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LvBMP-2 gene-modified BMSCs combined with calcium phosphate cement scaffolds for the repair of calvarial defects in rats

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Abstract The study aims to evaluate the effect of bone marrow stromal cells (BMSCs) expressing bone morphogenic protein-2 (BMP-2) mediated by lentiviral (Lv) gene transduction combined with calcium phosphate cement (CPC) scaffolds for the repair of critical size calvarial defects in rats. BMSCs derived from Fisher 344 rats were transduced with LvBMP-2 or lentivirus encoding enhanced green fluorescent protein (LvEGFP) in vitro. Obvious osteogenic differentiation of BMSCs in the LvBMP-2 group was demonstrated by alkaline phosphatase staining and alizarin red staining. Enzyme-linked immunosorbent assay results show that LvBMP-2 gene expression in vitro can last for at least 8 weeks. Gene-transduced or untransduced BMSCs were seeded onto CPC scaffolds to repair rat calvarial defects with a diameter of 5 mm. Scanning electron microscope analysis indicated that porous CPC scaffolds facilitated initial adhesion and spreading of BMSCs onto its surface. Calvarial defects were successfully repaired with LvBMP-2-transduced BMSCs/CPC constructs 8 weeks postoperatively. The percentage of new bone formation in the LvBMP-2 group was significantly higher than in other control groups. Lentiviral mediated BMP-2 gene therapy together with CPC scaffolds can be used successfully in calvarial repair and bone regeneration.

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1 Introduction

The treatment of bone defects caused by trauma and ablative surgery remains a challenging problem in clinical situations. Although autogenous bone grafting is the current gold standard for skeletal healing, it is limited by the amount of bone available, potential donor site morbidity, and difficulty in achieving the desired bone shape [1].

Alternative methods for bone repair, such as bone tissue engineering techniques, may avoid the above shortcomings. Regional gene therapy is one of the most promising of these alternatives. Since plasmid gene delivery is still inefficient, virus-based gene therapy has been widely used to stimulate bone formation in a series of animal models [2–4]. Among various viral vectors, lentiviral vectors can transduce both dividing and non-dividing cells and insert the target gene into the host genome, thus ensuring stable and prolonged gene expression [5]. Furthermore, lentiviral vectors have minimal immunogenicity [6] and have been through a number of optimizations to improve their safety [7]. Finally, the newly formed bone induced by lentiviral-

based gene therapy systems may be of superior quality as it has, for example, better mechanical properties [8].

The synthetic CPC scaffold, which has good biocompatibility, bioactivity, and osteoconductivity, has often been used in investigations of bone tissue engineering [9, 10]. The bone morphogenic proteins (BMPs) are currently considered to be the most powerful osteogenic growth factors [11]. Among them, BMP-2 is the most potent one to induce BMSCs' differentiation into osteoblastic cells. BMP-2/BMSCs based regional gene therapy has been successfully used in the treatment of different animal bony defect models [2–4, 12, 13]. However, it is not known whether critical size rat calvarial defects are responsive to lentiviral mediated BMP-2 regional gene therapy with a CPC scaffold carrier.

In this study, the bone healing effects of LvBMP-2-transduced BMSCs/CPC were compared with those of LvEGFP-transduced BMSCs/CPC, untransduced BMSCs/CPC and CPC alone. To our knowledge, this is the first time LvBMP-2-transduced BMSCs combined with CPC have been used to repair critical size calvarial defects.

2 Materials and methods

The whole experimental protocol was approved by the Animal Care and Experiment Committee of Ninth People's Hospital affiliated to Shanghai Jiao Tong University, School of Medicine (China).

2.1 Culture and validation of rat BMSCs

Rat BMSCs were isolated from the femurs of 6-week-old male Fisher 344 rats under sterile conditions according to the protocol reported by Maniatopoulos et al. [14]. Briefly, both ends of the femora were cut off at the epiphysis and the marrow was flushed out using Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) with 10% FBS (Hyclone, Logan, UT, USA) supplemented with 200 U/ml of heparin (Sigma, St. Louis, MO, USA). Cells were cultured in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 units/ml streptomycin, supplemented with 50 mg/ml ascorbic acid, 10 mM β-glycerolphosphate, and 10^{-8} M dexamethasone at 37°C in an atmosphere of 5% CO_2. The medium was changed after 24 h to remove non-adherent cells and was then renewed three times a week. When 90% confluence was reached, BMSCs were released from the culture substratum using trypsin/EDTA (0.25% w/v trypsin, 0.02% EDTA), and were moved to dishes (10 cm in diameter) at 1.0×10^5 cells/ml in 10 ml. The characterization of rat BMSCs was validated by flow cytometry assay of CD-markers as previously described [15].

2.2 Lentiviral transduction of rat BMSCs

LvEGFP or LvBMP-2 was produced by cotransfecting FUW (with EGFP or BMP-2 gene), HIV-1 packaging vector Delta8.9 and VSVG envelope glycoprotein into 293T cell line as described previously [6]. In FUW, human ubiquitin-C promoter is used to drive EGFP or BMP-2 gene expression [16]. The woodchuck hepatitis virus post-transcriptional regulatory element (WRE) is located at downstream of target gene to increase the transcription. Plasmid was harvested in competent cell TOP10 strain with ampicillin selection.

Rat BMSCs at passage 2 were infected with LvBMP-2 and LvEGFP in the presence of 8 μg/ml polybrene at a multiplicity of infection (MOI) of 35. After 24 h, the medium was replaced with fresh DMEM with 10% FBS. Gene transfer efficiency was determined by calculating the percentage of EGFP-expressing cells among all the cells observed in the LvEGFP group [17].

2.3 ELISA for BMP-2 production in vitro

The in vitro BMP-2 production in the culture medium in each group was determined using a BMP-2 ELISA kit (R&D Systems Inc, Minneapolis, MN, USA) according to the manufacturer's instructions at weeks 1, 2, 4 and 8. The culture medium was replaced with 10 ml of fresh DMEM 24 h before the assay, then the supernatant was collected for evaluation and cell number was counted.

2.4 ALP staining and alizarin red staining

BMSCs transduced with LvBMP-2, LvEGFP, or left untransduced were evaluated for ALP staining 14 days after transduction according to the manufacturer's instructions (ALP kit, Hongqiao, Shanghai, China). Alizarin red staining was also performed in each group 14 days after transduction to detect calcium rich deposits.

2.5 Preparation of BMSCs/CPC constructs and scanning electron microscopy evaluation

Porous CPC (Rebone, Shanghai, China) disks of 5-mm diameter and 1-mm thickness were used in this study. The CPC scaffolds were prepared using leaching method in the presence of sodium chloride (NaCl) particles as porogens for producing macroporosity. Briefly, The CPC powder was mixed with water using a spatula at a powder/liquid ratio of 3:1 (g/ml) to form a cement paste. NaCl particles sieved with the sizes of 400–500 μm were added into the CPC paste. The mixture of CPC paste/NaCl particles was placed into stainless steel molds and the mixture was modeled under a pressure of 2 MPa. After stored in beakers in a constant temperature oven at 37°C and 100% relative

humidity for 2 days, the samples were then immersed in deionized water to leach out NaCl particles. Finally, these samples were dried at 50°C in an oven to obtain sponge-like porous scaffolds.

One week after gene transduction, BMSCs were detached from their culture dishes, centrifuged to remove the supernatant, and then resuspended in serum-free medium at a density of 2×10^7 cells/ml. The CPCs were

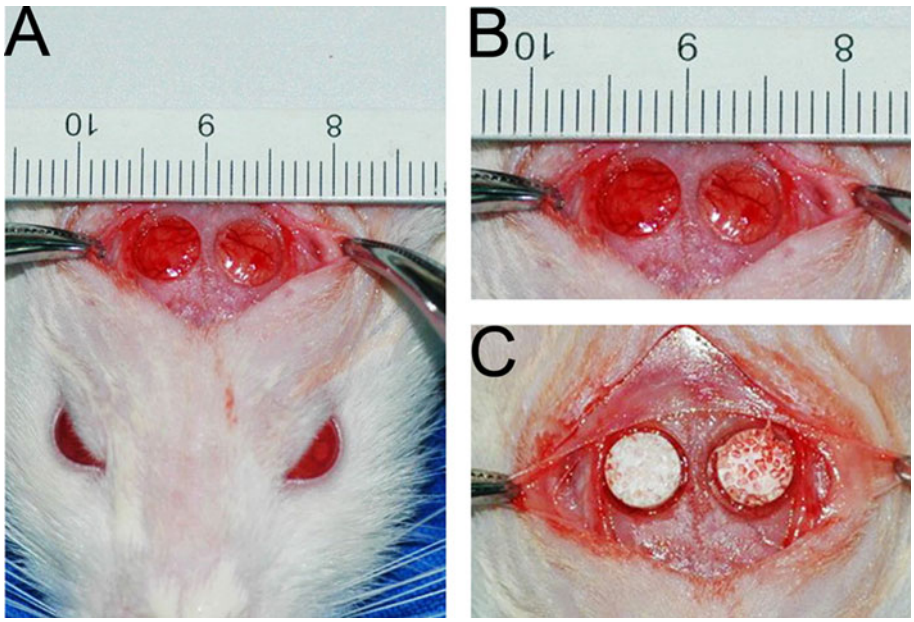

Fig. 1 Surgical procedure. **a**, **b** Calvarial defects were created and then **c** filled with different groups of CPC materials surgically

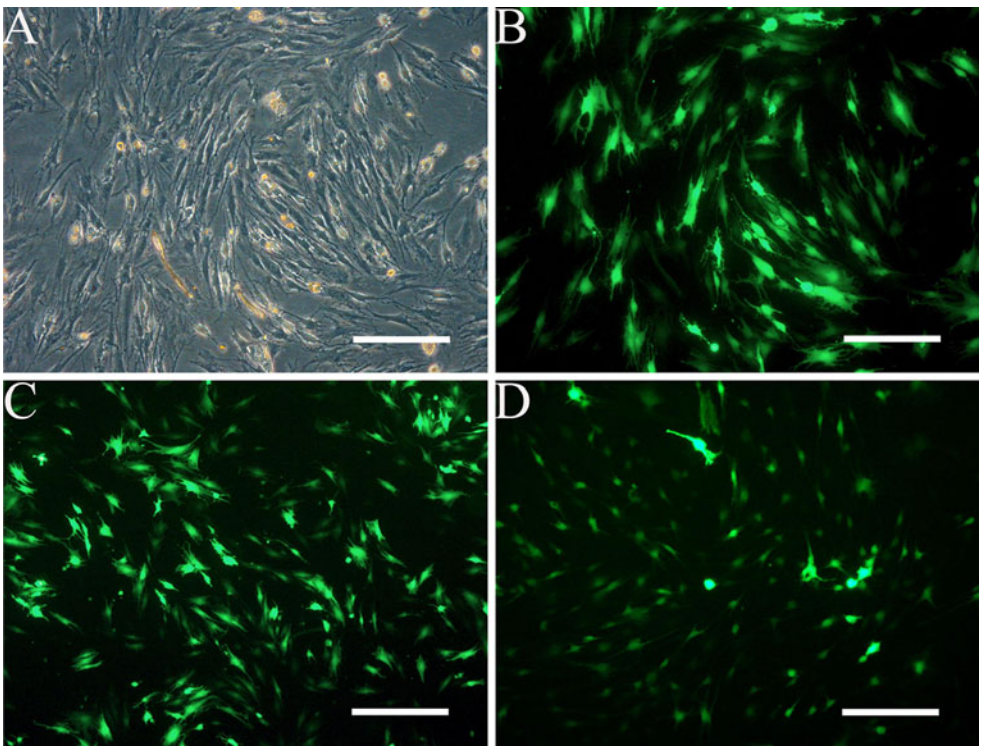

Fig. 2 EGFP expression in vitro. **a** The BMSCs grew well one week after LvEGFP transduction, with no obvious cell death observed. Over 80% of cells showed as EGFP positive at **b** 1 week, **c** 2 weeks and **d** 8 weeks after LvEGFP transduction (*Bar* 100 μm)

placed in sterile eppendorf tubes, and 15 μl cell suspension (containing 3×10^5 cells) was then dropped onto each scaffold to make it fully soaked. After an additional 4-h incubation to allow the initial attachment of cells, implantation surgeries were performed in vivo. In a parallel experiment, CPC disks of 5-mm diameter and 1-mm thickness were prepared and seeded with BMSCs at the same cell density. The extent of cell attachment and growth was assessed 4 and 24 h after cell seeding. The constructs were fixed in 2% glutaraldehyde for 2 h, cut into two halves, and then subjected for scanning electron microscopy examination (Philips SEM XL-30, Amsterdam, Netherlands).

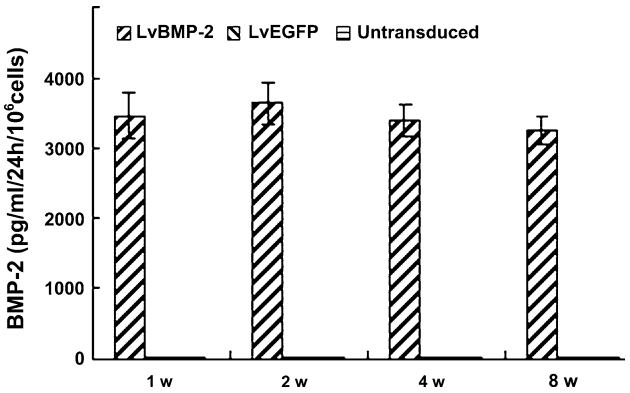

Fig. 3 ELISA for BMP-2 production in vitro. ELISA results demonstrated that the LvBMP-2 transduced BMSCs produced significantly higher BMP-2 at 1, 2, 4 and 8 weeks compared to those BMSCs transduced with LvEGFP or left untransduced ($P < 0.01$)

2.6 Surgical procedure

Fourteen 12-week-old male Fisher 344 rats were used for surgical operation. The animals were anesthetized by intraperitoneal injection of pentobarbital (Nembutal 3.5 mg/100 g). After an incision was made in the skin, bilateral full thickness defects (include the periosteum) of 5 mm diameter were created in the cranium (Fig. 1). A total of 28 calvarial defects were randomly repaired in the following five treatment groups: (1) LvBMP-2-transduced BMSCs/CPC ($n = 6$); (2) LvEGFP-transduced BMSCs/CPC ($n = 6$); (3) untransduced BMSCs/CPC ($n = 6$); (4) CPC alone ($n = 6$); and (5) blank control ($n = 4$). To compare the regenerates to the native calvarial bone, another two F344 rats without operation were left as normal control.

2.7 Micro-CT and bone mineral density analysis

All rats were sacrificed 8 weeks postoperatively. The craniums were then explanted and fixed in 4% phosphate-buffered formalin solution. The craniums of all rats were assessed using the eXplore Locus Micro-CT machine (GE Healthcare, Canada). After scanning, the micro-CT images were segmented using a nominal threshold value of 225 as reported previously [18], and a three dimensional (3D)

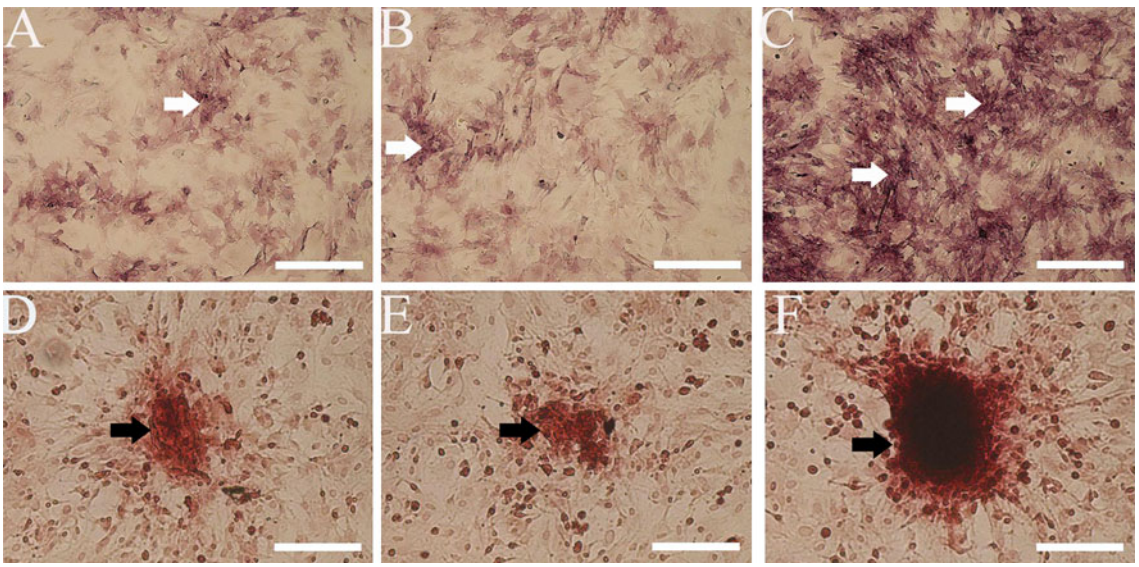

Fig. 4 ALP staining and alizarin red staining. Two weeks after gene transduction, ALP staining and alizarin red staining were apparently more pronounced in **c**, **f** LvBMP-2 transduced BMSCs than those in **b**, **e** LvEGFP transduced BMSCS or **a**, **d** untransduced BMSCs, which suggested that LvBMP-2 gene transduction promoted the BMSCs' differentiation into osteoblastic cells. *Black arrows* show ALP positive area, *white arrows* show mineralized calcium deposits (**a**–**c**, *Bar* 100 μm; **d**–**f**, *Bar* 200 μm)

morphometric analysis of bone mineral densities (the average bone mineral densities value of whole selected area)was analyzed automatically with the eXplore Micro-View v.2.2 Software (GE Healthcare, Canada). All specimens were analyzed and the average values of each group were used.

2.8 Histological and histomorphological analysis

After micro-CT analysis, all specimens were decalcified in 30% formic acid for 10 days. Samples were embedded in paraffin and serial horizontal cross sections were made. Three randomly selected cross-sections from each implant were stained with haematoxylin and eosin. Then the histologic images with magnification of 40× were analyzed by Image Pro 5.0 system (Media Cybernetics, USA). Percentages of new bone area were calculated by new bone area/whole defect area. All specimens were analyzed and the average values of each group were used.

2.9 Statistical analysis

Statistically significant differences ($P < 0.05$) among the various groups were measured using ANOVA and SNK post hoc. All statistical analysis was carried out using the SAS 6.12 statistical software package (Cary, NC, USA).

3 Results and discussion

BMP-2 is the most potent bone growth factor and was approved by USA. Food and Drug Administration (FDA) in 2002 for clinical trials in spinal fusion. However a short half-life, and high cost arising from large dose requirements in clinical situations, limit the application of these exogenous proteins [11]. Thus, effective alternative methods such as regional gene therapy are also being explored.

Adenovirus has been the most commonly used viral vector in gene therapy for bone healing. However, its application is limited by lack of long-term gene expression and complication of immune responses [8, 19, 20]. Lentivirus may be more efficient when used in a more biologically challenging environment where continuous protein production is needed. In this study, over 80% of cells were EGFP positive at one or two weeks after LvEGFP transduction (Fig. 2b, c) and a high level of EGFP expression was maintained until eight weeks (Fig. 2d). Other studies have even found that EGFP expression can last for up to 4 months [5]. Furthermore, ELISA results demonstrated that the LvBMP-2 transduced BMSCs in vitro could overexpress BMP-2 stably and continuously for at least 8 weeks (Fig. 3). Similar results were obtained by Hsu et al. [2]. Our gene expression study demonstrated that lentiviral vectors are capable of inducing stable and prolonged gene expression.

ALP is a marker of early osteogenic differentiation of BMSCs to the osteoblastic phenotype. In this study, the positive area of ALP staining in LvBMP-2-transduced

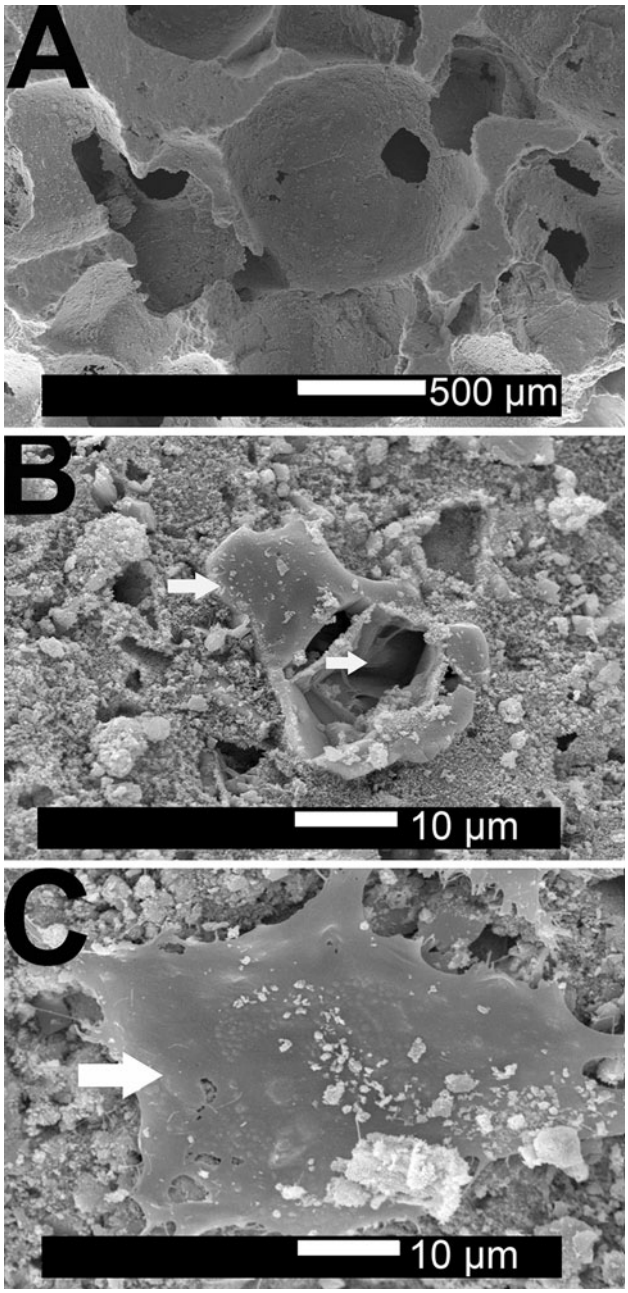

Fig. 5 Scanning electron microscopic evaluation of microstructure and biocompatibility of CPC scaffold. **a** Microstructure of porous CPC scaffold. (*Bar* 500 μm). **b** 4 h after the BMSCs were seeded onto CPC scaffold, cells could be seen attaching to the inner side of the scaffold. (*Bar* 10 μm). **c** 24 h after seeding, BMSCs were observed spreading along the scaffold surfaces (*Bar* 10 μm) *White arrows* show BMSCs

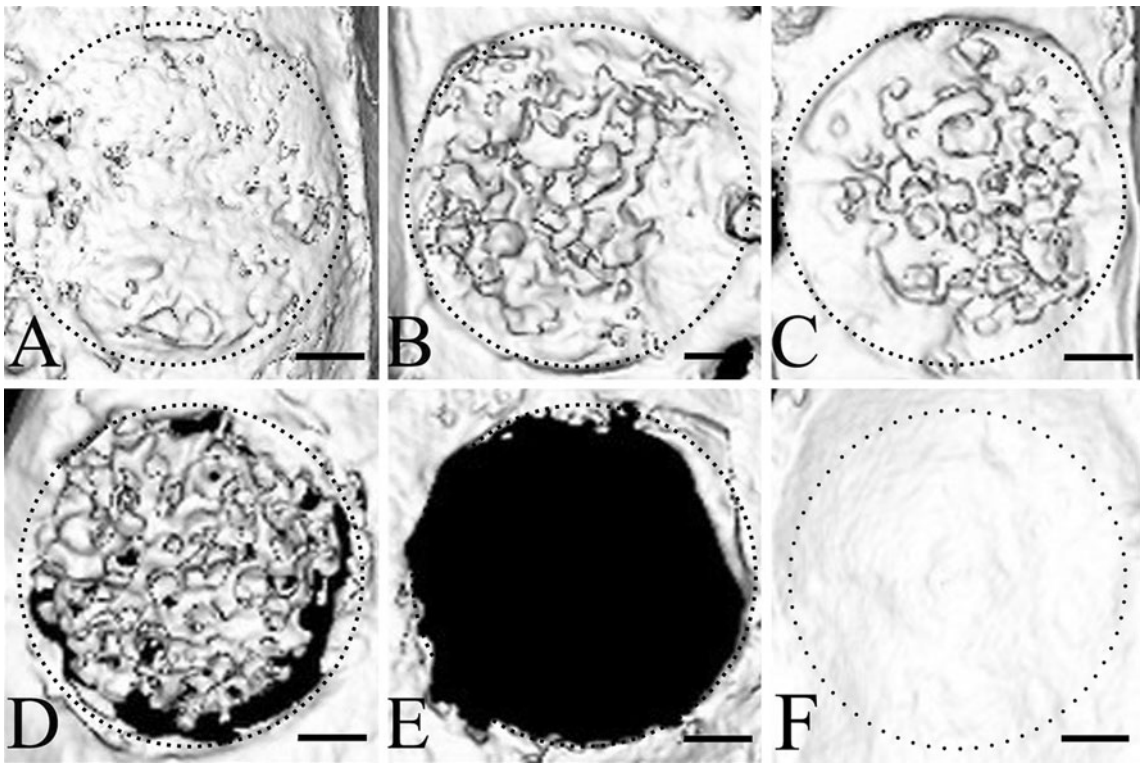

Fig. 6 Micro-CT photographs. **a** Substantial new bone formation was observed after 8 weeks in the defects which received LvBMP-2-transduced BMSCs/CPC constructs. Bone formation was less pronounced for defects filled with **b** LvEGFP-transduced BMSCs/CPC or **c** untreated BMSCs/CPC. **d** Less obvious bone formation was visible for defects treated with CPC alone. **e** In the blank control, untreated defects did not repair. **f** Normal calvarial bone. *Dotted circles* show original bone defects area (*Bar* 1 mm)

BMSCs was more pronounced compared with that in LvEGFP-transduced BMSCs and untransduced BMSCs (Fig. 4a–c). Furthermore, the mineralized nodules observed by alizarin red staining were more intense in LvBMP-2-transduced BMSCs than those in other groups (Fig. 4d–f). These results suggested that the lentiviral mediated BMP-2 gene transfer induced the BMSCs' osteogenic differentiation to osteoblastic cells.

CPC scaffold is an attractive material and has been widely investigated due to its excellent biological properties, potential resorbability, and osteoconductive capacity. In recent years, the increased macroporosity of porous CPC scaffolds may help facilitate bone in growth, implant fixation, and more rapid new bone formation [21]. In this study, scanning electron microscope analysis of CPC scaffolds indicated that the average pores were 400 ± 100 μm in size with interconnection pores of 100 ± 50 μm (Fig. 5a). Four hours after the BMSCs were seeded onto the scaffold, cells could be seen attaching to the inner surface of the scaffold (Fig. 5b). One day later, cells spreading on the implant surfaces were observed (Fig. 5c). These suggesting that CPC scaffolds has good biocompatibility and facilitated initial adhesion and spreading of BMSCs onto its surface. However, because of the mechanical weakness of porous CPC [11], more work needs to be done to evaluate the mechanical properties of this tissue engineered bone complex.

The results of micro-CT (Fig. 6) and histological analysis (Fig. 7) revealed that the LvBMP-2-transduced BMSCs/CPC construct promoted sufficient new bone formation 8 weeks after the operation. Furthermore, the bone mineral density analysis (Fig. 8) and the histomorphological analysis (Fig. 9) demonstrated that not only the quantity, but also the quality, of newly formed bone induced by LvBMP-2 gene therapy was superior to that found in the other groups. A similar study found that BMP-2-producing BMSCs mediated by lentiviral gene transfer induced more abundant bone formation within the fusion mass in a rat spinal fusion model than did BMSCs transduced via adenoviral gene transfer or recombinant protein therapy [20]. Another biomechanical testing study even demonstrated that the repaired bone at a rat femoral defect had a higher energy to failure when treated with LvBMP-2 gene transfer versus the adenoviral system [8]. The percentage of new bone area of normal bone was significantly higher than other groups

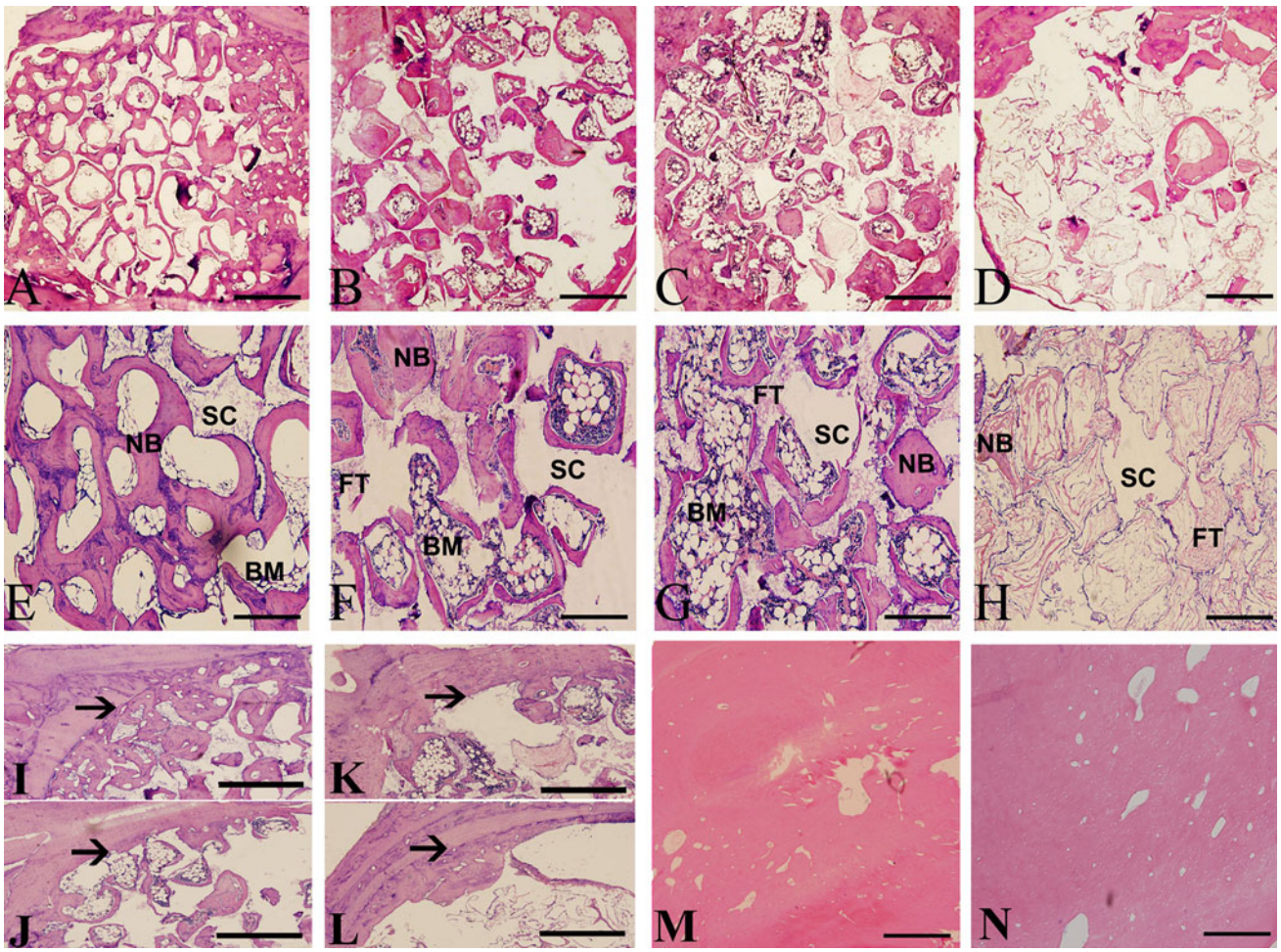

Fig. 7 Histological images. **a**, **e** Substantial mature newly formed bone tissue was observed in the LvBMP-2-transduced BMSCs/CPC group; **b**, **f** Less new bone formation were found in LvEGFP-transduced BMSCs/CPC or **c**, **g** untransduced BMSCs/CPC groups; **d**, **h** In CPC alone group, small amount of new bone formed at the edge area and more fibrous connective tissue with only a little immature bone formation was found in the central area; **m**, **n** Normal calvarial bone as control. Compared to **j** LvEGFP-transduced and **k** untransduced BMSCs groups, the integration of BMSCs/CPC construct and native bone was more closer in **i** LvBMP-2-transduced BMSCs group. Though a little new bone formed at the edge area in **l** CPC alone group, less integration could be found between scaffold and native bone. *BM* bone marrow, *FT* fibrous tissue, *NB* new bone, *SC* scaffold. *Black arrows* show the border line between scaffold and native bone. (**a-d/i–l/m**, *Bar* 1 mm; **e–h/n**, *Bar* 250 μm)

(Fig. 9) while the bone mineral density was lower than LvBMP-2 group (Fig. 8). This maybe caused by the natural radiopacity of CPC material which was not fully degraded.

Despite the advantages of LvBMP-2 gene therapy in animal models of bone regeneration, there is concern that long-term protein production may lead to heterotopic bone formation and potential oncogenic effects in surrounding cells. In this study, we did not note any serious side effects such as leukemia, immunorejection, or heterotopic bone formation. However, more preclinical experiments are required to guarantee the safety of lentiviral vectors before clinical trials.

In conclusion, the combination of CPC with LvBMP-2 gene-modified BMSCs achieved a greatly enhanced effect in repairing critical size rat calvarial defects. Lentiviral mediated BMP-2 gene therapy together with CPC scaffolds might therefore be a promising alternative approach for bone tissue engineering, especially in bone regeneration for large defects.

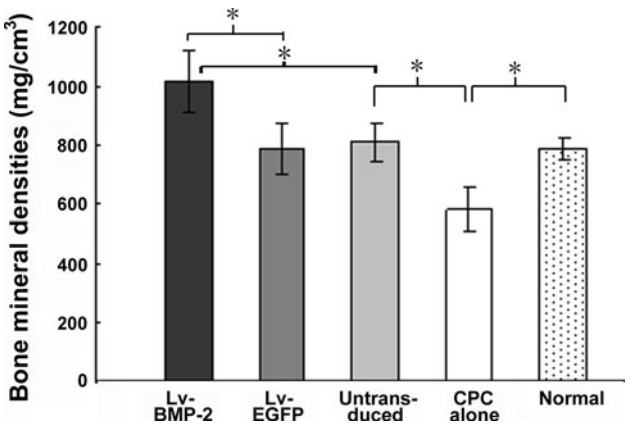

Fig. 8 Bone mineral density analysis. The bone mineral density in the LvBMP-2-transduced BMSCs/CPC group (1011.00 ± 104.63 mg/cm^3) was significantly higher than LvEGFP-transduced BMSCs/CPC group (789.75 ± 85.14 mg/cm^3, $P < 0.05$) and untransduced BMSCs/CPC group (804.50 ± 63.30 mg/cm^3, $P < 0.05$), which was also significantly higher than the CPC alone group (574.25 ± 74.85 mg/cm^3, $P < 0.05$) and normal calvarial bone (795.36 ± 36.15 mg/cm^3, $P < 0.05$). The differences between LvEGFP-transduced BMSCs/CPC group and untransduced BMSCs/CPC group were not statistically significant ($P > 0.05$), and both were comparable to the normal control ($P > 0.05$). The bone mineral densities of these three groups were both higher than the CPC alone group ($P < 0.05$). * $P < 0.05$

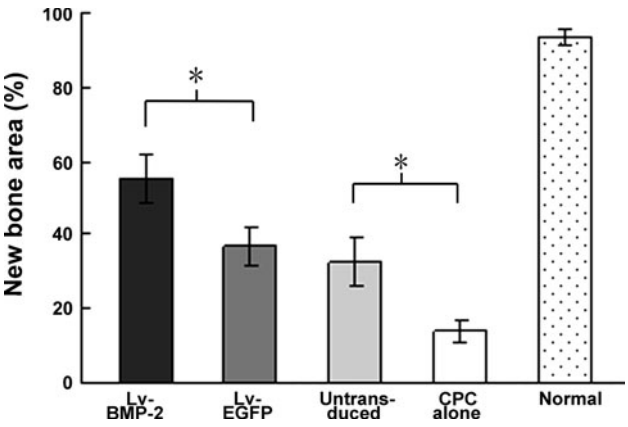

Fig. 9 Percentage of new bone area analysis. Though lower than the normal bone (93.49 ± 2.38%, $P < 0.01$), the percentage of new bone area in the LvBMP-2-transduced BMSCs/CPC group (56.70 ± 6.40%) was significantly higher than in other groups ($P < 0.05$). There was no significant difference between LvEGFP-transduced BMSCs/CPC group (36.41 ± 5.47%) and untransduced BMSCs/CPC group (32.60 ± 6.41%; $P > 0.05$), although both were higher than in the CPC alone group (13.89 ± 2.95%, P < 0.05). * $P < 0.05$

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