.

H.I. and H.K. (cofirst authors):

- Both authors had major contributions for the design of the work, acquisition of data, analysis, and interpretation of data for the work AND
- Drafting the work or revising it critically for important intellectual content AND
- Final approval of the version to be published AND
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

B.I.:

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Competing Interests: A competing interest exists when an individual (or the individual's institution) has financial or personal relationships that may inappropriately influence his actions. These competing interests may be potential or actual, financial or other.

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Other Competing Interests: Any personal relationship, which may inappropriately affect the integrity of the research reported (by an author) or the objectivity of the review of the article (by a reviewer or Editor), for example, competition between investigators, previous disagreements between investigators, or bias in professional judgment.

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References

1. Schemitsch, E.H. Size matters: defining critical in bone defect size! J Orthop Trauma **31 Suppl 5,** S20, 2017.
2. Sen, M.K., and Miclau, T. Autologous iliac crest bone graft: should it still be the gold standard for treating nonunions? Injury **38 Suppl 1,** S75, 2007.
3. Baldwin, P., Li, D.J., Auston, D.A., Mir, H.S., Yoon, R.S., and Koval, K.J. Autograft, allograft, and bone graft substitutes. J Orthop Trauma **33,** 203, 2019.
4. Kim, D.H., Rhim, R., Li, L., *et al.* Prospective study of iliac crest bone graft harvest site pain and morbidity. Spine J **9,** 886, 2009.
5. Glassman, S.D., Carreon, L.Y., Djurasovic, M., *et al.* RhBMP-2 versus iliac crest bone graft for lumbar spine fusion: a randomized, controlled trial in patients over sixty years of age. Spine (Phila Pa 1976) **33,** 2843, 2008.
6. Jones, A.L., Bucholz, R.W., Bosse, M.J., *et al.* Recombinant human BMP-2 and allograft compared with

autogenous bone graft for reconstruction of diaphyseal tibial fractures with cortical defects: A randomized, controlled trial. J Bone Joint Surg Am **88,** 1431, 2006.
7. McKay, W.F., Peckham, S.M., and Badura, J.M. A comprehensive clinical review of recombinant human bone morphogenetic protein-2 (INFUSE® Bone Graft). Int Orthop **31,** 729, 2007.
8. Fu, R., Selph, S., Mcdonagh, M., *et al.* Effectiveness and harms of recombinant human bone morphogenetic. Ann Intern Med **158,** 890, 2013.
9. Carragee, E.J., Hurwitz, E.L., and Weiner, B.K. A critical review of recombinant human bone morphogenetic protein-2 trials in spinal surgery: emerging safety concerns and lessons learned. Spine J **11,** 471, 2011.
10. Carragee, E.J., Mitsunaga, K.A., Hurwitz, E.L., and Scuderi, G.J. Retrograde ejaculation after anterior lumbar interbody fusion using rhBMP-2: A cohort controlled study. Spine J **11,** 511, 2011.
11. Boraiah, S., Paul, O., Hawkes, D., Wickham, M., and Lorich, D.G. Complications of recombinant human BMP-2 for treating complex tibial plateau fractures: A preliminary report. Clin Orthop Relat Res **467,** 3257, 2009.
12. Poynton, A.R., and Lane, J.M. Safety profile for the clinical use of bone morphogenetic proteins in the spine. Spine (Phila Pa 1976) **27(16 Suppl 1),** S40, 2002.
13. Uludag, H., D'Augusta, D., Palmer, R., Timony, G., and Wozney, J. Characterization of rhBMP-2 pharmacokinetics implanted with biomaterial carriers in the rat ectopic model. J Biomed Mater Res **46,** 193, 1999.
14. Bessa, P.C., Casal, M., and Reis, R.L. Bone morphogenetic proteins in tissue engineering: The road from laboratory to clinic, part II (BMP delivery). J Tissue Eng Regen Med **2,** 81, 2008.
15. Jeon, O., Song, S.J., Kang, S.W., Putnam, A.J., and Kim, B.S. Enhancement of ectopic bone formation by bone morphogenetic protein-2 released from a heparin-conjugated poly(l-lactic-co-glycolic acid) scaffold. Biomaterials **28,** 2763, 2007.
16. Bougioukli, S., Evans, C.H., Alluri, R.K., Ghivizzani, S.C., and Lieberman, J.R. Gene therapy to enhance bone and cartilage repair in orthopaedic surgery. Curr Gene Ther **18,** 154, 2018.
17. Bougioukli, S., Alluri, R., Pannell, W., *et al.* Ex vivo gene therapy using human bone marrow cells overexpressing BMP-2: ''next-day'' gene therapy versus standard ''two-step'' approach. Bone **128,** 115032, 2019.
18. Hsu, W.K., Sugiyama, O., Park, S.H., *et al.* Lentiviral-mediated BMP-2 gene transfer enhances healing of segmental femoral defects in rats. Bone **40,** 931, 2007.
19. Feeley, B.T., Conduah, A.H., Sugiyama, O., Krenek, L., Chen, I.S.Y., and Lieberman, J.R. In vivo molecular imaging of adenoviral versus lentiviral gene therapy in two bone formation models. J Orthop Res **24,** 1709, 2006.
20. Bougioukli, S., Sugiyama, O., Alluri, R.K., Yoho, R., Oakes, D.A., and Lieberman, J.R. In vitro evaluation of a lentiviral two-step transcriptional amplification system using GAL4FF transactivator for gene therapy applications in bone repair. Gene Ther **25,** 260, 2018.
21. Virk, M.S., Sugiyama, O., Park, S.H., *et al.* Same day ex-vivo regional gene therapy: A novel strategy to enhance bone repair. Mol Ther **19,** 960, 2011.
22. Alaee, F., Bartholomae, C., Sugiyama, O., *et al.* Biodistribution of LV-TSTA transduced rat bone marrow cells used for ''ex-vivo'' regional gene therapy for bone repair. Curr Gene Ther **15,** 481, 2015.
23. Bougioukli, S., Sugiyama, O., Pannell, W., *et al.* Gene therapy for bone repair using human cells: superior osteogenic potential of bone morphogenetic protein 2-transduced mesenchymal stem cells derived from adipose tissue compared to bone marrow. Hum Gene Ther **29,** 507, 2018.
24. Iyer, M., Wu, L., Carey, M., Wang, Y., Smallwood, A., and Gambhir, S.S. Two-step transcriptional amplification as a method for imaging reporter gene expression using weak promoters. Proc Natl Acad Sci U S A **98,** 14595, 2001.
25. Lieberman, J.R., Daluiski, A., Stevenson, S., *et al.* The effect of regional gene therapy with bone morphogenetic protein-2-producing bone-marrow cells on the repair of segmental femoral defects in rats. J Bone Joint Surg Am **81,** 905, 1999.
26. Virk, M.S., Alaee, F., Tang, H., Ominsky, M.S., Ke, H.Z., and Lieberman, J.R. Systemic administration of sclerostin antibody enhances bone repair in a critical-sized femoral defect in a rat model. J Bone Joint Surg Am **95,** 694, 2013.
27. Alluri, R., Song, X., Bougioukli, S., *et al.* Regional gene therapy with 3D printed scaffolds to heal critical sized bone defects in a rat model. J Biomed Mater Res A **107,** 2174, 2019.
28. High, K.A., and Roncarolo, M.G. Gene therapy. N Engl J Med **381,** 455, 2019.
29. Milone, M.C., and O'Doherty, U. Clinical use of lentiviral vectors. Leukemia **32,** 1529, 2018.
30. Ha, C.W., Cho, J.J., Elmallah, R.K., *et al.* A multicenter, single-blind, phase IIa clinical trial to evaluate the efficacy and safety of a cell-mediated gene therapy in degenerative knee arthritis patients. Hum Gene Ther Clin Dev **26,** 125, 2015.
31. Lee, M.C., Ha, C.W., Elmallah, R.K., *et al.* A placebo-controlled randomised trial to assess the effect of TGF-ß1-expressing chondrocytes in patients with arthritis of the knee. Bone Joint J **97-B(7),** 924, 2015.
32. Cherian, J.J., Parvizi, J., Bramlet, D., Lee, K.H., Romness, D.W., and Mont, M.A. Preliminary results of a phase II randomized study to determine the efficacy and safety of genetically engineered allogeneic human chondrocytes expressing TGF-β1 in patients with grade 3 chronic degenerative joint disease of the knee. Osteoarthritis Cartilage **23,** 2109, 2015.
33. Tsuchida, H., Hashimoto, J., Crawford, E., Manske, P., and Lou, J. Engineered allogeneic mesenchymal stem cells repair femoral segmental defect in rats. J Orthop Res **21,** 44, 2003.
34. Hanes, M.A. The Laboratory Rat: Nude Rat. 2nd ed., Amsterdam, Elsevier, 2006.

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