

## Original Article

# Gene gun transferring-bone morphogenetic protein 2 (BMP-2) gene enhanced bone fracture healing in rabbits

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**Abstract:** Purpose: Transferring the bone morphogenetic protein 2 (BMP-2) genes into the tissues or cells can improve the bone healing of the fracture has been widely accepted. We evaluated the efficiency of using gene gun to transfer the BMP-2 gene thereby affected the healing of a fractured bone. Methods: The vector coding for BMP-2 was constructed by a non-replicating encephalo-myocarditis virus (ECMV)-based vector. The segmental bone defect (1.5 cm) model was created by a wire-saw at the middle part of the radius bone of the New Zealand white rabbits. Then either BMP-2 gene or control vector without BMP-2 gene was injected into the tissues around the fracture site. Healing of the defects was monitored radiographically for 9 weeks, bone consolidation was determined by the Lane-Sandhu score pre- and post-operatively, which can evaluated bone formation, bone connect and bone plasticity. Results: The radiographic score and bone consolidation rates were significantly higher in animals injected with BMP-2 gene group as compared with control vector-injected animals ( $P < 0.05$ ). The control group still showed no radiological signs of stable healing. Western-blot and RT-PCR showed BMP-2 expression was significant increase in the tissues around the site of osseous lesions in comparison with the control vector-injected animals ( $P < 0.05$ ). Conclusions: Our results suggested that BMP-2 gene transferred by gene gun could increase the expression of BMP-2 protein and improved the bone callus formation therefore shortened the time of bone defect healing.

**Keywords:** Bone morphogenetic protein 2, gene gun, bone fracture, healing

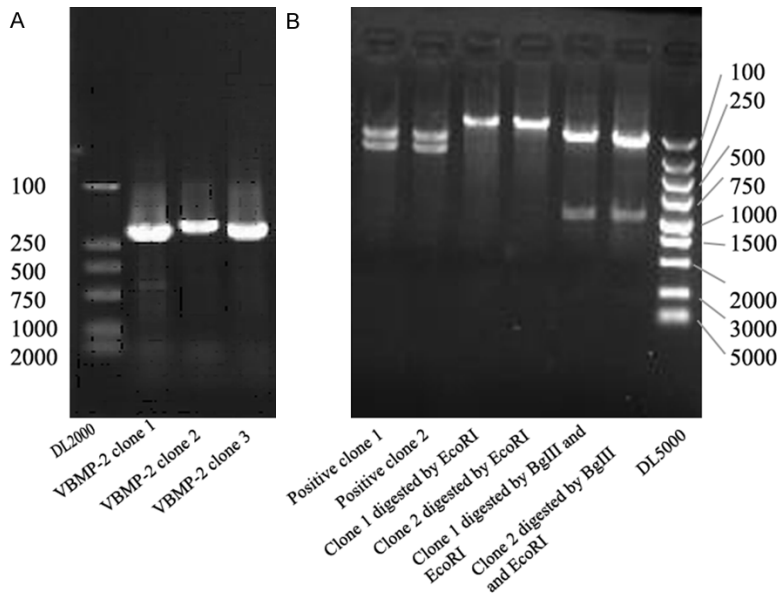
## Introduction

Bone loss caused by congenital defects, traumatic injury, cancer, reconstructive surgery, or periodontal disease has aroused widespread concern all over the world. Regeneration of bone is a common problem in clinical settings. It remains an important clinical challenge because the regeneration potential of human bone appears to be limited [1]. Bone morphogenetic proteins (BMPs) are well known as osteo-inductive growth factors that play important roles in the bone regeneration process [2]. Since the discovery of BMPs by Urist in 1965, many studies have shown that these proteins

are capable of inducing the osteogenic differentiation of mesenchymal cells and ectopic new bone formation and healing bone defects of critical size [3, 4].

The BMP-2 protein has been recognized as the powerful cytokine for improving the healing of the bone [5, 6]. With the development of the biological technology, transferring the BMP-2 gene into the tissues or cells to increase the expression of BMP-2 protein and improve the bone healing of the fracture has become the reality [7-9]. But the method of transferring the BMP-2 gene into the tissues or cells is a little tedious and inefficient [9-11]. A gene gun or a biolistic particle delivery system, originally

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**Figure 1.** pIRES2-EGFP-BMP-2 plasmid was screened and confirmed. A: Three pIRES2-EGFP-BMP-2 plasmid clones were screened by reverse transcription-polymerase chain reaction (RT-PCR) method. B: Two out of the three positive pIRES2-EGFP-BMP-2 plasmids were confirmed by the Electrophoresis. VBMP-2: pIRES2-EGFP-BMP-2.

designed for plant transformation, is a device for injecting cells with genetic information [12]. The payload is an elemental particle of a heavy metal coated with plasmid DNA. This technique is often simply referred to as bioballistics or biolistics. It has become a new approach of the gene transferring [13] since it was first used in living cells by Sanford et al. in 1987 [14]. This method of gene transfer has a lot of advantages: there is no need to choose the target cell type, no need to choose the phase of the cell differentiation, in addition, the operation procedure is relatively simple. So it has been used to transfer genes into the animals and human beings to express some special protein to treat some kind of diseases [15].

In our present study, we injected the non-replicating encephalomyocarditis (ECMV)-based vectors which expressed human BMP-2 (hBMP-2) by gene gun into surrounding area of the bone defect at the middle part of the radius bone of rabbits. We found that gene gun-mediated BMP-2 gene transfer increased the expression of BMP-2 protein at the bone fracture site and improved bone callus formation, thereafter shortened the time of the bone fracture healing when compared to animals treated with control vector. These improvements might be related

to the increase of the BMP-2 protein expression in local.

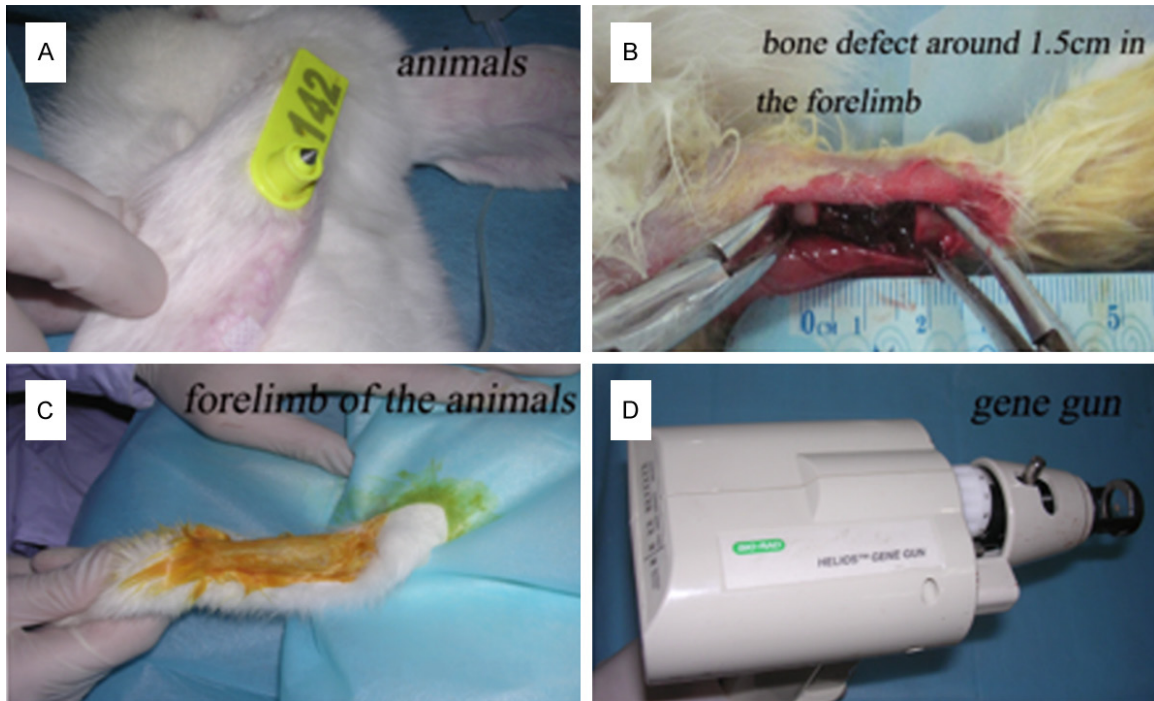
### Materials and methods

#### Vector construct

The recombinant genomic ECMV-based vector expressing BMP-2 was constructed as our previous work [16]. In brief, Full length human BMP-2 was obtained and amplified from a cDNA library which was collected by total RNA extracted from human bone sarcoma specimen by using Trizol Reagent (Invitrogen Corporation). The signal region of human BMP-2 protein was amplified by using the primers BMP-F: ACT-CAGATCTGCCACCATGGTGG-CCGGGACCCGCTG and BMP-R: CTTCGAATTCCTAGCGA-

CACCCACAACCCTC. Both the BMP-2 gene fragments and the pIRES2-EGFP plasmid were digested by BglII and EcoRI enzyme. pIRES2-EGFP contained the internal ribosome entry site (IRES; 1, 2) of the encephalomyocarditis virus (ECMV) between the multiple cloning site (MCS) and the enhanced green fluorescent protein (EGFP) coding region. Then the two digested products were recombined by the T4 DNA ligase so that the BMP-2 gene was ligated to the MCS of the pIRES2-EGFP vector. Then the pIRES2-EGFP-BMP-2 plasmid was screened and confirmed by reverse transcription-polymerase chain reaction (RT-PCR) method (Figure 1). This plasmid (pIRES2-EGFP-BMP-2) was also sequenced to ensure the correct reading-frame. Expression of BMP-2 from the vector was confirmed by western blot test. The clone (Clone 1) which expressed the highest levels of BMP-2 infection of 293 cells (designated hBMP-2) was propagated to high titer ( $4 \times 10^{10}$  pfu/ml) and used in the experiments. Control vector (VC) treatment is identical to VBMP-2 vector except that no specific gene was inserted in the expression cassette. The EGFP gene was carried by the plasmid itself, which served as a reporter gene to indicate that the plasmid had been transferred into the target cells or tissue (Figure 3).

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**Figure 2.** The 1.5 cm bone defection fracture was performed at the middle part of the radius bone at the left upper forearm of the rabbit. A: Animals for study; B: The left upper forearm was prepared for operation; C: The 1.5 cm bone defection fracture was performed; D: Gene gun used in this study.

### *Animal preparation*

Male or Female 4-month-old New Zealand rabbits, weighing 2.5-3.0 kg were housed one per cage approximately 5 days prior to the study, with free access to food and water and maintained on a 12:12, light-dark schedule at 20°C and 40% humidity. This experiment was approved by the Institutional Animal Care and Use Committee of Xinjiang Medical University (20100723008). All housing conditions and experimental procedures were carried out in accordance with Guidance Suggestions for the Care and Use of Laboratory Animals of the People's Republic of China. Under sodium pentobarbital anesthesia with the concentration of 30-35 mg/kg, the left forelimb area of each rabbit was shaved and disinfected with povidone-iodine (**Figure 2A, 2B**), a 2 cm longitudinal incision was then made at the left upper forearm of each rabbit at the middle part of the radius bone. Soft tissues around the middle part of the diaphysis of the radius were dissected, and the left radius was exposed. Under copious sterile saline irrigation, a 1.5 cm bone defection fracture was performed by the wire saw and a ruler so as to create a 1.5 cm osseous lesion (**Figure 2C**). The bone defection frac-

ture site was washed three times with the saline and penicillin G potassium solution. The surrounding muscle was closed around the lesion, producing a closed chamber between the cut ends of the bone.

Then the plasmid of pIRES2-EGFP-BMP-2 or VC was bombarded into the bone fracture site with a Helios Gene Gun (Bio-Rad, **Figure 2D**). For this purpose, 1 µg of DNA was precipitated on 0.5 mg of gold as described in the manufacturer's instructions. Briefly, for gold-coated DNA vaccination, plasmid DNA was coated on gold particles (Bio-Rad, Hercules, CA, USA) at the ratio of 1-2 µg of DNA per mg of gold particles, and was dissolved in 20 µl of 100% ethanol. The gold-coated DNA was delivered to the region of operation using a helium-driven gene gun (Bio-Rad, Hercules, CA, USA) with a discharge pressure of 40 psi. Each time 1 µg of DNA with the help of the gene gun and helium gas at 350 to 400 lb/in<sup>2</sup> pressure was bombard into the tissues around the bone fracture site. The tissues contained the bone membrane, skeletal muscles and other connective tissue. Then the incision was closed without any other interference. After conscious again from anesthesia, the animals were put back into their

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cage without restriction of movement. Postoperatively, rabbits were administered 1 mg/kg of penicillin three times daily for three days to protect the animals from infection intramuscularly. Rabbits were individually caged, and received food and water freely.

In our present study, there were 15 rabbits in each group (VBMP-2 and VC) at every time point of 0, 1, 3, 5 and 9 weeks after bone fracture operation. After the rabbits were anesthetized by the sodium pentobarbital with the concentration of 30-35 mg/kg, the anterior-posterior (AP) and lateral view of the X-ray examination of the animal's left forelimb were performed. To confirm this by independent criteria, representative three rabbits were selected in each group (VBMP-2 and VC) and sacrificed with the air embolism by injecting 20 ml air through the ear-edge veins. Then the bone fracture/defection site was exposed to identify the healing of the osseous lesions by gross observation. The tissues between the two extreme ends of the bone fracture were excised and quickly frozen in dry-ice and kept in -80°C refrigerator for protein analysis.

### *General observation*

During the experimental period, the survival, eating situation, resting posture, movement, posture and activities of rabbits were observed, and the reddish swelling, infection, liquid oozing and abscess in surgical site were observed.

### *Gross observation*

At the 9<sup>th</sup> week after surgery, 3 rabbits were executed both in VBMP-2 and VC group. According to original surgical approach, the skin and subcutaneous tissue was incised layer-by-layer, and the scar tissue was separated. The inflammation response in tissue around defect area was observed. The central defect site was opened, and the soft tissue around defect area and repair of defect were observed.

### *Radiological analysis*

The animals were examined by X-ray machine (Faxitron MX-20, USA) before and after the bone defect surgery to evaluate the bone defect repairing process. The radiological examination of the radius bone of the animals was also performed at 1<sup>st</sup>, 3<sup>rd</sup>, 5<sup>th</sup> and 9<sup>th</sup> week after the operation. Bone consolidation was

determined in preoperative and postoperative X-rays. For radiographic outcome and bone healing, the Lane-Sandhu scoring system [17] was applied in both BMP-2 gene and control vector-injected animals. Radiographic scores were compared between two groups. All radiographic films were evaluated in randomized and double-blind conditions by 2 radiologists with more than 10 years of experience to analyze the repair of bone defect by using the Lane-Sandhu criteria [17].

### *Primary cell culture*

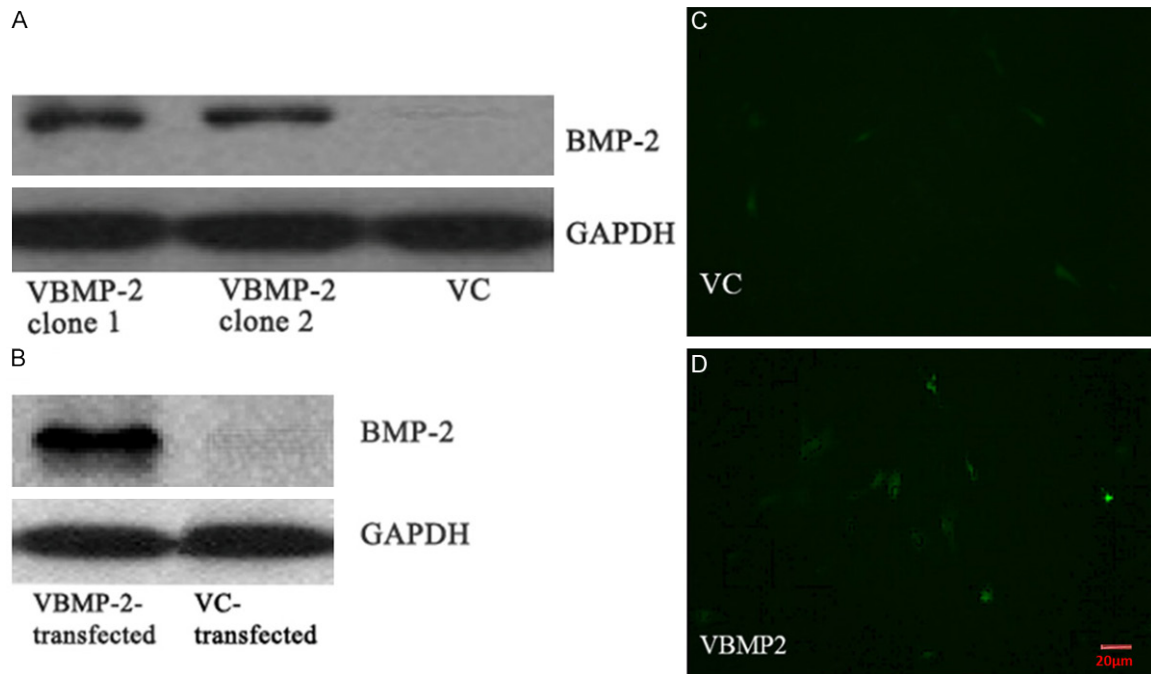
Primary skeletal muscle cells were dissected from rabbit muscle of the quadriceps and were placed on 12 well plates coated with poly-L-lysine, at a seeding density of 5000 cells/cm<sup>2</sup> and cultured in skeletal muscle cell medium (SkMCM, ScienCell). DPBS, fetal bovine serum, trypsin neutralization solution and trypsin/EDTA were used to subculture the cells when their confluent were over 90%. The concentration was adjusted to 3×10<sup>5</sup> cells per well finally. At day 14 in vitro, vectors VBMP-2 or VC at a multiplicity of infection (MOI) of 2 and lipofectamin2000 were added to the wells for 2 hours. Two and three days after infection, cell lysates of skeletal muscle cells were collected for western blot to analyze the transgene expression. Each experiment was repeated three times.

### *Western blot*

The gross observation of the bone fracture/defection was made when the animals was sacrificed for harvesting the tissues between the two fracture/defection ends for protein analysis. The soft tissues were carefully removed without destroying the tissues\bone callus around the bone fracture/defection site. The tissue in 5 mm length around epicenter of the bone defection site were dissected and homogenized in lysis buffer (Amresco). Cultured skeletal muscle cells were collected in the same lysis buffer after being dislodged from the culture plates with a cell scraper. Cell lysates and tissue homogenates were sonicated and centrifuged at 14,000×g for 5 min at 4°C. Medium from cell cultures was centrifuged at 1,000×g for 10 min at 4°C. Protein concentration in tissue homogenates and cell lysates was determined using the BCA assay (Pierce) and spectrophotometry (AD340; Beckman Coulter). Aliquots containing 20 µg of protein were dis-



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**Figure 3.** Western blot or fluorescence microscopy confirmed that VBMP-2 was successfully transfected in to the primary skeletal muscle cells at 72 hours post-infection. A: Two positive pIRES2-EGFP-BMP-2 plasmid clones (clone 1, clone 2) and VC vector expressed the different levels of BMP-2. B: Western blot analysis of the primary skeletal cells transfected by pIRES2-EGFP-BMP-2, infection of primary skeletal muscle cells resulted in robust BMP-2 protein expression, while there was no transgene BMP-2 detected under basal conditions or from cells transfected with VC. VBMP-2: pIRES2-EGFP-BMP-2 plasmid, VC: pIRES2-EGFP plasmid. C and D: Fluorescence microscopy detected EGFP protein expression in 293 cells transfected by VC or VBMP-2 (scale bar=20 µm).

solved in Laemmli buffer and boiled at 95°C for 5 min. Proteins were separated on 12% Tris-Glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and then transferred onto a polyvinylidene difluoride membrane (Millipore, Medford, MA, USA). Immunoblots were blocked and incubated with anti-BMP-2 (ab6285, ABCAM USA) primary antibodies at 4°C overnight, then incubated with horseradish peroxidase-conjugated secondary antibody (1:10000 JACKSON USA), followed by enhanced chemiluminescence detection (Pierce USA). Protein bands were visualized using X-OMAT AR film (Kodak) after chemiluminescence (Pierce USA). The membranes were stripped and re-probed with anti-GAPDH (1:5000 Earthox USA) as a loading control. The intensity of each band was determined by quantitative densitometry using a PC-based image analysis system (ChemiDoc XRS System, BioRad).

### Reverse transcription-polymerase chain reaction (RT-PCR)

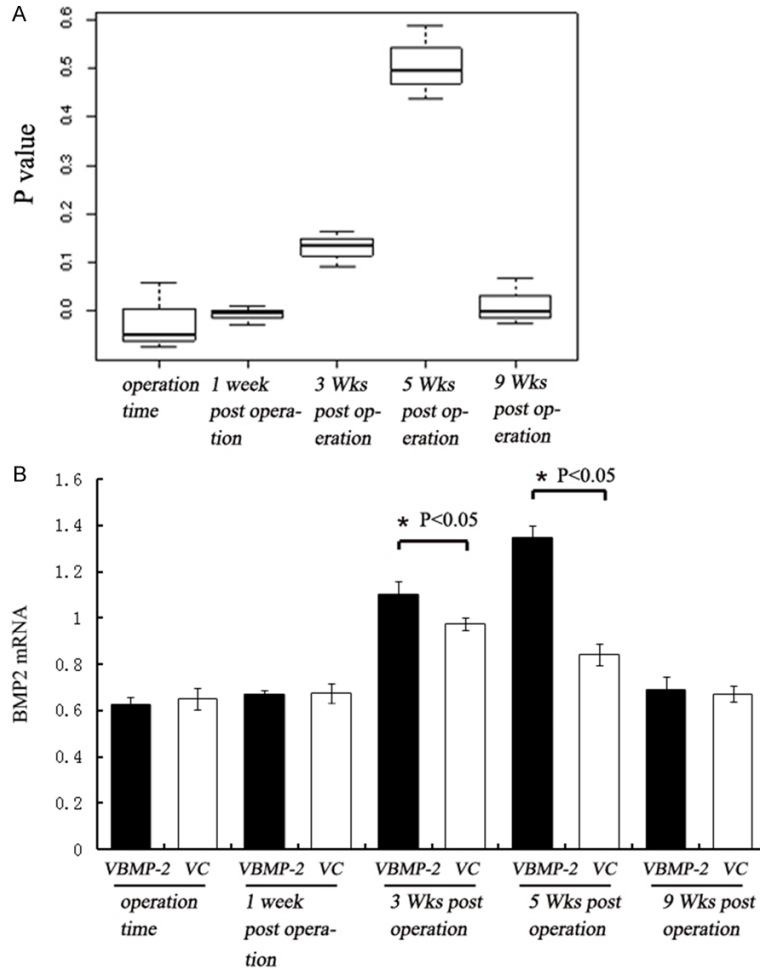
Total RNA was isolated from the specimens that were taken between the two ends of the

bone defect by Trizol (Invitrogen, USA). cDNA prepared from mRNA isolated and was amplified using the following primer sets: BMP-forward (5'-ACTCAGATCTGCCACCATGGTGCCGGACCCGCTG-3') and BMP-reverse (5'-CTT CGAATTCCTAGCGACACCCACAACCCTC-3'). Amplification was carried out by denaturation at 94°C for 5 min followed by 40 cycles (95°C for 10 sec, 60°C for 20 sec, 72°C for 20 sec, and 99°C for 15 sec using a Roche Light cycle 480. Each vitro experiment was repeated three times in three different animals in each group.

### Statistical analysis

Statistical difference between gene gun mediated and vector-treated animals was determined by one-way ANOVA with post-hoc comparisons where appropriate and parametric statistics, using the general linear model for repeated measures, were used to identify significant effects of treatment on the bone healing over time. All the analyses were performed on SPSS 12.0 for Windows (SPSS Inc.). Data are expressed as mean  $\pm$  standard error of mean (SEM), with  $P < 0.05$  considered significant.

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**Figure 4.** RT-PCR analysis of the BMP-2 gene expression in the bone fracture/defection surrounding site at different time point. BMP-2 mRNA expression of bone fracture/defection site was significantly increased in the animals treated with vBMP-2 at 3 and 5 wks. Post-bone defection in comparison with the control-vector-treated-animals. VBMP-2: pIRES2-EGFP-BMP-2 plasmid; VC: pIRES2-EGFP plasmid. \*P<0.05 vs VC control group.

### Results

#### Identification of the BMP-2-expressing ECMV-vector

The 293 cells in vitro transfected with VBMP-2 (MOI 2) expressed EGFP and BMP-2 protein in cell lysate when detected by western blot (Figure 3A, 3B) or fluorescence microscopy (Figure 3C, 3D) at 72 hours post-infection. Both approaches verified BMP-2 gene was successfully transfected. No transgene BMP-2 was detected under basal conditions or from cells transfected with VC. In vitro, infection of primary skeletal muscle cells resulted in robust BMP-2 protein expression.

#### Gene gun injection of VBMP-2 increases the BMP-2 mRNA expression and the secretion of BMP-2 protein

BMP-2 mRNA expression of bone fracture/defection site was significantly increased in the animals treated with VBMP-2 at 3<sup>rd</sup> (VBMP-2 vs VC=0.3019±0.0070 vs 0.2538±0.0118, P<0.05, and 5<sup>th</sup> week (VBMP-2 vs VC=0.3309±0.0083 vs 0.2746±0.0078, P<0.05) post-bone defection in comparison with the control-vector-treated animals (Figure 4). Western blot showed BMP-2 protein was significantly increased also in the bone fracture/defection site when compared to animals treated with VC-injected animals at 3<sup>rd</sup> (VBMP-2 vs VC=1.1034±0.0542 vs 0.9730±0.0281, P<0.05) and 5<sup>th</sup> week (VBMP-2 vs VC=1.3484±0.0501 vs 0.8412±0.0486, P<0.05) post-bone lesion surgery (Figure 5).

#### General observation

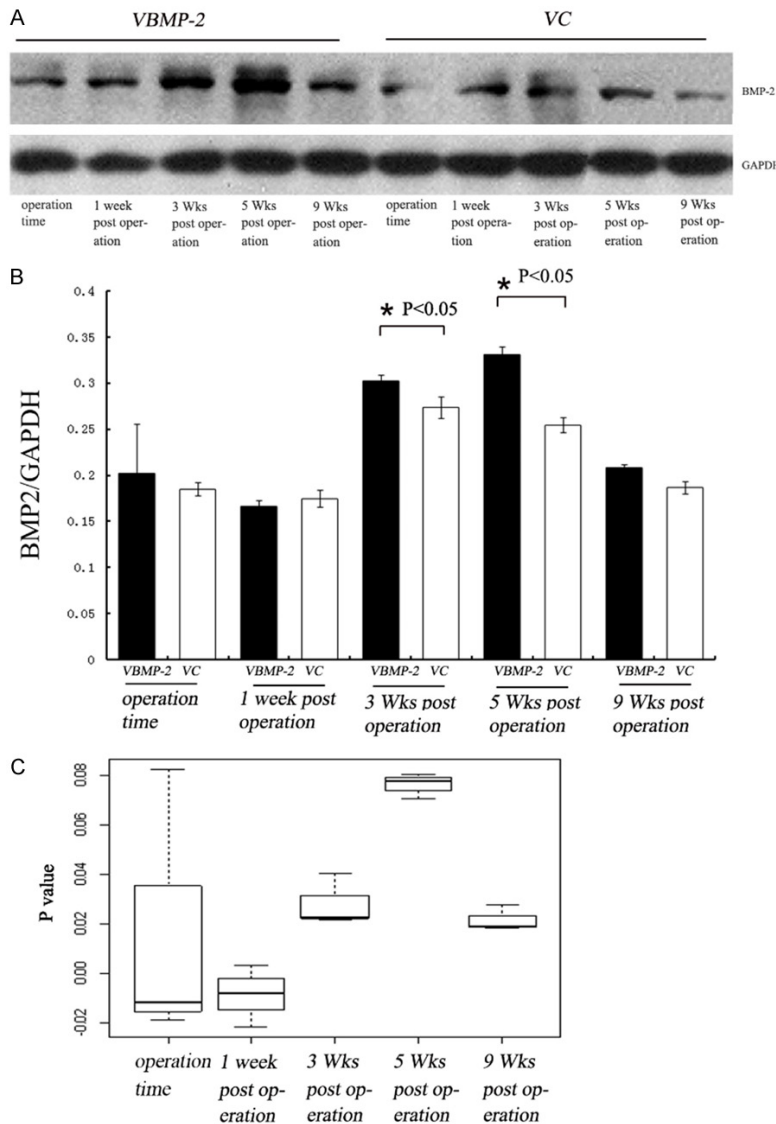
After surgery, there was no reddish swelling, infection, liquid oozing or abscess in incision in all rabbits, with good apposition of skin flap. At the 2<sup>nd</sup> postoperative week,

the incision was healed. At the 4<sup>th</sup> week, all sutures fell off naturally. The rabbit fur was shiny and supple, with erection of both ears. Rabbits had frequent nocturnal activities, with good eating situation. No rabbit died unexpectedly after surgery.

#### Gross observation

At different time point in experiment, there was no inflammatory lesion in tissue around defect area in experimental and control side, respectively. During the experiment, the manifestations of radius bone both in BMP-2 and VC group were different from normal ones. At the 9<sup>th</sup> postoperative week, the defect area was

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**Figure 5.** Western blot analysis of the BMP-2 protein expression in the bone fracture/defection surrounding site at different time point. A: The representative bands of BMP-2 protein in the defect tissues by Western blot analysis. B: Statistical results of BMP-2 protein levels at different time point. Animals treated with vBMP-2 showed significantly increased in the bone fracture/defection site as compared with animals treated with VC. VBMP-2: pIRES2-EGFP-BMP-2 plasmid; VC: pIRES2-EGFP plasmid. \* $P < 0.05$  vs VC control group. C:  $P$  values analysis were shown when BMP-2 protein expressions were compared between groups at different post-operation time point.

filled with a large amount of white fiber and fibrous callus, with unclear boundary with surrounding normal bone. The fiber and fibrous callus touched flexible. In the bone defect area of control side, the boundary between new bone and surrounding normal bone was clear. The normal bone edge was slightly disheveled. In VBMP-2 experimental group, the defect orifice was closed with new bone smoothly extending from surrounding normal bone, with attachment of a small amount of fibrous tissue. The

boundary between new bone and normal bone was not clear (Figure 6).

Radiological analysis showed that the animal treated with VBMP-2 manifested improved healing of radius bone fracture/defection

Bone healing was assessed by scoring system of the Lane-Sandhu X-ray of bone union scale [17]. Animals injected VBMP-2 showed a significantly higher rate of the bone healing compared with the animals injected with control vector (Figure 7). There was a significant improvement in the bone formation, bone link and bone reconstruction, which was seen already at 3<sup>rd</sup> (VBMP-2 vs VC=2.1333±0.1527 vs 0.9666±0.1527,  $P < 0.05$ ), 5<sup>th</sup> (VBMP-2 vs VC=2.6666±0.1527 vs 0.9666±0.1527,  $P < 0.05$ ) and 9<sup>th</sup> week (VBMP-2 vs VC=1.166±0.1527 vs 3.6666±0.1527,  $P < 0.05$ ) in animals treated with VBMP-2 in comparison with the control vector injected. The representative of X-ray observation of the radius bone in the rabbit which was taken at 1 week, 3, 5 and 9 weeks post-surgical of bone fracture were shown in Figure 7.

### Discussion

Although the human beings or animals can have their bone fracture or defection healed themselves, but it will take a little long time. And sometimes the bone fracture or defection even can't heal although the doctors have tried their best to treat the fracture [18]. Different osteoinductive and osteoconductive methods are currently under investigation for developing adequate alternatives to bone grafting. Because bone defection always causes non-union, pain, disability and high cost, many

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**Figure 6.** Gross observation of the repairing of radius bone defection. At the 9<sup>th</sup> postoperative week, in both groups, the defect area was filled with a large amount of white fiber and fibrous callus, with unclear boundary with surrounding normal bone. The fiber and fibrous callus touched flexible. In the bone defect area of VC group, the boundary between new bone and surrounding normal bone was clear (yellow arrow). The normal bone edge was slightly disheveled. In the VBMP-2 experimental group, the defect orifice was closed with new bone smoothly extending from surrounding normal bone (green arrow), with attachment of a small amount of fibrous tissue. The boundary between new bone and normal bone was not clear.

methods have been devised to solve this problem [18, 19]. It has been widely accepted that the autologous bone grafting is still the clinical gold standard for the treatment of large defects or fracture non-unions in patients. However, the limited amount of bone tissue available and donor site morbidity are still the significant drawbacks of this method [20-22].

Regeneration of bone defects is accomplished by cells, bioactive factors, and ECM (extracellular matrix), which continue to work together to regulate the proliferation, differentiation, and migration of osteoprogenitor cells. So they have been put into clinical or laboratory test and some of them have been proved to be effective. BMP-2 protein is one of these proteins which has the ability of facilitating the healing of the bone fracture or bone defection and has been recognized as one of the most powerful proteins of which can induce the bone formation and then improve the bone heal [22]. The secreted BMP-2 protein plays a role in inducing

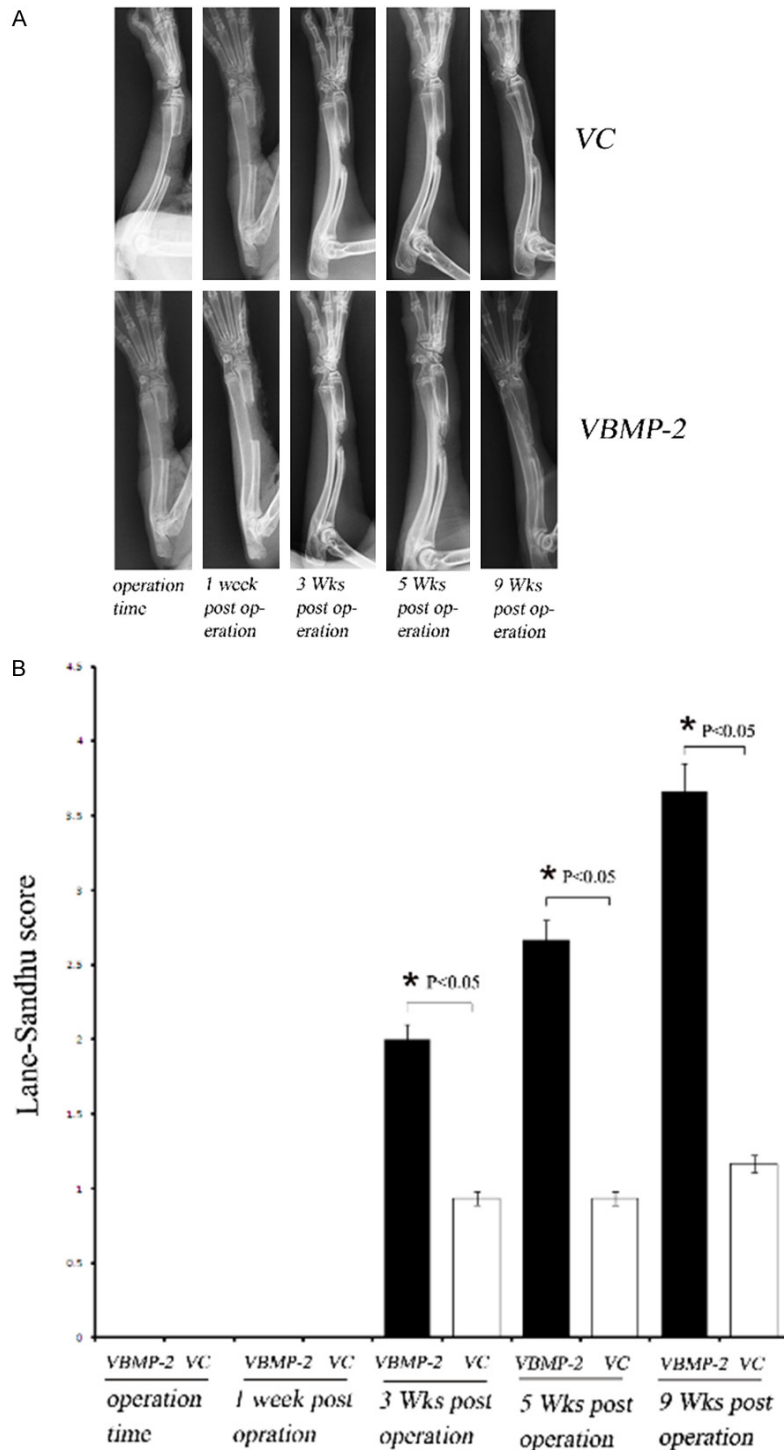
bone formation via autocrine and paracrine pathways. The mechanism inducing the bone formation is that the BMP-2 has the ability to increase the blood supply of the bone fracture site and the strong osteoinduction ability, by acting of the Smad signaling pathway and the inducing of Runx2 expression [23, 24]. And this ability is dose-dependent and time-dependent.

With the development of the technology tissue engineering, there are two major methods have been devised to make the BMP-2 to work as the facilitator of bone fracture/defect healing. One is to mix the BMP-2 protein into a scaffold, then the scaffold is put into the site of bone defection/fracture and then BMP-2 can be released slowly to satisfy the requirement of dose-dependent and time-dependent. While the scaffold work as the linkage between the two ends of the bone fracture or defect. Inorganic calcium phosphate-based scaffolds such as hydroxyapatite (HAP) and tricalcium phosphate (TCP) have already been investigated as carriers of BMP individually. Previous studies demonstrated that the incorporation of BMP into these ceramics greatly accelerates the bone formation [4, 25-27]. Another way is to transfer the BMP-2 gene into the cells or tissues to secret BMP-2 protein themselves which will help the healing of the bone. It does not need of scaffold and the BMP-2 protein is secreted by the creature itself which is more effective and no antigen rejection. AWBaltzer et al. showed that adenoviral vectors can transfer marker genes into the bone defect model of the lapine femur [28], with high levels of local transgene expression. Gene expression persisted up to 6 weeks in bone, and up to 4 weeks in the surrounding soft tissues. They found that by in vivo gene therapy based on adenoviral vectors carrying the BMP-2 gene could accelerate callus formation, as well as ossification and mineralization, within a segmental long bone defect, and improve the biomechanical properties of the newly formed bone [28]. But the method of transferring the BMP-2 gene into the tissues or cells is a little tedious and inefficient. Usually the tissue engineering products need a complex procedure, which make the use of the tissue engineering products unpopular.

Gene gun, a kind of ballistic tool, has been used to change the gene character of the plants or to immune human beings from some diseases. Briefly, for gold-coated DNA vaccination, plas-



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**Figure 7.** The union scale of the bone was assessed by scoring system of the Lane-Sandhu\* in the radiological films. A: The representative of X-ray observation of the radius bone in the rabbit which was taken at 1 week, 3 weeks, 5 weeks and 9 weeks post-surgical of bone fracture. B: Statistical results of Lane-Sandhu scoring at different time point post-surgical of bone fracture. VBMP-2: pIRES2-EGFP-BMP-2 plasmid, VC: pIRES2-EGFP plasmid. \* $P < 0.05$  vs VC control group. \*Lane-Sandhu scoring system describes the radiologic findings: Score 0 (No callus); score 1 (Minimal callus); score 2 (Callus evident but healing incomplete); score 3 (Callus evident with stability expected); score 4 (Complete healing with bone remodeling).

mid DNA was coated on gold particles at the ratio of 1-2  $\mu\text{g}$  of DNA per mg of gold particles, and was dissolved in 20  $\mu\text{l}$  of 100% ethanol. The gold-coated DNA was delivered to the region of operation using a helium-driven gene gun with a discharge pressure of 40 psi. High efficiency and stability are two key points in these DNA vaccines coated with gold particles for in vivo use. The protocol and delivery device for DNA vaccinations by gene gun have been described previously [8, 11, 13, 14]. Generally, gold particle-mediated epidermal delivery of DNA vaccines is based on the acceleration of DNA-coated gold directly into the cytoplasm of antigen-presenting cells (APCs) in the epidermis, resulting in antigen presentation via direct transfection and cross-priming mechanisms [29].

With the development of its technology [30], it has become a new star of the gene transferring method to transfer the gene into the animals and human beings to expression some special protein to treat some kind of disease. It has the advantage of no selection of the kind of the target cell, the phase of the cell differentiation and relative simple operation procedure. And most important, it can transfect a huge amount of the cells at the same time and all this transfect can be done in vivo. There is no need for preparation of the agents to be used in the operation [31]. A suitable vector for

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the purpose of inducing bone growth in a lesion is encephalomyocarditis virus as it is a non-integrating vector with transient expression without the risk of gene mutation. Additionally, the gene-related protein expression in vivo will cease after gene being transfected about two or three weeks, which might just provide enough proteins for bone repair without the fear of excessive growth of bone [32-34].

In our study, we evaluate the availability of using the gene gun to improve the efficiency of gene transfer so as to improve the healing of a fractured bone which was created by a wire-saw at the middle part of the radius bone of the New Zealand white rabbits. The vector coding for BMP-2 was constructed by a non-replicating encephalo-myocarditis virus (ECMV)-based vector [35]. We found that secretion of the BMP-2 protein was increased and last for about 3 weeks after the plasmid was bombard into the tissues around the bone fracture/defect site comparing to that of the control group. Radiographic and gross observations showed that bridging of the segmental defect was gained after 8 weeks in the BMP-2 gene treated, whereas there was only a faint bridging or no bridging in the control group.

Our results suggested that BMP-2 gene transferred by gene gun after radius bone defect could increase the expression of BMP-2 protein and improved the bone callus formation and shortened the time of bone defect healing. There after non-coating BMP-2 gene administered via gene gun might be one of the potent gene transferring approaches for improving the healing of bone fracture/defect. In addition, gene gun administration using non-coating BMP-2 gene could generate high concentration of BMP-2 protein and enhance the activity of bone formation in vivo. It might attribute to both the sustained fashion of BMP-2 delivering and a natural degradable scaffold by implanting muscle tissue.

Local delivery of growth factor genes is likely to be more effective in bone healing than the application of pure recombinant proteins [36-38]. Moreover, the application of gene therapy vectors and gene guns, unlike present surgical approaches to non-unions, does not further destroy the integrity of the injured tissue. The local overexpression of growth factors at week 5 might explain the strong acceleration of new

bone formation seen in our study. The experiments presented here demonstrate the ability of the BMP-2 gene delivered locally by a gene gun, to enhance healing of a long bone defect.

Therapeutic applications of BMPs as they are presently available and suggests future applications based on a lot of researches. Among the future directions are percutaneous injections, protein carriers, advances in gene transfer technology and the use of BMPs to engineer the regeneration of skeletal parts [39].

In conclusion, our results suggested that BMP-2 gene transferred by gene gun could increase the expression of BMP-2 protein and improved the bone callus formation therefore shortened the time of bone defect healing. These promising data encourage the further investigation of genetic approaches to enhance bone healing.

### Acknowledgements

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### Disclosure of conflict of interest

None.

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