Original Article

Bone formation in rabbit's leg muscle after autologous transplantation of bone marrow-derived mesenchymal stem cells expressing human bone morphogenic protein-2

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Abstract

Background: To test whether autologous transplantation of bone marrow-derived mesenchymal stem cells (BM-MSCs) expressing human bone morphogenic protein-2 (hBMP-2) can produce bone in rabbit leg muscles.

Materials and Methods: MSCs were isolated from BM of the iliac crest of rabbits and then infected with lentiviral vectors (LVs) bearing hBMP-2 and green fluorescent protein under the control of the cytomegalovirus (immediate early promoter). Differentiation of transduced MSCs to osteoblasts *in vitro* was evaluated with an alkaline phosphatase activity assay and immuohistochemistry against osteoblast specific markers. MSCs expressing hBMP-2 were placed in an absorbable gelatin sponge, which was then transplanted into the gastrocnemius of rabbits from which MSCs were isolated. Bone formation was examined by X-ray and histological analysis.

Results: LVs efficiently mediated hBMP-2 gene expression in rabbit BM-MSCs. Ectopic expression of hBMP in these MSCs induced osteoblastic differentiation *in vitro*. Bone was formed after the MSCs expressing hBMP-2 were transplanted into rabbit muscles.

Conclusion: Ectopic expression of hBMP-2 in rabbit MSCs induces them to differentiate into osteoblasts *in vitro* and to form a bone *in vivo*.

Key words: Autologous transplantation, bone morphogenic protein-2, lentiviral vector, mesenchymal stem cells
MeSH terms: Autologus, transplantation, stem cells, mesenchymal, bone marrow

Introduction

Bone defects from congenital anomalies, trauma, infection, or tumors are common clinical problems. Autologous or allogeneic transplantation of osteoblasts are popular strategies to treat bone defects. However, osteoblast transplantation has been limited due to the lack of available transplantable cells. In recent times, tissue engineered transplantable bones have been developed to meet the clinical needs to treat bone defects.[1-3] This strategy requires the use of various growth and differentiation factors, which unfortunately have short half-lives *in vivo*. Thus, cells over-expressing the genes encoding these factors have been implemented. For example, cells transfected with the bone morphogenic protein-2 (BMP-2) gene stably express BMP-2 in artificial tissues.[4] A successful tissue engineering technique requires transplantable cells, signaling molecules for bone formation and a scaffold to support cell proliferation.

Mesenchymal stem cells (MSCs) can be isolated from various adult tissues, such as bone marrow (BM), fat, bone, placenta, and skeletal muscle.[5-7] Under appropriate conditions, MSCs can differentiate into osteogenic, chondrogenic, and adipogenic cells.[8-11] For example, the over-expression of osteogenic factors, including BMP-2, induces MSCs to differentiate into osteoblasts.[12] Thus, MSCs have been thought as a useful cell source for bone tissue engineering.

BMP-2 is a member of the transforming growth factor-β superfamily. It stimulates heterotopic bone growth, enhances the healing of bone defects, and strongly induces *de novo* bone formation at orthotopic and heterotopic sites.[13-17] Expression of BMP-2 in MSCs has been shown to induce

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MSC differentiation into osteoblasts *in vitro* and bone formation *in vivo* in mice, rats, rabbits.[18] Cheng *et al.*[19] used a recombinant adenoviral vector to deliver the human BMP-2 (hBMP-2) gene into rabbit BM-derived MSCs. These BM-MSCs expressed BMP-2, differentiated into osteoblasts expressing osteogenic marker genes, such as alkaline phosphatase (ALP), osteocalcin and collagen I, *in vitro*, as well as formed new bones when transplanted *in vivo*. Sugiyama *et al.*[20] used a lentiviral vector (LV) to deliver hBMP-2 into rat BM-MSCs and achieved a long term BMP-2 expression, which resulted in bone formation in immune deficient mice. LVs can efficiently infect relatively quiescent stem cells and incorporate into the host genome, achieving long term transgene expression. Furthermore, LVs have been shown to be less immunogenic.[21-24] Artificially synthesized biodegradable poly (lactic acid) has been widely used as the scaffold for bone construction *in vitro* and *in vivo* because it can be hydrolytically degraded by de-esterification.[25-29] In contrast, absorbable gelatin sponge is composed of purified porcine derived gelatin.[30] This material is cheap, easy to obtain and has been extensively used in the clinics for years.

In this study, we used rabbits as an animal model to develop a simple and efficient MSC autologous transplantation approach to generate new bone. This approach should be suitable for future clinical application.

Materials and Methods

New Zealand white rabbits (8-10-month-old) were used for this study. Rabbits were housed in a clean environment according to the criteria set by the Ministry of Science and Technology of the People's Republic of China.[31] The experimental procedure was approved by the animal ethics committee at the Central South University of China (approval ID: SCXK2009-0012).

Isolation and culture of MSCs

MSCs were isolated from the BM of rabbits as described previously.[32] Briefly, a rabbit was anesthetized by 2% pentobarbital sodium. BM was aspirated from its iliac crests using 18G needle. After lysing the red blood cells, we cultured cells in DMEM/F12 supplemented with 10% fetal bovine serum (Gibco BRL) at a density of 1×10^5 cells/cm^2. Cells were incubated at 37°C with 5% CO_2. Culture medium was changed every 3 days to remove nonadherent cells. After 7-10 days, adherent cells were trypsinized and replated in plates according to the experimental design.

Flow cytometry

After passaging the third time, MSCs were harvested for flow cytometry analysis to determine whether they expressed MSC surface markers. Cells were stained with primary mouse anti rabbit monoclonal antibodies (anti-CD29, anti-CD44, anti-CD105, anti-CD34, anti-CD45, anti-CD146, and anti-HLA-DR) and then stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG. An identical antibody isotype was used as a negative control. All antibodies were purchased from Roche Biotechnology Inc., (Roche, Switzerland). Stained cells were then subjected to flow cytometry (EPICS® ALTRA™, USA). All analyses were performed in triplicate.

Viral production and tittering

A LV bearing hBMP-2-green fluorescent protein (GFP) under the control of the cytomegalovirus (CMV) immediate early promoter was kindly provided by Dr. Pu Qin (Department of Biochemistry, The Fourth Military Medical University, PR of China). Viral particles were produced and tittered as described previously.[33] Briefly, LV-hBMP-2-GFP viral vectors were produced in 293 cells by co-transfecting cells with the LV-hBMP-GFP transfer plasmid and three packaging plasmids. Viral vectors were purified by centrifugation, and viral tittering was assayed by measuring the ability to infect 293 cells.

Lentiviral transduction

MSCs (5×10^4 cells/well) were seeded into a 6-well plate and cultured for 24 h before lentiviral infection. Viral infection was carried out in 1 ml of serum-free growth medium in the presence of 6 g of polybrene (Sigma, USA) at multiplicities of infection (MOI) of 1, 10 and 100. After the cells were incubated with medium mixed with viral vectors at 37°C and 5% CO_2 for 1 h, viral medium was replaced with 3 ml of fresh growth medium. The GFP expression in infected cells was monitored with a fluorescence microscope to estimate transduction efficiency. An optimal MOI had a high transduction efficiency and low rate of cell death.

ALP activity assay

The ALP activity of the MSCs was measured using an ALP detection kit (Sigma, St. Louis, MO) according to manufacturer's instruction. MSCs were trypsinized and lysed with TritonX-100 lysis buffer. The ALP activity was expressed as the optical density times 100.

Immunohistochemical staining

Immunohistochemical staining was performed as previously described.[34] Briefly, cultured cells were rinsed with phosphate-buffered saline, and then fixed in cold acetone for 5-10 min. After blocking with normal horse serum (1:20) for 10 min, the cells were incubated with primary antibodies at 37°C for 60 min followed by incubation with biotinylated anti-goat IgG (1:1000) at room temperature for 30 min. To detect hBMP-2, a goat anti-human BMP-2 antibody (1:800) was used, and a goat anti-rabbit osteocalcin antibody (1:400) was used to detect osteocalcin.

Labeled cells were then stained with 3, 3-diaminobenzidine solution at room temperature for 3-10 min. Samples were also counterstained with hematoxylin, and then dehydrated and mounted. Stained cells were imaged with an Olympus microscope (Olympus, Japan) connected to an Olympus camera.

In situ hybridization

To determine type I collagen expression in MSCs, we performed *in situ* hybridization as described previously.[35] The type I collagen specific probe used in the *in situ* hybridization was 5'-GATTGTGGGATGTCTTCGTCTT-3'.

Cell transplantation and examination of bone formation

At 3 weeks after viral infection, MSCs transduced with LV-hBMP-2-GFP or mock from each rabbit were harvested and transplanted, respectively, into each gastrocnemius of the same rabbit. For transplantation, rabbits were anesthetized with Phenobarbital (150 ~ 200 mg/kg), and a 4-cm incision was created in each hind limb. Ten million cells were loaded into an absorbable gelatin sponge, which was then implanted into a gastrocnemius muscle. Rabbits were administered Gentamicin antibiotics for the following 3 days.

New bone formation in the hind limb of each rabbit was examined using X-ray every 2 weeks for up to 12 weeks post cell transplantation. Rabbits were then sacrificed using air embolism, and ossified tissues were harvested for histological analyses. To prepare tissue sections, the harvested tissues were cut into cubes of 1.0 cm × 1.0 cm × 0.3 cm in size and fixed in 10% neutral formalin. After decalcifying in 10% nitric acid and 2% sodium hydroxide, the tissues cubes were dehydrated in a gradient series of alcohol solutions, embedded in paraffin, and cut into 5 μm sections. The sections were stained with H and E dyes before being imaged [Figure 1].

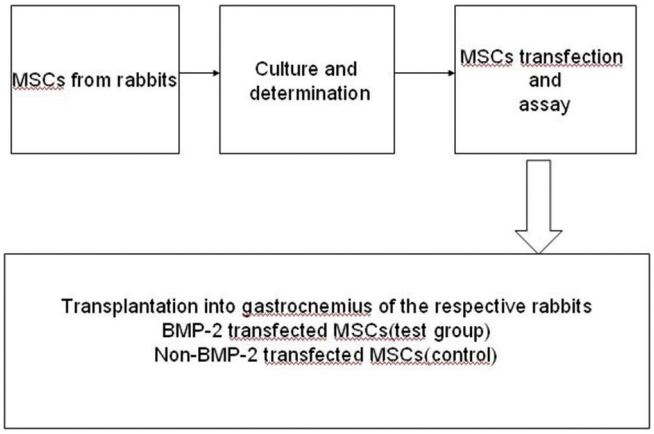

Figure 1: Flow diagram

Statistical analyses

Statistical analyses were performed using Statistical Product and Service Solutions 17.0. Paired-Student's *t*-test was used. $P < 0.05$ was considered as statistically significant.

Results

Characterization of MSCs

MSCs were isolated from six rabbits (three males and three females). They were long and spindle-like. After three passages, MSCs from each rabbit were harvested and analyzed with flow cytometry for the expression of MSC surface markers. Most cells (70%) expressed high levels of CD29, CD44 and CD105 and low levels of CD34, CD45 and HLA-DR (data not shown), indicating that they were MSCs.

LVs efficiently transduce MSCs

To determine whether LV-hBMP-2-GFP could transduce MSCs efficiently, MSCs (5×10^4) were infected with LV-hBMP-2-GFP at MOI of 1, 10 and 100 in 6-well plates. At day 3, percentages of cells expressing GFP were 30%, 50%, and 60% at a MOI of 1, 10, and 100, respectively, when analyzed by flow cytometry. A representative of fields of MSCs expressing GFP which infected with LVs at MOI of 100 [Figure 2a].

hBMP-2 expression in MSCs transduced with LV-hBMP-2-GFP

Next, we examined the expression of hBMP-2 in MSCs infected with LV-hBMP-2-GFP at MOI of 100 by immunohistochemical analysis. Nearly 85% cells expressed hBMP-2 [Figure 2b and c], suggesting that the LVs efficiently induced ectopic expression of hBMP-2 in MSCs.

Osteogenic activity in MSCs transduced with LV-hBMP-2-GFP

To determine whether the ectopic expression of hBMP-2 in MSCs induced osteogenic differentiation, we first measured the ALP activities in LV-hBMP-2-GFP or mock transduced cells at various time points post viral infection. As shown in Figure 3a, the ALP activity increased in LV-hBMP-2-GFP transduced cells gradually with time and reached a maximum at day 18 post viral infection.

We then examined whether osteoblast-specific markers, osteocalcin and type I collagen, were expressed in LV-hBMP-2-GFP transduced cells using immunohistochemical staining and *in situ* hybridization, respectively. At 4 weeks post viral infection, 75% LV-hBMP-2-GFP transduced cells expressed osteocalcin and type I collage. Taken together, these data suggest that ectopic expression of hBMP-2 induced osteogenic differentiation of MSCs [Figure 3b].

MSCs expressing hBMP-2 form bones *in vivo*

We then investigated whether MSCs expressing hBMP-2 could form bones *in vivo*. For this purpose, MSCs transduced with LV-hBMP-2-GFP were loaded in an absorbable gelatin sponge, and then the sponge was transplanted into gastrocnemius muscles of the rabbit from which the MSCs were isolated. The vital signs of six rabbits receiving transplantation were monitored every day. No apparent immune reactions, such as redness of transplantation site, fever, wound infection and local inflammatory secretions, were observed. X-ray examination revealed new bone formation in the left gastrocnemius muscles (receiving LV-hBMP-2-GFP transduced MSCs), but not in the right gastrocnemius muscles (receiving mock transduced MSCs) of six rabbits [Figure 4a]. At 12 weeks, the newly formed bones were confirmed by histological analysis [Figure 4b and 4c].

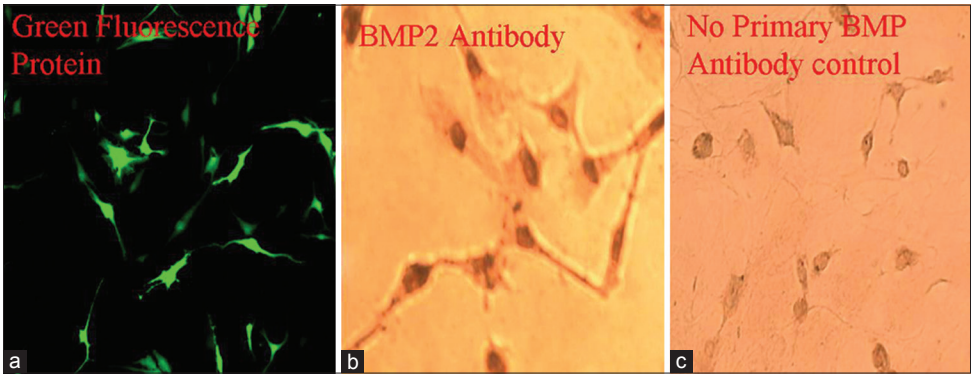

Figure 2: Lentiviral vector (LV)-human bone morphogenic protein-2 (hBMP-2)-green fluorescent protein (GFP) efficiently transduced mesenchymal stem cells, which were infected with LV-hBMP-2-GFP at multiplicities of infection of 100 (a) Expressions of transgenes were observed by fluorescence microscopy and immunohistochemical analysis against hBMP-2 (b and c)

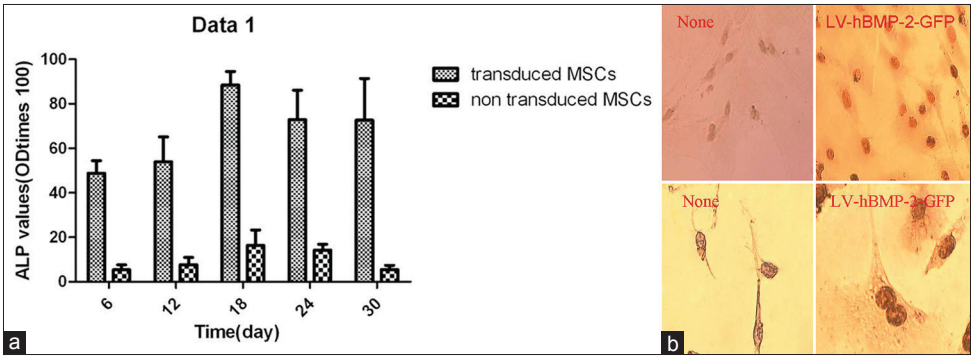

Figure 3: Osteogenic activity of lentiviral vector (LV)-human bone morphogenic protein-2 (hBMP-2) transduced mesenchymal stem cells *in vitro*. Mesenchymal stem cells were infected with LV-hBMP-2-green fluorescent protein at multiplicities of infection of 100. (a) Bar diagram showing ALP activity at various time points were measured and reported as optical density (OD) times 100. The values are shown as the average of the OD plus standard derivation from six samples. (b) Immunohistochemical analysis was used to detect osteocalcin and *in situ* hybridization was used to detect type I collagen

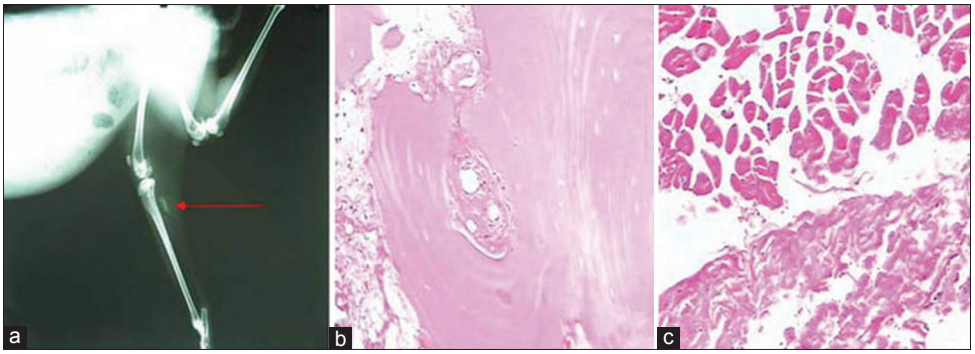

Figure 4: Lentiviral vector (LV)-human bone morphogenic protein (hBMP) transduced mesenchymal stem cells (MSCs) form bones *in vivo*. Ten million mesenchymal stem cells (MSCs) transduced with LV-hBMP-2-green fluorescent protein (left muscle) or mock (right muscle) were loaded into an absorbable gelatin sponge, which was then transplanted into a gastrocneminus muscle of the same rabbit from which the MSCs were isolated. (a) Bone formation shown by X-ray rabbits hind foot. The red arrow points to newly formed bone. (b) Newly formed bone stained with H and E. (c) Normal muscle tissue stained with H and E

Discussion

Musgrave *et al*.[36] have compared five different cell types include a BM stromal cell line, primary muscle derived cells, primary BM stromal cells, primary articular chondrocytes, and primary fibroblasts in *ex vivo* gene therapy to produce bone, and demonstrated that BM stromal cells showed more responsiveness to recombinant human bone morphogenetic protein-2. In this study, we demonstrated that ectopic expression of hBMP-2 in rabbit BM-derived MSCs could induce the MSCs toward osteogenic differentiation *in vitro* and *in vivo*. Furthermore, we successfully used a simple and economic scaffold to induce new bone formation in the muscles of rabbits after transplanting MSCs expressing hBMP-2. Isolated MSCs were long and spindle-like and expressed MSC-specific surface markers, including CD29, CD44, CD105, CD34, CD45, CD146 and HLA-DR (data not shown), which is consistent with other groups.[37] These MSCs were transduced efficiently with LVs bearing two transgenes, hBMP-2 and GFP, and they expressed both transgenes [Figure 1]. Transduced cells altered their morphology from long spindle-like to triangle-like. Moreover, these cells had increased ALP activity and expressed osteocalcin and type I collagen, which are specific markers for osteoblasts[38] [Figure 2]. After the cells were transplanted into the same rabbits from which the MSCs were isolated, new bone formation was observed [Figure 3]. Our data were consistent with another report, concluding that hBMP-2 is an osteoinductive factor.[39]

Ectopic expression of hBMP-2 in MSCs from various tissues of mouse, rat and rabbit has been mediated with adenoviral, AAV, and LVs. In this study, we successfully used LVs to mediate both hBMP-2 and GFP simultaneously. The CMV promoter once was used as the standard promoter in most BMP transgene expression systems.[40] However, Ferreira *et al*.[41] have proved a fact that elongation factor-1α, (EF-1α), β-actin and GAPDH promoters are more efficient than the viral promoters such as CMV in driving gene expression, and provided the first *in vitro* evidence for a safe alternative to viral methods that permit efficient BMP-2 gene delivery and expression in MSCs. We have no experiences about above cellular promoters. Based on our laboratory experience and convenience, we always used to apply CMV as a promoter since 2000. Moreover in practice, we also acquired satisfactory results.

At a MOI of 100, 65% MSCs expressed both transgenes [Figure 1], suggesting that LVs can efficiently transduce BM-derived MSCs *in vitro*. At day 28 post viral infection, GFP expression was still apparent, suggesting that long term transgene expression was obtained. Long term hBMP-2 expression is one of the advantages of using LVs, but may cause a safety concern due to its integration into transcriptional active regions in chromosomes.

In this study, we applied lentiviruses as transgene vehicle. Many authors have explicitly addressed the advantages and disadvantages about nonviral and viral gene transfer.[42,43] Nonviral vector has advantages such as low immunogenicity, relative safety. However, the efficiency of transfection is relative lower and short transgene expression. Transgene electrotransfer is first highly efficient nonviral gene transfer method for most primary cells and for hard-to-transfect cell lines; it looks like a promising method. Ferreira *et al*.[41] have compared a BMP-2 secretion rate of the control under CMV, eIF4A1, EF-1α, β-actin, GAPDH, fibronectin or osteocalcin promoters using electrotransfer into rat MSCs and showed that BMP-2 production correlated with sustained (up to 21 d) hBMP-2 mRNA expression. But some authors also pointed out that the major demerits of which are the cost and the induced cell mortality, and improvements must be made to increase the expression efficiency of the transfected cells stable expression of transgene.[44] Furthermore, in the past recombinant adeno-associated virus and lipidosome transfection reagent Fugene6 were used in our laboratory, we found the transfection efficiency of lipidosome transfection reagent Fugene6 was relatively lower. In this study, we did not observe oncogenicity in rabbits until 12 weeks after autologous cell transplantation. Another safety concern of using LVs is the potential to generate replication-competent lentiviruses. We carefully recommended that LV is a high efficiency and relative safety transgene vehicle. Meanwhile, further investigations to evaluate the bio-safety of using LVs are needed in large-scale animal experiments before they can be implemented in the clinics.

Previous studies often used complicated and expensive scaffolds to load cells for bone formation.[45] And Lü *et al*.[46] have described the osteogenic differentiation after β-tricalcium phosphate calcium phosphate cement seeded with pBMP-2 modified canine MSCs through polyethylenimine transgene. Fujita *et al*.[47] also have found no significant difference between the gelatin sponge incorporating BMP-2 and the gelatin-β TCP sponge incorporating BMP-2 groups and although gelatin has been applied in a number of tissue-engineering studies and clinical practices. To our limited knowledge, sole application of absorbable gelatin sponge as a scaffold in gene-mediated bone tissue engineering is rare. Mehanna *et al*.[48] have reported a clinical case about used recombinant human morphogenetic protein-2 on absorbable gelatin sponge for augmentation of severe lateral ridge defects. In this study, an absorbable gelatin sponge was used as a scaffold to load MSCs expressing hBMP-2 for bone formation *in vivo*. Although, this scaffold was simple and economic, it was efficient in our procedure. In our study, all six rabbits formed bones of 10-15 mm^3 after transplantation with 10^7

LV-hBMP-2-GFP transduced MSCs, suggesting that the gelatin sponge functioned as an efficient scaffold for bone formation. Absorbable gelatin sponge used as Scaffolds is degradable and has good biocompatibility. However, we also acknowledge the main limitation of it is less osteoconductivity compared with calcium phosphate cement.

In our study, efficient bone formation may also be ascribed to the autologous cell transplantation used in our experiments. Autologous cell transplantation can prevent the immune response, which commonly occurs during allogenic cell transplantation.

Using rabbit as an animal model, we demonstrated that ectopic expression of hBMP-2 in rabbit BM-derived MSCs induced MSCs toward osteogenic differentiation and formed a bone *in vivo*. In addition, we developed a simple and economic absorbable gelatin sponge scaffold to induce new bone formation *in vivo*. The procedure established in the rabbit could be translated for human clinical applications for bone defects.

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