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Ex vivo gene therapy using human bone marrow cells overexpressing BMP-2: "Next-day" gene therapy versus standard "two-step" approach

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ABSTRACT

Traditionally, ex vivo gene therapy involves a two-step approach, with culture expansion of cells prior to transduction and implantation. We have tried to simplify this strategy and eliminate the time and cost associated with culture expansion, by introducing "next-day" regional gene therapy using human bone marrow cells. The purpose of this study was to determine whether a lentiviral vector (LV) carrying the cDNA for BMP-2 can transduce freshly isolated human BM cells, leading to abundant BMP production and bone formation in vivo, and evaluate the in vivo osteoinductive potential of "next-day" gene therapy and the standard "two-step" tissue culture expansion approach. To this end, human bone marrow cells (HBMC) from patients undergoing total hip arthroplasty were harvested, transduced with a BMP-2-expressing LV either overnight ("next day" gene therapy; ND) or after culture expansion (cultured "two-step" approach; C) and then implanted into a rat critical-sized femoral defect. The animals were randomly assigned to one of the following groups: I; ND-HBMC transduced with LV-TSTA BMP-2, II; ND-HBMC transduced with LV-TSTA GFP, III; non-transduced ND-HBMC; IV; C-HBMC transduced with LV-TSTA BMP-2, V; C-HBMC transduced with LV-TSTA-GFP, VI; non-transduced C-HBMC. Treatment with either "next-day" or cultured HBMC demonstrated a significant increase in new bone formation compared with all negative control groups as seen in plain radiographs, microCT and histologic/histomorphometric analysis. At 12 weeks post-op, complete defect union on plain X-rays occurred in 7/14 animals in the ND-HBMC/BMP-2 group and 12/14 in the C-HBMC/BMP-2 treated rats. The two-step approach was associated with more consistent results, a higher union rate, and superiority with regards to all of the studied bone healing parameters. In this study we demonstrate proof of concept that BMP-2-transduced human bone marrow cells can be used to enhance bone healing in segmental bone defects, and that regional gene therapy using lentiviral transduction has the osteoinductive potential to heal large bone defects in clinical settings.

1. Introduction

The majority of fractures heal uneventfully, but it has been reported that approximately 5% [1] of all fractures either fail to unite or demonstrate a delay in healing. Bone loss and inadequate bone repair is also associated with trauma, revision total joint replacement and spinal fusion. In such settings, options for reconstruction include autologous bone graft, bone allografts, demineralized bone matrices, and biologic therapies that employ delivery of stem cells and/or growth factors [2,3]. Each of these treatment options have their own advantages and limitations [2–5]. In general, iliac crest bone graft is considered the gold standard bone graft agent, but it has been associated with significant donor site morbidity and limited availability [6,7]. RhBMP-2 is an osteoinductive agent that has been FDA-approved for use in spine fusion and tibia fractures [8]. Although rhBMP-2 has demonstrated efficacy in several clinical scenarios [8,9], the clinical results have been variable [10,11]. In addition it has been associated with side effects including heterotopic ossification, wound complications and significant soft tissue swelling, mostly stemming from the fact that supraphysiologic doses of the protein are needed to promote adequate bone repair [12,13]. There are also concerns that in a clinical situation with significant bone loss, abundant scar tissue or limited vascularity, a single dose of recombinant protein may not yield an adequate osteogenic response. Thus there is clearly an unmet clinical need for consistently

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successful treatment of these injuries, especially in compromised biological environments.

There has been substantial preclinical research activity in the field of gene therapy for tissue engineering, with major advances in understanding the biological aspects of gene therapy and providing proof of concept for potential clinical applications. Ex vivo regional gene therapy has emerged as a promising alternative for use in bone repair applications [14–18]. The advantage of such a strategy is that osteoprogenitor cells producing an osteoinductive factor and loaded on an osteoconductive carrier can be delivered to a specific anatomic site to promote bone healing. Sustained production of BMP-2 by the transduced cells leads to longer half-life of the osteoinductive signal and thus increased recruitment and differentiation of osteoprogenitor cells leading to enhanced formation of new bone [19].

Traditionally, ex vivo gene therapy involves a two-step approach, with culture expansion of cells prior to transduction and implantation. Although this approach has been used successfully to promote bone formation in various animal models in our laboratory and others [14,16], it has the disadvantage of being complex, expensive and time-consuming, as it requires extensive culture of autologous cells under good manufacturing practice conditions in special facilities prior to transduction [14,17]. We have tried to simplify this approach by introducing an expedited single-step strategy that eliminates the rate-limiting step of cell expansion. The success of single-step “same day” regional gene therapy has been previously demonstrated in a rat critical-sized femoral defect, using buffy coat cells from rat bone marrow transduced for 2 h with a lentiviral vector (LV) expressing BMP-2 [20]. However, in order to adapt regional gene therapy for clinical applications it is essential to assess the osteogenic potential of transduced human cells. In this study we assess human bone marrow (BM), since it can be easily obtained and has been studied extensively as a source of multipotent stem cells. When using human bone marrow cells in our preliminary experiments we noted that BMP production is significantly enhanced by transducing the cells overnight instead of only 3 h, leading to the development of the “next-day” gene therapy concept. In this strategy, the cells are harvested, transduced overnight and implanted the following day. This strategy is highly clinically relevant, since it would eliminate the need for costly and lengthy cell culture expansion, which would risk possible contamination and gene mutations, prolong the period of time between initial autologous tissue harvest and re-implantation back into the patient's bone defect and significantly increase the overall cost of the therapy.

The purpose of this study was to determine whether a two-step transcriptional lentiviral system carrying the cDNA for BMP-2 could successfully transduce freshly isolated human BM cells, leading to abundant BMP-2 production. We also evaluated the in vivo osteoinductive potential of lentiviral-mediated BMP-2 delivery via human bone marrow cells in a segmental critical-sized femoral defect by comparing two different strategies: “next-day” gene therapy, where freshly isolated human bone marrow cells are transduced overnight and implanted the following day, or standard “two-step” approach, where the cells are expanded in culture for a few passages prior to viral transduction and implantation.

2. Material and methods

2.1. Isolation and culture of human bone marrow cells

Human bone marrow cells (HBMC) were harvested from the intramedullary femoral canal of 18 healthy patients (13 male, 5 female), 34–77 years of age, undergoing primary total hip arthroplasty (THA) for osteoarthritis of the hip at our institution. The study protocol was reviewed and approved by the Institutional Review Board as a coded specimens study before the commencement of any experiments involving human cells. Three total joint replacement surgeons from the same institution participated in the sample collection. During THA the bone marrow from the femoral intramedullary canal is cleared and discarded so that an implant can be placed into the femur. This bone marrow, that would normally be discarded, was collected for use in our experiment. During the preparation of the femoral canal for a THA successively large broaches are used. After each broach the canal was aspirated with suction connected to a canister so as to collect marrow in a sterile manner.

The patients underwent THA solely for medical treatment; no additional research-related procedures were performed. Patients with significant co-morbidities, known history of human immunodeficiency viruses (HIV) infection, hepatitis B or hepatitis C or taking immunosuppressive or disease modifying agents were excluded. The decision was made by the surgeons alone based on the patient's history. The rest of the research team members did not have any access to patients' information, other than the patient's sex and age.

The collected bone marrow was transferred into sterile 50 cc tubes. PBS was added in a 1:1 ratio. The bone marrow/PBS suspension was carefully layered on top of 5-ml of Histopaque 1077 (Sigma-Aldrich, St. Louis, MO), and centrifuged for 30 min at 200*g*. The band of cells between the histopaque and plasma/PBS interface was aspirated to acquire the mononuclear cell fraction. These cells were then washed twice and resuspended in Dulbecco's modified Eagle medium (DMEM, Corning Mediatech, Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS, Omega Scientific, Tarzana, CA, USA). The isolated mononuclear cells were counted with an automated cell counter (Bio-Rad, Hercules, CA) using trypan blue. Half of the freshly isolated mononuclear cells were used immediately in the “next day” group, whereas the other half were plated and expanded in culture for 5 passages before transduction for use in the “two-step” approach according to previously established protocols [21,22]. In brief, for the two-step approach, cells were plated in DMEM/10% FBS at a concentration of 50×10^6 cells per 10 cm plate. The cells were maintained in a 5% CO_2 humidified atmosphere, at 37 °C. The culture medium was replaced every 3–4 days, with all non-adherent cells and contaminating red blood cells being removed. The adherent cells were passaged when > 90% confluent at a density of $0.8–1.0 \times 10^6$ cells per 10 cm plate.

2.2. Viral transduction

A two-step transcriptional amplification (TSTA) lentiviral system overexpressing BMP-2 (LV-TSTA-BMP-2) or GFP (LV-TSTA-GFP) was used in the study. As previously described [20,22,23], the TSTA system is comprised of 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 293 T cells (American Type Culture Collection, Manassas, VA), based on prior established protocols [20,24]. The LV titers were determined by quantifying p24 protein in vector supernatant using ELISA (ELISA, Quantikine, R&D Systems, Minneapolis, MN, USA). Only vectors with titers > 0.50 lfu/ml were used in the study.

Our original plan was to evaluate the osteoinductive potential of transduced human bone marrow cells using either “same-day” gene therapy, as previously described with rat bone marrow cells [20], or a standard “two-step” approach with culture expansion of the cells prior to transduction and implantation. However in a preliminary study we noted that the “same-day” approach was associated with low BMP levels, and thus an overnight transduction was introduced. The overnight transduction led to a 3–4× higher BMP production compared with the “same-day” strategy. Therefore instead of a 3-hour transduction we decided to use an overnight transduction to increase BMP production by transduced human bone marrow cells (Table 1). We call this strategy “next-day” gene therapy and the transduced cells “next-day” HBMC (ND-HBMC).

The freshly isolated human bone marrow cells or passage 5 HBMCs were plated in DMEM/10% FBS and transduced overnight with LV-

Table 1
BMP production by HBMC/LV-TSTA-BMP-2 (expressed in ng/million cells/24 h).

Donor	Age, Sex	3 h	ON
THA 1	52, M	6.8	20.4
THA 2	68, M	6.9	17.8
THA 3	60, F	6.8	28.9
THA 4	34, M	6.5	28.4
THA 5	42, M	5.1	23
THA 6	79, M	5.1	25.3
THA 7	63, M	5	19.1
THA 8	77, M	4.7	22.3

THA: total hip arthroplasty, 3 h: 3-hour transduction, ON: overnight transduction.

RhMLV-GAL4-VP16 and Lenti-G5-BMP-2 at MOI of 25/25 in the presence of 8 μg/ml polybrene. Following transduction the cells were washed with PBS three times to eradicate any extracellular virus. The cells were then resuspended in 10 μl of PBS and placed on ice until implantation into the femoral defect (< 3 h since harvesting from the plate) (Fig. 1).

2.3. In vitro BMP-2 production

Following overnight transduction, LV-TSTA-BMP-2-transduced HBMCs (freshly isolated or cultured) were washed twice and then incubated with fresh media for an additional 24 h period. The culture supernatant was subsequently harvested for evaluation of in vitro BMP-2 production, using ELISA (Quantikine, R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Each sample was run in triplicate. Results were standardized by cell number and reported as nanograms of BMP-2 per 24 h per 1×10^6 cells. Non-transduced HBMCs were used as negative control.

2.4. Animal model

The animal study was reviewed and approved by the university's Institutional Animal Care and Use Committee. A total of forty-eight, 12–14 week old, male nude rats were used in this study. Nude rats were selected to avoid immune reaction from the implantation of human cells. An internally stabilized, critical-sized full-thickness femoral defect (6 mm) was created unilaterally using an established model [25–27]. In brief, after induction of anesthesia using 2–3% isoflurane, the left hind limb of the animals was shaved and prepared with alternating scrubs of 70% ethanol and betadine. Using sterile techniques, an anterolateral approach of the thigh was used to access the femur. The vastus lateralis and biceps femoris were split exposing the femur. All muscles were dissected off the femur and the periosteum was incised along the length of the diaphysis. A 23 mm × 4 mm × 4 mm polyethylene four-hole plate (Findlay's Machine Shop, Los Angeles, CA) was secured to the femur using 0.90 mm K-wires (Zimmer Biomet, La Verne, CA) and two 1.0 surgical steel cerclage wires (Zimmer Biomet, La Verne, CA) proximally and distally. A 6-mm mid-diaphyseal, full thickness defect was then created with a high-speed burr. The area was irrigated with copious amounts of normal saline. A compression resistant matrix carrier (Mastergraft strip, Medtronic, Minneapolis, MN), made from collagen type I, hydroxyapatite and β-tricalcium phosphate [28], was cut to size and loaded with HBMCs. The carrier was then implanted in the defect, followed by a multi-layer closure with absorbable sutures. The rats received buprenorphine SR right after the procedure and antibiotics in drinking water for 5 days post-operatively. Animals had no restrictions with regards to weight bearing and diet.

The animals were randomly assigned to one of the following groups: ND-HBMC transduced with LV-TSTA BMP-2 (group I; $n = 14$); ND-HBMC transduced with LV-TSTA GFP (group II; $n = 5$); non-transduced ND-HBMC (group III; n = 5); C-HBMC transduced with LV-TSTA BMP-2 (group IV; n = 14); C-HBMC transduced with LV-TSTA-GFP (group V; n = 5); non-transduced C-HBMC (group VI; n = 5). Each donor's cells were used in at least 3–4 different groups to control for variability in cell properties between the different donors. Groups I–III received 18×10^6 cells, whereas groups IV–VI received $5x10^6$cells. All cells were delivered on a compressed resistant matrix carrier. Animals were euthanized at 12 weeks. Bone healing was assessed with plain radiographs (4, 8 and 12 weeks post-operatively) and microCT, biomechanical

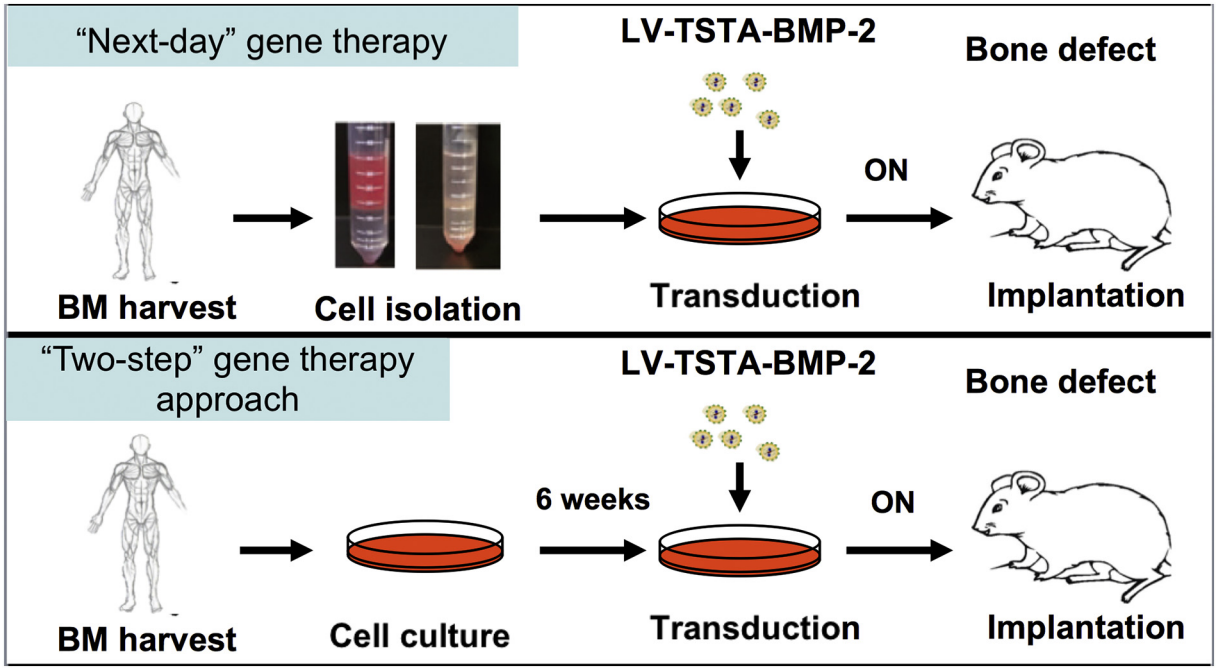

Fig. 1. Preparation of HBMC for implantation. Bone marrow was harvested from the intramedullary femoral canal of THA patients. Mononuclear cells were isolated with density gradient centrifugation and then either transduced immediately (A; "next-day" gene therapy) or culture expanded for 5 passages before transduction (B; "two-step" approach). After transduction the cells were loaded on the carrier and implanted into the femoral defect. BM; Bone marrow. ON; overnight.

Table 2
Animal groups and study design.

Group	N of animals	Treatment	X-rays	microCT	Histo	Biomechanics
I	14	ND HBMC/LV-TSTA-BMP-2	14	14	5	3
II	5	ND HBMC/LV-TSTA-GFP	5	5	5	0
III	5	ND HBMC/non transduced	5	5	5	0
IV	14	C HBMC/LV-TSTA-BMP-2	14	14	5	7
V	5	C HBMC/LV-TSTA-BMP-2	5	5	5	0
VI	5	C HBMC/non transduced	5	5	5	0

testing, histologic (H&E and Masson's Trichrome), and histomorphometric analyses post-mortem as described in the following sections (Table 2). In the two treatment groups (groups I and IV), the animals were randomized to undergo either histology/histomorphometry (5 animals/group) or biomechanical testing (9 animals/group).

2.5. Radiographic evaluation

Bone healing was evaluated with plain radiographs at 4, 8 and 12 weeks post-operatively using an Ultrafocus 60 X-ray device (Faxitron Bioptics, Tucson, AZ). A defect was considered completely healed when a bony bridge across both cortices of the defect was formed, restoring the osseous continuity of the femur. The 12-week X-rays were scored by 3 blinded independent observers, using a previously established protocol (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: complete healing) [25]. Inter-observer agreement was evaluated using kappa statistic.

2.6. MicroCT imaging

All of the operated femora were harvested post mortem and imaged using microCT to evaluate bone formation within the defect area (μCT40, Scanco Medical, Bassersdorf, Switzerland) as previously described [29,30]. All hardware was removed prior to imaging. Serial tomographic images (500 slices per sample) within the area of the bone defect were acquired at 12 um, 55 kV, and 145 mA, collecting 1000 projections per rotation at 300 ms integration time. Three-dimensional 16-bit grayscale. Images were reconstructed and rendered within a 16.4 mm field of view at a discrete density of 244,141 voxels/mm^3. Segmentation of bone from marrow and soft tissue was performed applying hydroxyapatite-equivalent density thresholds of 375 mg/cm^3 for bone, and 750 mg/cm^3 for the scaffold. Bone volume (BV), total volume (TV), and bone volume fraction (BVF:BV/TV) within the defect area were then calculated using Scanco Medical Bone Density Analysis software.

2.7. Histologic and histomorphometric analyses

Five femora per group were processed for histologic and histomorphometric analysis following microCT imaging. These specimens were fixed in 10% formalin in 4 °C for 1 week followed by decalcification in 10% EDTA for 4 weeks with gentle mechanical stirring at room temperature, and then paraffin embedding. Both transverse and longitudinal sections were acquired, using a previously described protocol [20,29]. In brief, each femur was cut transversely through the center of the defect, thus creating two distinct specimens, containing either the proximal or the distal aspect of the femoral defect. Transverse cuts were first obtained, followed by longitudinal sections. Sections were stained with Hematoxilin & Eosin (H&E) or Mason's Trichrome, using standard staining protocols, and then imaged using a Nikon's AZ100 Multizoom microscope (Nikon Instruments Inc., Melville, NY) at 1 × magnification. Bone formation within the area of the bone defect was evaluated with the Bioquant analysis software (Bioquant Image Analysis, Nashville, TN) using two transverse Masson's trichrome sections per sample (proximal and distal end). The region of interest (ROI) represented the total tissue area (TA) and was selected to include the defect, along with the scaffold and any newly formed bone. The amount of new bone within this area was then quantified (Bone area-BA). Finally, the BA/TA ratio was calculated for each end of the specimen, and then averaged for use in statistical analysis.

2.8. Biomechanical testing

The rest of the femora in groups I and IV were processed for biomechanical testing following microCT, using an established biomechanical testing protocol [26,27]. After limb harvesting and removal of the fixator and adjacent soft tissue, the specimens were wrapped in a wet (0.9% NaCl solution) 4 × 4 gauze and then frozen in -80oC. Specimens were thawed gradually the morning of testing. On the day of biomechanical testing, the proximal and distal ends of the specimens were embedded in polymethylmethacrylate, making sure to center the longitudinal bone axis with the axis of torsion. Torsional testing was performed by mounting each femur in a torsional testing fixture attached to a mechanical test machine (Mini Bionix, MTS, Minneapolis, MN). Each femur was subjected to external rotation, with the distal end rotating relative to the proximal aspect of the femur at 15°/min until failure. The contralateral, intact femora were used as controls. Peak torque and displacement, energy to failure, and torsional stiffness were calculated from the torque-rotation curves.

2.9. Statistical analysis

Statistical analysis was done with IBM SPSS 21 software. The significance level was set at 0.05. The power analysis was designed to detect differences in torsional failure between the two experimental groups. Assuming a pooled standard deviation of 0.07 units, the study would require a sample size of 9 animals for each group, to achieve a power of 80% and a level of significance of 5% (two sided), for detecting a true difference in means between the two treatment groups of 0.1 units. Five animals per group were added for histologic and histomorphometric analyses, numbers that can detect between-group differences with regards to BA/TA as previously described [20,26,27]. Thus we used a total of 14 animals per experimental group (groups I and IV) and 5 per control group (groups II, III, V and VI).

One way ANOVA and post hoc analysis with Tukey's range test was used to compare radiographic scores, microCT data (BV and BVF), and quantitative histomorphometric analysis results (BA and BA/TA) between the 6 groups. A student *t*-test was used to compare in vitro BMP-2 production between freshly isolated and cultured samples and biomechanical parameters between operated and contralateral unoperated femora. A Welch test was used for X-ray scores, and post-CT analysis and biomechanics comparisons between healed femora in groups I and IV.

3. Results

3.1. In vitro BMP-2 production

In vitro BMP-2 production following overnight transduction was

Table 3
BMP-2 production by ND-HBMC (next day gene therapy) and C-HBMC (two-step gene therapy approach) transduced with LV-TSTA-BMP-2 (expressed in ng/million cells/24 h).

Donor	Age	Sex	Next day	Two step
THA 9	52	M	20.9	NA
THA 10	51	F	23.7	79.4
THA 11	57	M	19.9	104.7
THA 12	72	M	NA	82.9
THA 13	75	F	NA	91.4/79
THA 14	57	M	17.3	90.5
THA 15	62	F	18	105.8
THA 16	56	M	23.2	NA
THA 17	59	M	17.4	NA
THA 18	60	F	NA	83.2

THA: Total hip arthroplasty, ND: next-day, C: Cultured, HBMC: Human bone marrow cells, M: male, F: female, NA: Not applicable.

quantified for study groups I and IV. ELISA confirmed successful transduction and abundant BMP-2 production by both freshly isolated and cultured HBMC transduced with LV-TSTA-BMP-2. (Table 3) Mean BMP-2 production following transduction was 20.05 ± 2.67 ng/24 h/million cells for ND-HBMC/BMP-2 and 89.6 ± 10.7 ng/24 h/million cells for C-HBMC/BMP-2 ($p < 0.001$). Non-transduced cells did not produce any BMP-2 (0.00 ng).

3.2. Radiographic evaluation of bone healing

Treatment with BMP-2 transduced "next-day" (Fig. 2) or cultured HBMC (Fig. 3) for twelve weeks demonstrated a significant increase in new bone formation compared with all negative control groups (Groups II, III, V, VI). Bone formation within the area of the bone defect was seen as early as 4 weeks post-operatively in animals implanted with BMP-2 transduced HBMCs on plain X-rays. A bony bridge spanning the defect, consistent with bony union, was noted at 8 weeks in the majority of C-HBMCs/BMP-2 treated animals (12/14; 86%) and five of the ND-HBMCs/BMP-2 defects (5/14; 36%). At 12 weeks post-op, two additional defects of group I went on to heal (7/14; 50%). No heterotopic bone formation was noted in the BMP-2 treated animals. Minimal or no bone formation was seen in defects implanted with GFP-transduced (groups II&V) or non-transduced (groups III&VI) HBMCs.

The group of animals treated with C-BMSC/BMP-2 had a higher average radiographic score (4.42 ± 1.29) versus C-BMSC/GFP (0.53 ± 0.45, $p < 0.001$) and C-BMSC/Non transduced (0.47 ± 0.30, $p < 0.001$) treated animals. ND/BMP-2 treated animals were also noted to have better radiographic healing at the 12 week time-point compared to group II ($p = 0.032$), V ($p = 0.019$) and VI ($p = 0.025$) animals. In addition, radiographic healing scores at 12 weeks were significantly higher in the cultured BMSC/BMP-2 group compared with ND BMSC/BMP-2 (mean score of 4.42 ± 1.29 vs 2.81 ± 1.99 respectively, $p = 0.031$). Finally, there was no significant difference in the radiographic scores between GFP-transduced and non-transduced next-day or cultured bone marrow groups. Inter-observer agreement for 12-week X-ray radiographic healing was strong, with a kappa statistic of $\kappa = 0.94$.

3.3. Bone formation on microCT

Treatment with BMP-2 transduced next-day or cultured BMSCs for twelve weeks demonstrated a significant increase in new bone formation compared with all negative control groups as seen on microCT. In the healed femora of groups I and IV, formation of continuous bony cortex spanning the length of the defect was noted (Fig. 4). In non-healed animals in the next-day group (7/14), two patterns were

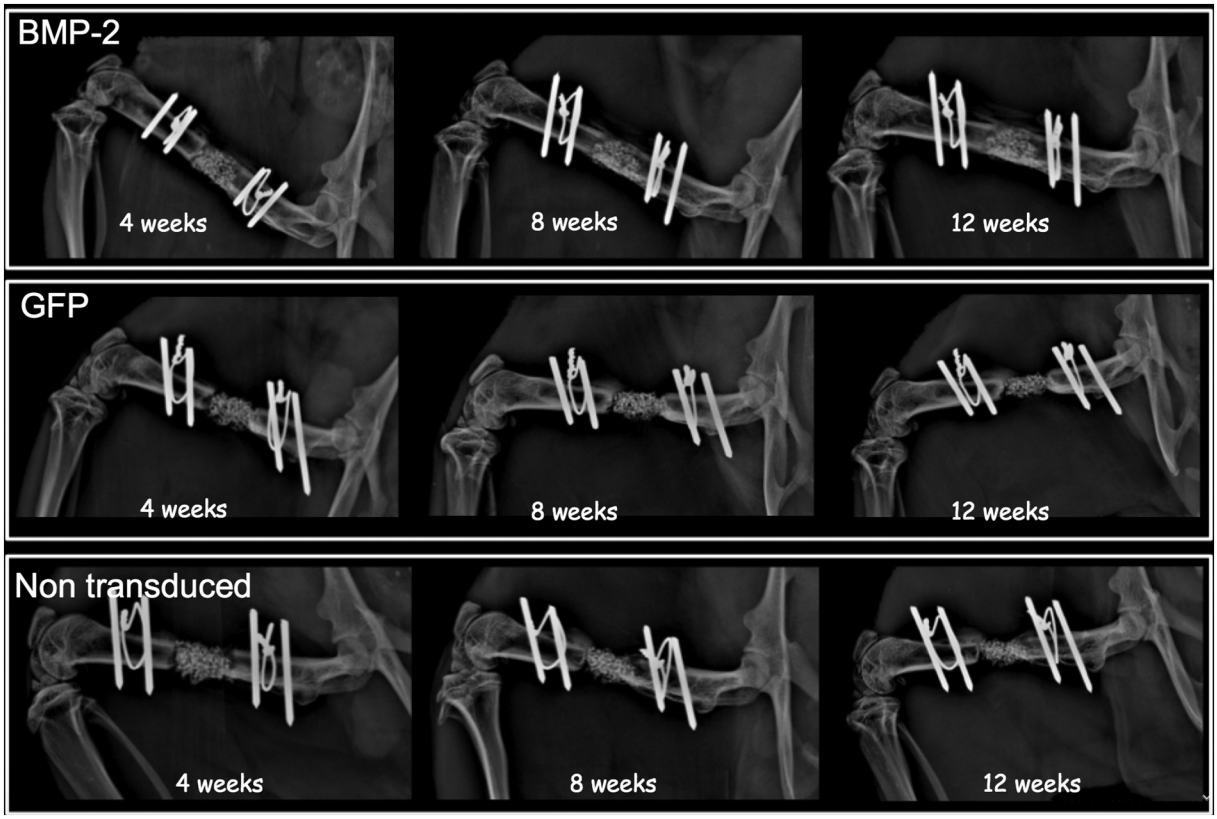

Fig. 2. Radiographic imaging of "next-day" gene therapy groups at 4-week intervals. At 4 weeks, some bone formation in the area of the bone defect, without bridging of the defect, is seen in ND-HBMC/BMP-2 treated animals. Complete healing of the femoral defect is seen in ND-HBMC/LV-TSTA-BMP2 at 8 and 12 weeks post-op. In contrast little bone formation and lack of bridging are evident in control groups II and III at all time points.

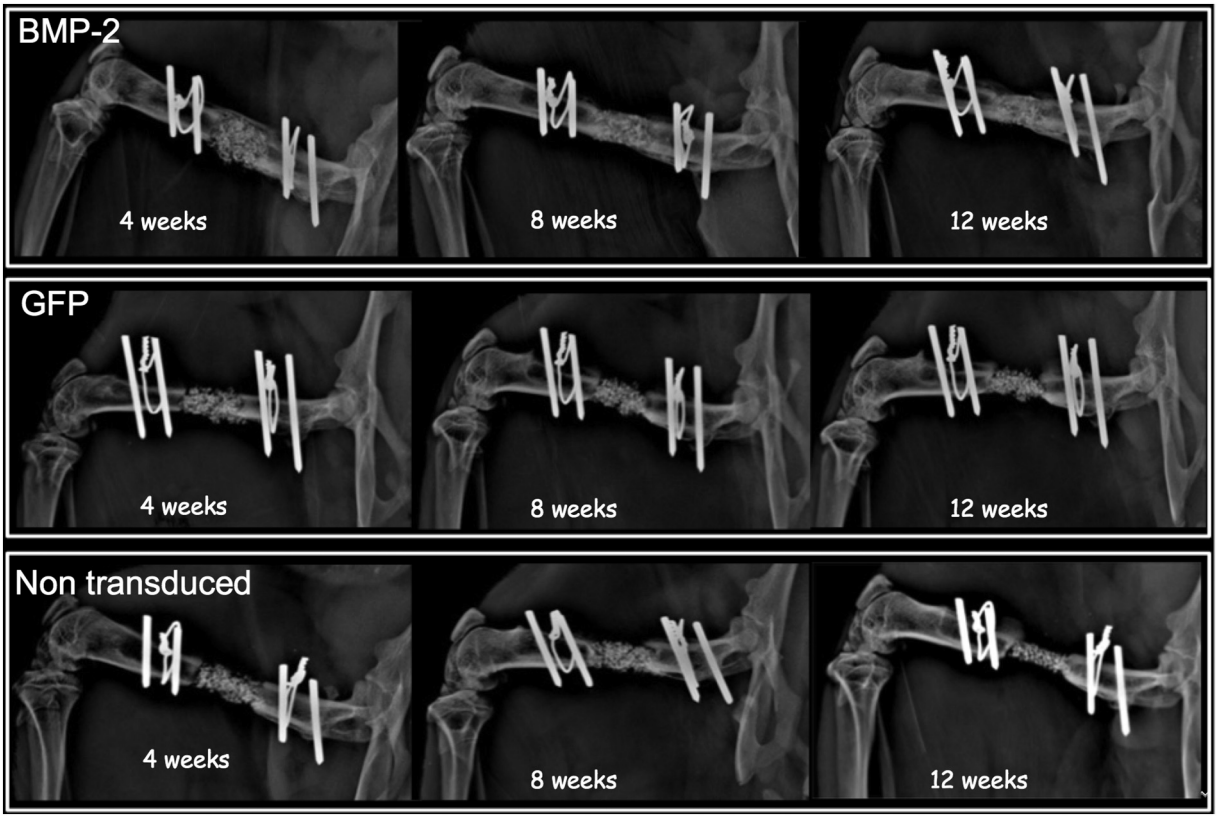

Fig. 3. Radiographic imaging of "two-step" gene therapy approach at 4, 8 and 12 weeks post-op. Bone formation within the area of the bone defect was seen as early as 4 weeks post-operatively, with complete healing achieved by 8 weeks in animals implanted with BMP-2 transduced HBMCs. Scant bone formation at the bony edges of the defect is noted in the C-HBMC control groups.

observed. In 4 animals significant new bone formation was noted outside the defect distal to the distal pin in the proximal end and proximal to the proximal pin in the distal end, but not contributing to healing of the actual defect (Supplementary Fig. 1). In the rest of the group I animals, minimal new bone formation was noted, similar to the one seen in control defects. In the 2 defects that failed to heal in the BMP-2 transduced cultured cells (group IV), no osteogenesis was noted within the defect. Finally, groups treated with the GFP-transduced HBMCs or HBMCs alone exhibited minimal bone formation within the femoral defect, and without bridging of the defect. (Fig. 4).

Post imaging volumetric assessment of new bone formation via micro-CT revealed significantly higher bone volume fractions (BVF) in C-BMSC/BMP-2 (25.1 ± 4.4%) and ND-HBMC/BMP-2 (17.3 ± 7.5%) versus all control groups. When comparing the two experimental groups, animals treated with C-BMSC/BMP-2 cells had a higher bone volume and bone volume fraction as compared to ND-BMSC/BMP-2 implanted rats ($p = 0.015$). (Table 4).

Post imaging analysis eliminating the femora that did not heal in the ND-HBMC/BMP-2 and C-HBMC/BMP-2 groups showed similar BV and BVF between the 2 groups. In detail, healed ND-HBMC/BMP-2-treated femora had an average BV: 50.02 ± 10.01 mm^3 and BVF: 22.29% ± 4.68% compared to 47.05 ± 6.75 mm^3 and 25.83% ± 3.49% in the healed C-HBMC/BMP-2 group ($p = 0.531$ and $p = 0.112$ respectively).

3.4. Histologic and histomorphometric analyses of bone formation

Histologic evaluation of the femora that healed completely in groups I and IV demonstrated abundant new bone formation at the host defect interface and across the defect, as well as reconstitution of the medullary canal. In the one femur that did not heal in the next-day gene therapy group a pattern similar to the one seen in non-unions with bony "caps" over the distal and proximal ends and fibrous tissue interposed in the defect area was noted. Finally, longitudinal sections of defects in groups II, III, V and VI demonstrated minimal bone formation in the form of bony caps at the host defect interface, scant bone within the composite carrier and fibrous tissue within the defect. (Fig. 5).

Histomorphometric analysis (Fig. 6) of transverse sections revealed similar results with regards to bone formation as seen with plain radiographs, CT and qualitative histologic examination. Group I defects were associated with a significantly higher bone area to tissue area ratio (BA/TA) when compared to groups II, V and VI ($p < 0.01$). There was also a trend towards higher BA/TA versus group III implanted animals but the difference did not reach statistical significance ($p = 0.078$). Animals treated with BMP-2 transduced C-BMSC exhibited significantly higher BA/TA compared to all negative control defects ($p \leq 0.001$). No difference in bone formation as analyzed with quantitative histomorphometry was observed between the two BMP-2 groups (groups I and IV).

3.5. Biomechanical analysis

As previously described in the material and methods section, nine animals per group for groups I and IV were randomized to undergo torsional biomechanical testing. However, only 3/9 in group I and 7/9 in group IV were considered healed and thus underwent biomechanical testing (Table 5). The contralateral intact femora were used as a control. For the 3 healed femora in group I, there were no differences with regards to stiffness ($p = 0.26$) and energy to failure ($p = 0.086$) when compared to the contralateral unoperated femora. Peak torque and displacement were significantly greater in the intact femur ($p = 0.001$ and $p = 0.003$ respectively). In group IV animals, average stiffness between the healed femur and intact contralateral limb were similar ($p = 0.418$). On the other hand, a significantly greater peak torque

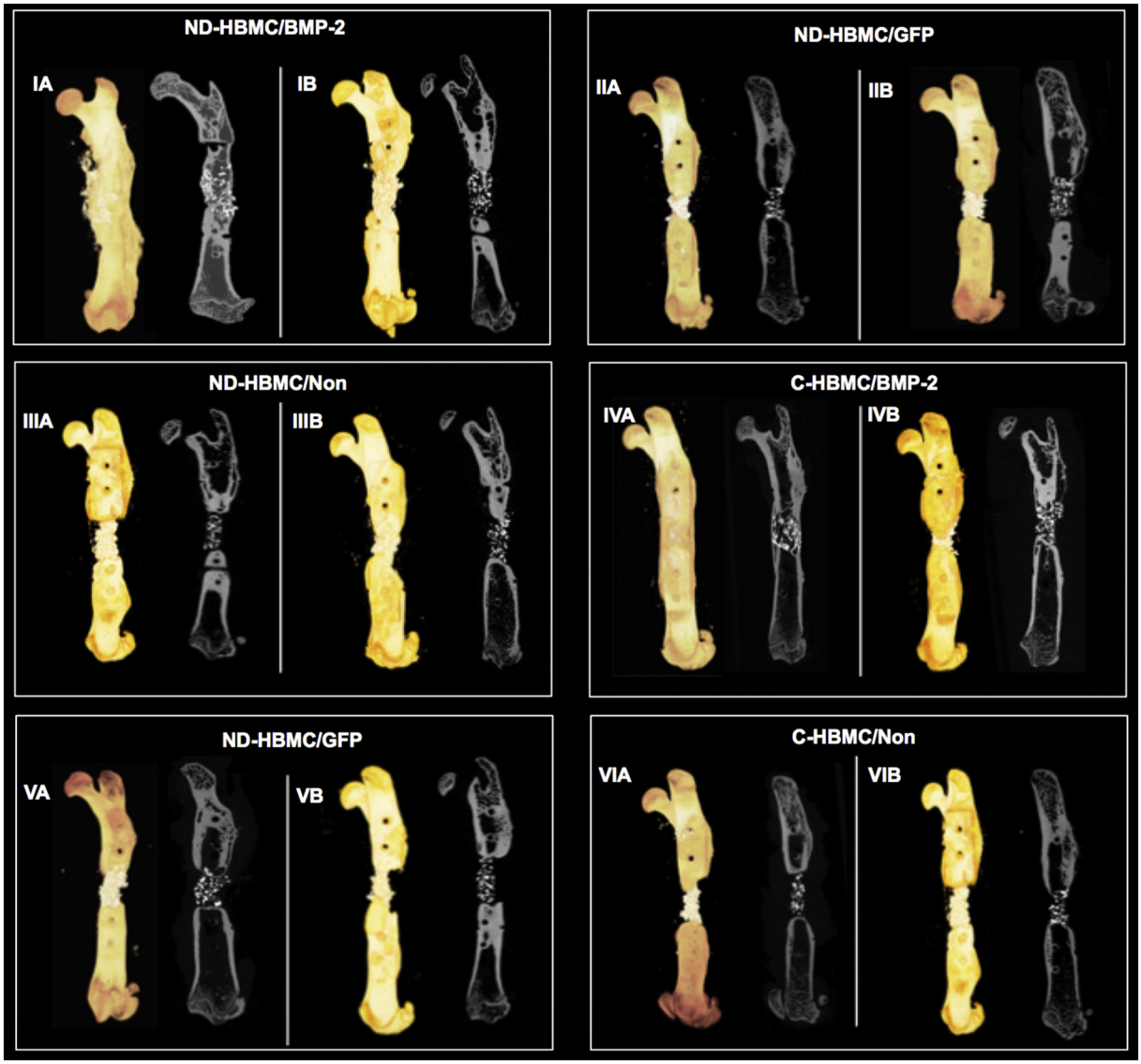

Fig. 4. MicroCT images (3D rendering and single coronal slice) of femoral defects 12 weeks following cell implantation. The best (left panel) and worst (right panel) CT outcome is presented for each group. In Group I (A), the best outcome was associated with complete bridging of the defect, while the worst one presented with only traces of newly formed bone within the defect. In control groups II and III minimal new osteogenesis is noted both in best and worst CT images (B and C respectively). In Group IV (D), the best outcome showed robust healing with reconstitution of the bony cortex at the area of the bone defect, whereas the worst outcome showed minimal -if any- bone formation across the length of the defect. Finally in groups V (E) and VI (F), both the best and worst outcomes were associated with little new bone formation.

Table 4
Post CT imaging volumetric analysis.

Groups	Bone volume (mm^3)	Bone volume fraction (%)
Group I; ND-HBMC/BMP-2	32.37 ± 17.84*	17.27 ± 7.53*
Group II; ND-HBMC/GFP	11.08 ± 5.70	5.06 ± 1.83
Group III; ND-HBMC/Non	17.67 ± 6.29	8.23 ± 2.50
Group IV; C-HBMC/BMP-2	43.84 ± 10.33*	24.24 ± 5.36*,†
Group V; C-HBMC/GFP	9.86 ± 4.56	5.38 ± 2.54
Group VI; C-HBMC/Non	9.70 ± 3.22	5.70 ± 2.29

MicroCT volumetric analysis, including calculation of BV and BVF within the defect area, for groups I–VI.

* $p < 0.05$ vs groups II, III, V, VI.

† $p < 0.001$ vs SD-HBMC/BMP-2.

($p = 0.002$), peak displacement ($p = 0.007$) and energy to failure ($p = 0.004$) were demonstrated in the intact femora. No statistically significant differences with regards to any of the biomechanical parameters were noted between healed defects in the next-day versus the two step gene therapy treated animals (groups I and IV), but this comparison was underpowered due to the low union rate in the next-day gene therapy group.

3.6. BMP-2 production and association with healing

An association between BMP-2 production and healing of the defect in ND-HBMC/BMP-2 treated animals was noted. "Next-day" gene therapy with freshly isolated cells from donors 14, 15 and 17 consistently failed to heal the defect in all implanted animals (seven). The overall levels of BMP-2 produced by the donors that did not heal the defect (D14, D15 and D17) was significantly lower compared to the donors that led to robust bone formation (D9, D10, D11 and D16) after implantation (17.6 ± 0.38 vs 21.9 ± 1.8, $p = 0.015$).

In contrast, it is unclear why 2/14 animals implanted with C-HBMC/BMP-2 did not demonstrate union, since no direct association between BMP-2 production and these two non-unions was noted in this

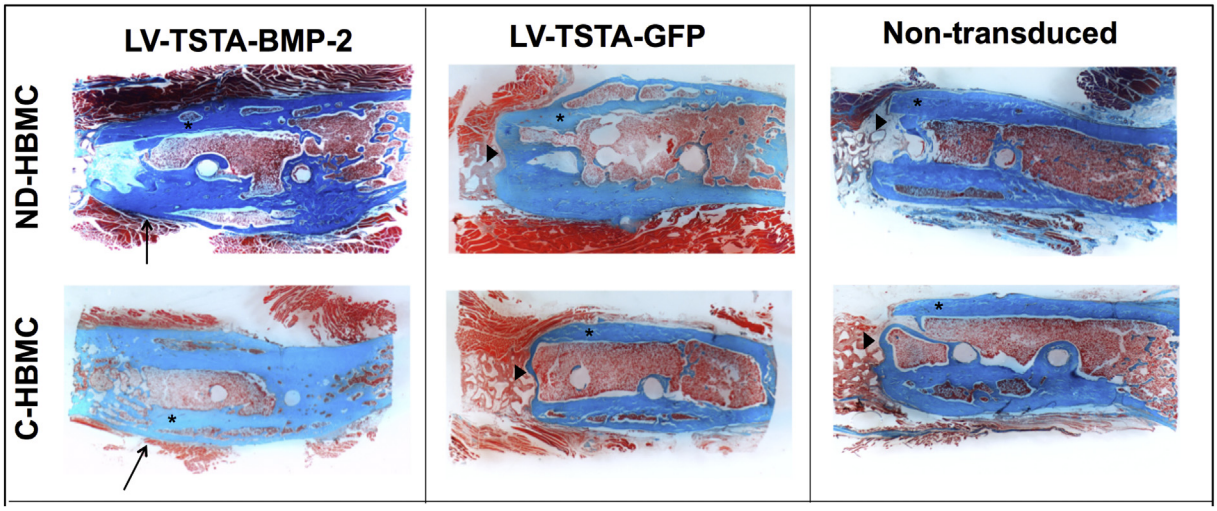

Fig. 5. Representative Masson's Trichrome stained longitudinal sections of rat femora at 12 weeks post-op. The original host cortex is labeled with an asterisk. The healed femora in group I and group IV demonstrated abundant new bone formation at the host defect interface and across the defect (arrows), as well as reconstitution of medullary canal. In contrast, defects in groups II, III, V and VI demonstrated minimal bone formation in the form of bony caps at the host defect interface (arrow heads) and fibrous tissue mixed with the composite carrier within the defect.

group; 1 animal was treated with D13 C-HBMC (BMP-2 = 91.4 ng/24 h/million cells) and the second one with D18 cells (BMP-2 = 83.2 ng/24 h/million cells). The rest of the animals implanted with cultured cells from the exact same donors (D13 and D18) healed the defect without any issues. Moreover, the implantation of cultured cells associated with lower BMP production (e.g. D10; 79.4 ng/24 h/million cells) resulted in successful bone healing.

4. Discussion

To our knowledge, this is the first study to evaluate the use of lentiviral-mediated BMP-2 delivery via human bone marrow cells to induce bone formation. The osteoinductive potential of a "next-day" and a standard "two-step" approach were assessed. Our results demonstrated that both "next-day" and cultured HBMC transduced with LV-TSTA-BMP-2 can enhance bone healing in an athymic nude rat critical-sized bone defect. In contrast, freshly isolated bone marrow cells alone and cultured bone marrow mesenchymal stem cells alone consistently failed to heal the defect. When comparing the two different gene therapy strategies, the two-step approach was associated with more consistent results, a higher union rate, and superiority with regards to all of the studied bone healing parameters. The two-step approach is associated with culture-expansion prior to transduction and implantation and it performed better than "next-day" gene therapy. The "two step" approach can be cost-effective for the treatment of difficult bone repair scenarios where there is no consistently successful treatment and amputation is often required.

Cell-based therapies using freshly isolated or cultured HBMC have been recently introduced for clinical use in bone repair applications [31–33]. The buffy coat from autologous bone marrow aspirated from patients' iliac crest, concentrated with centrifugation, and injected at the non-union site has shown promising results for the treatment of tibial diaphyseal nonunions [34,35]. Percutaneous autologous bone marrow injection has also been used in the treatment of distal metadiaphyseal tibial nonunions or delayed unions [36]. Recently, a multicenter international study evaluated the use of culture-expanded autologous BM-MSCs to augment non-union healing [37]. Twenty-eight patients with diaphyseal or metaphyseal-diaphyseal non-unions following open or closed fractures of the femur, tibia or humerus were recruited. Patients underwent BM harvesting from the iliac crest, followed by culture expansion at GMP facilities. BM-MSCs were mixed with bioceramic granules and re-implanted at the debrided non-union site with an open approach. The aim of the study was to assess the feasibility and safety of implementing such a treatment protocol, and

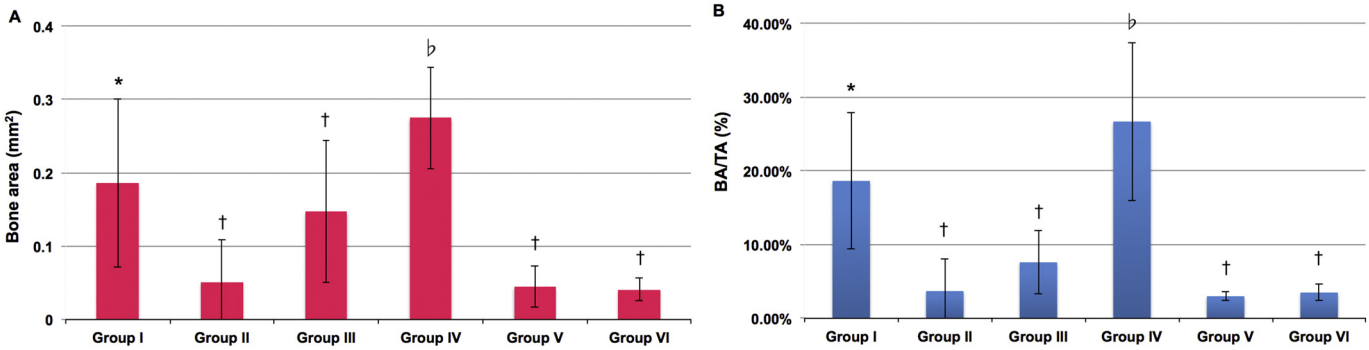

Fig. 6. Quantitative histomorphometric analysis of Masson's Trichrome transverse sections. Bone area (BA) and Bone area/Tissue area (BA/TA) for all groups are presented. BMP-2-treated defects had a higher average BA and BA/TA compared to animals treated with cells alone or GFP-transduced cells. No difference with regards to histomorphometric results was seen between the two BMP-2 groups.
* $p < 0.01$ versus groups II, V and VI, $p > 0.05$ vs groups III and IV.
♭$p \leq 0.001$ versus groups II, III, V and VI, $p > 0.05$ vs group I.
† $p < 0.05$ compared to the rest of the control groups.

Table 5
Biomechanical analysis.

Group	No of femora	Peak torque (Nm)	Peak displacement (deg)	Stiffness (Nm/deg)	Total energy to failure (Nm·deg)
Group I; ND-HBMC/BMP-2	3	125.33 ± 69.8	6.04 ± 1.22	38.35 ± 12.77	1.26 ± 1.38
Contralateral group I	3	571.29 ± 47.09	10.64 ± 0.25	54.75 ± 13.08	3.12 ± 0.34
Group IV; C-HBMC/BMP-2	7	249.72 ± 136.5	7.58 ± 2.32	38.13 ± 18.3	1.57 ± 0.5
Contralateral Group IV	7	514.09 ± 116.5	10.1 ± 1.56	45.47.29 ± 14.2	2.96 ± 0.92

not to determine efficacy. However, the authors did report that 26/28 patients demonstrated radiographic union following implantation, although 2 patients dropped out early and 3 were associated with protocol deviations. No complications following the implantation were reported. Though promising, these studies are underpowered, have a case-series design and lack control groups. In addition, BM preparation protocols are highly variable and the authors generally do not provide sufficient information to allow for protocol reproduction [33]. Moreover these cases did not include the treatment of large bone defects with compromised soft tissue envelope, in which there are concerns that HBMCs alone will not have the biologic ability to heal the defect [35].

In difficult bone repair scenarios, characterized by large segmental bone defects, limited vascularity or otherwise compromised biological environment, and in cases where other treatment modalities fail, combined delivery of osteogenic cells and an osteoinductive signal may be necessary to successfully enhance bone formation. Ex vivo regional gene therapy can serve this exact purpose by combining multipotent cells and osteoinductive factors loaded on osteoconductive scaffolds and delivered at the injury site to promote osteogenesis. Prior studies have clearly established the efficacy of ex vivo gene therapy using lentivirally-mediated BMP-2 transduction and rodent bone marrow stem cells in critical-sized long bone defects [26,38–40]. Furthermore, we have recently demonstrated that cultured human bone marrow MSCs can be successfully transduced with our lentiviral vector and lead to significant enhancement of these cells' osteogenic capacity in vitro [21,22]. Compared to recombinant proteins, that are released with an early burst and diffuse rapidly from the implantation site in the first 4 days following implantation [11], lentivirally-mediated BMP-2 production is associated with superior protein release kinetics. Ex vivo regional gene therapy has been shown to induce a sustained, low level (100–150 ng/ml/day/mg of protein) protein release lasting for at least 12 weeks after implantation [19]. Previous studies from our laboratory have clearly demonstrated the ability of ex vivo gene therapy to produce healing equal or superior to rhBMP-2 in preclinical models [20,25,26].

In a prior study we demonstrated the success of “same day” regional gene therapy in a rat critical-sized femoral defect [20]. In the present study, when comparing levels of BMP production following 3 h vs overnight transduction in 8 BM samples, it was demonstrated that BMP-2 production was at least 3× higher with the overnight transduction as compared to the 3-hour transduction ($p < 0.001$). Thus a “next-day” gene therapy strategy using an overnight transduction protocol was developed for use in this experiment. A standard “two-step” approach was also used to compare the efficacy of the two gene therapy strategies and determine which one could be more clinically relevant.

Cultured HBMC/BMP-2 were associated with a higher union rate and more consistent results compared to ND-HBMC/BMP-2. It is unclear why 2/14 animals implanted with C-HBMC/BMP-2 did not demonstrate union, especially since other animals in this group implanted with the cultured cells from the exact same donor (and thus same levels of BMP-2) healed the defect. A subacute infection cannot be ruled out, since these are nude animals and thus very susceptible to infections. Alternatively, differences in bone healing could also be a result of an immune response to the xenografted cells. In cases of discordant xenografts (e.g. human tissue or cells in a rodent host), an important aspect of the model is to minimize the rejection response by the host immune system in order to ensure survival of the transplanted xenografted cells/tissue [41]. Although, human cell engraftment has been shown to be more successful in immunodeficient compared to immunocompetent animals, previous studies have shown that certain athymic nude rats can still mount significant immune responses towards the xenografted implanted tissue [41–43]. In the study by Liu et al., it was shown that differences in healing following implantation of genetically modified sheep muscle correlated with the level of anti-donor IgG present in the serum, with high levels of anti-sheep IgG being associated with impaired healing [43].

In the “next-day” group, animals that failed to heal were the ones implanted with HBMC with a BMP-2 production of < 20 ng/24 h/million cells. These findings indicate that there may be a threshold in the amount of BMP needed to induce osteogenic differentiation in vivo and that the “next-day” implanted defects that did not heal were the ones characterized by lower BMP-2 production that failed to reach the required threshold. It is not clear why certain donors did not make adequate BMP. One explanation could be that the overall health of the cells, transduction efficiency or innate variability between cell populations obtained from different patients may affect levels of BMP production and overall osteogenic capacity of the cells. Prior studies have also reported that there is a significant variability in concentration of progenitor cells between different donors, differences that can translate to variable osteogenic responses [5,35]. In the study by Hernigou percutaneous injection of freshly isolated autologous BM in 60 tibial nonunions led to an 88% union rate [35]. The 7 nonunions that failed to heal were the ones that were characterized by a significantly lower number and concentration of progenitor cells. It is also possible that the failure of certain ND-HBMC/BMP-2 treated animals to heal may have been secondary to some type of inflammatory response associated with the high number of cells implanted into the femoral defect.

More consistent results regarding BMP levels and bone repair were observed in the cultured HBMC group. This may be because C-HBMC comprise a more homogeneous cell population. Moreover the majority of passage 5 cultured HBMC are true MSCs [44,45] and thus when transduced they may have the ability to not only secrete BMP, but also respond to the BMP in an autocrine way. In addition, differences in terms of the level, patterns and duration of transgene expression in the defects of ND-HBMC and C-HBMC groups may be present, as hinted at by a prior biodistribution study done in our laboratory using freshly isolated (SD-RBMC) and cultured rat bone marrow cells (C-RBMC) [23]. Finally the BMP-2 levels produced by the cultured HBMC were consistently higher compared to freshly isolated cells. Thus although the two-step approach is associated with culture-expansion prior to transduction and implantation, and may be considered more costly and inconvenient, it performed more consistently than “next-day” gene therapy with regards to bone healing and quality of bone formation.

It should be noted that despite the overall lower union rate in the “next-day” group, no statistically significant difference was observed between the two BMP-2 groups with respect to X-rays, microCT, biomechanical and histomorphometric analyses when only the healed femora were compared. This shows that “next-day” gene therapy may not be effective for everyone, but it has clinical potential for patients who produce adequate amounts of BMP. Therefore patients could be screened for high progenitor counts and a BMP threshold before being eligible for “next-day” gene therapy.

A limitation of this study is that we have not assessed the potential of progenitors obtained from the iliac crest versus the medullary canal. It is true that in a clinical scenario with a fracture non-union or significant bone loss in the settings of trauma or joint replacement, the bone marrow for use in ex vivo gene therapy would be harvested from the iliac crest, as is the norm. However, comparison of bone marrow harvested from the femoral canal versus the iliac crest would require a second invasive procedure (i.e. iliac crest bone marrow harvesting), unrelated to clinical care of these patients and for research purposes only. Therefore this comparison was not done in this study. Second, the comparison between "next-day" and "two-step" approach with regards to biomechanical parameters was underpowered. Our hypothesis was that both BMP-2 groups would heal the defect, with differences in torsional failure of 0.1 units. Thus we would require a sample size of 9 animals for each group, to achieve a power of 80%. Due to the lower union rate in the "next-day" group only 3 specimens were available for biomechanical analysis. However, X-rays, microCT and histomorphometry were adequately powered and detected differences between experimental and control groups. Another limitation is that a CFU assay was not performed to determine the concentration of progenitor cells in each bone marrow sample; this information could potentially explain the variability between the different donors with regards to BMP production and osteogenic potential. Finally, we did not assess the ultimate fate of the transduced cells and whether differences between cultured and freshly isolated cells exist with respect to in vivo survival, duration of BMP production, and their specific contribution to bone healing.

In conclusion, we demonstrated that human bone marrow cells, freshly isolated or culture-expanded, can be transduced with a lentiviral vector expressing BMP-2 and lead to enhanced osteogenesis in vivo as compared to non-transduced or GFP-transduced cells. The two-step approach was associated with more consistent results and a higher union rate, making this strategy a potential candidate for use in clinical practice where other clinical strategies fail. We believe that "next-day" gene therapy has clinical potential but further refinement of the protocol is necessary. We consider regional gene therapy to represent one aspect of a comprehensive tissue engineering approach to heal large bone defects. Patients with small to medium sized defects could be treated with either autologous bone graft, rhBMP or cell-based therapies with an osteoconductive scaffold. Regional gene therapy would be reserved for patients with large bone defects or those associated with a compromised biological environment that need a more prolonged osteoinductive signal to induce healing.

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Declaration of Competing Interest

None.

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