# Construction and identification of pIRES2-EGFP-hBMP-2 bicistronic eukaryotic expression vector*★

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**Abstract**

**BACKGROUND:** Bone morphogenetic protein (BMP) and its derived scaffold or vector here been widely used in experiment and gradually applied in the clinic. However, the tracing method challenges the therapeutic effect. The discovery and application of enhanced green fluorescent protein (EGFP) can solve this problem.

**OBJECTIVE:** To construct the bicistronic eukaryotic vector pIRES2-EGFP-hBMP-2 and to observe its expression in human embryo kidney (HEK) 293 cells.

**METHODS:** hMBP-2 gene was extracted from human osteosarcoma by RT-PCR and inserted into PMD18-T vector. Following the DNA sequence verification, it was then sub-cloned into the eukaryotic vector pIRES2-EGFP. After restriction enzyme analysis, the pIRES2-EGFP-BMP-2 was transfected into HEK 293 cells. Then its expression was observed using fluorescence microscopy and Western blot.

**RESULTS AND CONCLUSION:** hBMP-2 gene was amplified and the eukaryotic vector pIRES2-EGFP-hBMP-2 was constructed successfully. At 48 hours after tranfection of pIRES2-EGFP-hBMP-2 into HEK 239 cells, approximately 30% of cells emitted green fluorescence under a fluorescence microscope. Western blot results demonstrated that there was a specific BMP strap at $M_r$46×10$^6$ side, which indicated that the target gene could be expressed in constructed pIRES2-EGFP-hBMP-2 vector.

## INTRODUCTION

Repair of articular cartilage defect is a puzzle in orthopedics at present. Orally taking or injection of chondroitin sulfate, as well as intraarticular injection of related stimulating factor, are utilized in treating articular cartilage defects[1-2]. Currently, tissue engineered technology is gradually applied in treating bone and articular cartilage defects[3-5], which arose more and more attention. Insulin-like growth factor, transforming growth factor, bone morphogenetic protein (BMP), and tumor necrosis factor or the derived scaffolds or vectors constructed based on these factors have been widely used in experiment[6-8] and gradually applied in the clinic. The gene therapy is preferred for researchers[9-12]. However, the tracing method challenges the therapeutic effect. The discovery and application of enhanced green fluorescent protein (EGFP) can solve this problem[13-15]. In the experiment, human BMP-2 (hBMP-2) was used as target gene, EGFP in the pIRES2-EGFP vector used as tracing gene to observe whether the two genes can be co-expressed in human embryo kidney (HEK) 293 cells.

## MATERIALS AND METHODS

### Design

Single sample observation.

### Time and setting

The experiment was performed at the Laboratory of Microbiology, Qingdao University Medical College in November 2009.

### Materials

| Reagent and equipment | Source |
|---|---|
| pIRES2-EGFP | BD Biosciences, USA |
| Total RNA extraction kit, reverse transcription kit | Invitrogen, USA |
| High-purity gel extraction kit, DNA Marker | QIAGEN, Germany |
| $T_4$DNA ligase, $T_4$DNA polymerase, *Bam*H Ⅰ and *Kpn* Ⅰ restriction enzyme | Takara Biotechnology (Dalian) Co., Ltd., China |
| Competent *E. coli* DH5α | Beijing TransGen Biotech, China |
| DMEM culture medium, Western blot kit | Hyclone, USA |
| HEK 293 cells | Presented by professor Luo Bing |
| BMP-2 human polyclonal antibody, goat anti-human IgG | DAKO, Denmark |
| Type 2323 $CO_2$ incubator | SHEL-LAB, USA |
| PM-10AD inverted fluorescence microscope | OLYMPUS, Japan |

### Methods

#### *Clone of hBMP-2 gene*

Totally 0.5 g resected human osteosarcoma was obtained, and 2 mL RNA isolation reagent was added. After grinding, the total RNA was extracted using Trizol regent, qualified, and synthesized into cDNA according to reverse transcription kit instructions. BMP-2 gene sequence (M22489) in Genebank served as template and DNA Star software was used to design primers. The primer sequence was listed as follows:

Upstream primer:
5'-GGG GTA CCG AAG GAG GAG GCA AAG AAA-3'
Downstream primer:
5'-CGG GAT CCG GGA GCC ACA ATC CAG TCA-3'

*Kpn* Ⅰ restriction enzyme cut site and protective base

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were added in the upstream primer, and *Bam*H Ⅰ restriction enzyme cut site and protective base were added in the downstream primer, the length of amplified fragment was 1 092 bp. A total of 25 mL PCR reaction system was added, and the reaction conditions were: 94 ℃ for 5 minutes, 94 ℃ for 30 seconds, 53 ℃ for 45 seconds, and 72 ℃ for 1 minute, 35 cycles, finally, 72 ℃ maintained for 10 minutes. The agarose gel electrophoresis was performed following amplification.

### *Construction of PMD18-T-BMP-2 vector*

Target DNA fragment was retrieved from gel electrophoresis strap based on high-purity gel extraction kit. And then 10 μL system, including PMD18-T 1 μL, solution 15 μL, cDNA (BMP) 4 μL was added at 16 ℃ overnight. *E. coli* DH5α was added to transform the NDA fragment and placed on lB plate at 37 ℃ in incubator for a night. Monoclonal colony was picked up and shaken for 12−16 hours at 37 ℃ with a speed of 180 r/minutes. The plasmid was extracted and identified by using B*am*H Ⅰ and *Kpn* Ⅰ double enzyme digestion. The correct clones were sequenced by Nanjing GenScript Corporation.

### *Construction of bicistronic eukaryotic vector pIRES2-EGFP-hBMP-2*

Plasmid PMD18-T-BMP-2 and pIRES2-EGFP were digested by *Kpn* Ⅰ and *Bam*H Ⅰ and analyzed by electrophoresis. The hBMP-2 fragment and linearized pIRES2-EGFP vector were linked to transform competent *E. coli* DH5α and were spread on the kanamycin resistant LB flat plate. The plasmid was extracted and identified by using *Bam*H Ⅰ and *Kpn* Ⅰ double enzyme digestion and the recombinant plasmid pIRES2-EGFP-hBMP-2 was obtained.

### *Identification of pIRES2-EGFP-hBMP-2 plasmid expression*

pIRES2-EGFP-hBMP-2 was transfected into HEK 293 cells using liposome Lipofectamime 2000. At 48 hours after transfection, the protein expression was observed under a fluorescence microscope, followed by total protein extraction using RIPA lysate (400 μL RIPA+4 μL PMSF). A total of 50 μg protein sample were treated subsequently by 12%SDS-PAGE electrophoresis, trarsmembrane and blocking. The samples were incubated with BMP-2 human polyclonal antibody (1:150) at room temperature for 2 hours, and placed overnight at 4 ℃. After washing with TBS for three times (10 minutes per time), followed by incubation with horseradish peroxidase conjugated goat anti-human IgG (1:5 000) at room temperature for 2 hours, TBS washing (3 times, with 15 minutes per time), and performed X-ray film to observe the final results.

### Main outcome measures

The expression of constructed vector in HEK 239 cells was observed by fluorescence microscopy and Western blot.

### Statistical analysis

Data were analyzed by the first author using SPSS 13.0 software.

## RESULTS

### Amplification of hBMP-2 gene from human osteosarcoma

hMBP-2 gene was amplified from retrieved total RNA followed by reverse transcription and PCR amplification, the size of which was corresponding to 1 092 bp fragment (Figure 1).

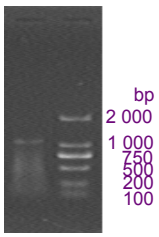



Figure 1 The band at 1 108 bp was the gene segment of human bone morphogenetic protein 2

### Clone and sequencing of PCR product

PCR product was linked with PMD18-T vector, and transformed by competent *E. coli* DH5α. Two colonies were selected and incubated in LB culture medium containing 40 mg/L ampicillin for a night, followed by *Kpn* Ⅰ and *Bam*H Ⅰ double enzyme digestion. A gene fragment with 1 092 length was obtained, which was in full agreement with reported sequence (Figure 2).

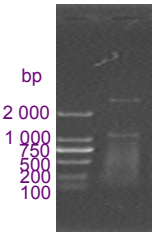



Figure 2 Restriction analysis results of PMD18-T-hBMP-2, and the above was linearized PMD18-T-hBMP vector

### Identification of pIRES2-EGFP-BMP-2

Enzyme digestion and electrophoresis: Straps were presented at the 5.3 kb and 1.1 kb following *Kpn* Ⅰ and *Bam*H Ⅰ double enzyme digestion and electrophoresis, which demonstrated that the vector was successfully constructed (Figure 3).

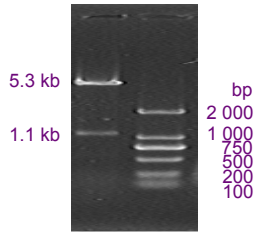



Figure 3 Restriction analysis results of pIRES2-EGFP-hBMP-2, and the above was linearized pIRES2-EGFP vector

Identification of pIRES2-EGFP-hBMP-2 expression: Approximately 30% of cells emitted green fluorescence under a fluorescence microscope at 48 hours after tranfection of pIRES2-EGFP-hBMP-2 into HEK 239 cells (Figure 4). Western blot results demonstrated that there was a specific BMP strap at $M_r$46×10$^6$ side, which indicated that the target gene could be expressed in constructed pIRES2-EGFP-hBMP-2 vector (Figure 5).

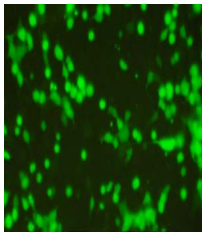
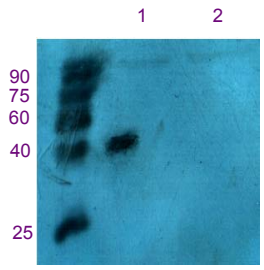


Figure 4 Expression of green fluorescent protein under a fluorescent microscope (×200)




1: transfected cells; 2: blank control cells

Figure 5 Expression of human bone morphogenetic protein 2 protein in human embryo kidney 293 cells by Western blot

## DISCUSSION

BMP-2 is one member of the transforming growth factor β superfamily, which is a factor that has highest osteogenic potential to cause cartilage and bone formation[16-19]. Experiments have indentified that BMP-2 plays a significant role in the process of pyramid fusion, bone healing, as well as ectopic osteogenesis[20-23], which affects the biological behavior of tumor and generative cells[24-27]. There are many problems when introducing hBMP-2 protein into human body directly, such as the spatial structures differences, potential induction of immunologic rejection, repetitive application due to short half life period, and high economic expenditure. Here, BMP-2 gene was cloned into self-replicative vectors to play local therapeutic effects *via* transfected cells expression and secretion.

The diseased region tracing and self secreted BMP-2 discrimination following transplantation of reconstituted cells carrying target genes play an important role in evaluating therapeutic efficacy. Therefore, in the experiment, pIRES2-EGFP vector was selected to labeling the effects of target gene. pIRES2-EGFP vector was a bicistronic eukaryotic expression vector, which carried EGFP gene and expressed GFP in competent cells. The green fluorescence can be seen under a fluorescence microscope. There were internal ribozyme entry sites between multiple clone sites and GFP coding gene, which allowed co-expression of exogenous gene and EGFP. Target gene hBMJP-2 possesses its own initiator codon and termination codon, thus, it can express protein individually with EGFP rather than fusion protein. Though expressed fusion protein can promote coexpression of tracing gene and target gene, the activity of target protein still needs to be verified. The activity of target protein was approach to natural protein in this paper.

Selection of gene transfer method also affects clinical effectiveness. Transgenic vectors comprised viral and non-viral vectors. The transfection efficiency of former was higher than the latter, but the rudimental immunogenicity or mutagenic potential limited its clinical application in certain degrees[28]. However, non-viral vector can surmount this potential discrepancy, especially cationic liposomes, which are used extensively[29]. The maximum transfection efficiency of liposome was 30%, which far from virus vector (> 80%)[30-32], but the liposome was preferred due to economical cost and highly safety.

The fluorescence microscope and Western blot were used here to detect the vector expression, it is simple to use to observe whether the tracing gene and target gene can be co-expressed in the recombination vector, and to determine the correct of expressed proteins. However, HEK 293 cells were not target cells for animal experiment, and whether BMP can be secreted needs to be further identified by transfecting with bone marrow mesenchymal cells. Zhan *et al*[33] transfected the constructed vector into 3T3 cells, and Zou *et al*[34] transfected the vector into human bone marrow mesenchymal cells. Due to the diversity of target cells and transfection methods, whether target protein can be secreted was not detected in previous studies.

BMP-2 gene with strongest induced osteogenesis was served as target gene are widely common in previous studies, which achieved statistical significance results though the selection of vector and transfection methods are diversity. With the maturation of transfection technology, electroblot and lentivirus, with high transfection efficiency and safety, are favored gradually. Additionally, the synchronization of target gene and objective gene as well as the control studies need to further explored. The expression and secretion of recombination vector in bone marrow mesenchymal cells and its *in vivo* action effect is worthy of exception.

## REFERENCES

[1] Herrero-Beaumont G, Marcos ME, Sánchez-Pernaute O, et al. Effect of chondroitin sulphate in a rabbit model of atherosclerosis aggravated by chronic arthritis. Br J Pharmacol. 2008;154(4):843-851.
[2] Schwartz Z, Fisher M, Lohmann CH, et al. Osteoprotegerin (OPG) production by cells in the osteoblast lineage is regulated by pulsed electromagnetic fields in cultures grown on calcium phosphate substrates. Ann Biomed Eng. 2009;37(3):437-444.
[3] Gelse K, Mühle C, Franke O, et al. Cell-based resurfacing of large cartilage defects: long-term evaluation of grafts from autologous transgene-activated periosteal cells in a porcine model of osteoarthritis. Arthritis Rheum. 2008;58(2):475-488.
[4] Kubo S, Cooper GM, Matsumoto T, et al. Blocking vascular endothelial growth factor with soluble Flt-1 improves the chondrogenic potential of mouse skeletal muscle-derived stem cells. Arthritis Rheum. 2009;60(1):155-165.
[5] Xu SQ, Xu YB. Recent progress of BMSCs acting as seeding cell for tissue engineered cartilage. Zhongguo Xiufu Chongjian Waike Zazhi. 2008;22(2):163-166.
[6] Matsumoto T, Cooper GM, Gharaibeh B, et al. Blocking VEGF as a potential approach to improve cartilage healing after osteoarthritis. J Musculoskelet Neuronal Interact. 2008;8(4):316-317.
[7] Chen WJ, Li GT, Luo DX, et al. Application of mesenchymal stem cell combining with bone morphogenic protein to improve spine interbody fusion. Zhongguo Jiaoxing Waike Zazhi. 2007;15(3):1012-1014.
[8] Jancár J, Slovíková A, Amler E, et al. Mechanical response of porous scaffolds for cartilage engineering. Physiol Res. 2007;56 Suppl 1:S17-25.

[9] Guo T, Zhao J, Chang J, et al. Porous chitosan-gelatin scaffold containing plasmid DNA encoding transforming growth factor-beta1 for chondrocytes proliferation. Biomaterials. 2006;27(7):1095-1103.
[10] Ulici V, James CG, Hoenselaar KD, et al. Regulation of gene expression by PI3K in mouse growth plate chondrocytes. PLoS One. 2010;5(1):e8866.
[11] Prochazkova M, Zanvit P, Dolezal T, et al. Increased gene expression and production of spinal cyclooxygenase 1 and 2 during experimental osteoarthritis pain. Physiol Res. 2009;58(3):419-425.
[12] Steinert AF, Nöth U, Tuan RS. Concepts in gene therapy for cartilage repair. Injury. 2008;39 Suppl 1:S97-113.
[13] Ahmed MM, Huang TH, Xie QD. A sensitive and rapid assay for investigating vertical transmission of hepatitis B virus via male germ line using EGFP Vector as reporter. J Biomed Biotechnol. 2008;2008:495-436.
[14] Marjamaa K, Kukkola EM, Fagerstedt KV. The role of xylem class III peroxidases in lignification. J Exp Bot. 2009;60(2):367-376.
[15] Shitara H, Shimanuki M, Hayashi J, et al. Global imaging of mitochondrial morphology in tissues using transgenic mice expressing mitochondrially targeted enhanced green fluorescent protein. Exp Anim. 2010;59(1):99-103.
[16] Reddi AH. Interplay between bone morphogenetic proteins and cognate binding proteins in bone and cartilage development: noggin, chordin and DAN. Arthritis Res. 2001;3(1):1-5.
[17] Avsian-Kretchmer O, Hsueh AJ. Comparative genomic analysis of the eight-membered ring cystine knot-containing bone morphogenetic protein antagonists. Mol Endocrinol. 2004;18(1):1-12.
[18] Lu L, Ma J, Wang X, et al. Synergistic effect of TGF-beta superfamily members on the induction of Foxp3+ Treg. Eur J Immunol. 2010;40(1):142-152.
[19] Andriopoulos B Jr, Corradini E, Xia Y, et al. BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. Nat Genet. 2009;41(4):482-487.
[20] Kamiya N, Ye L, Kobayashi T, et al. Disruption of BMP signaling in osteoblasts through type IA receptor (BMPRIA) increases bone mass. J Bone Miner Res. 2008;23(12):2007-2017.
[21] Samee M, Kasugai S, Kondo H, et al. Bone morphogenetic protein-2 (BMP-2) and vascular endothelial growth factor (VEGF) transfection to human periosteal cells enhances osteoblast differentiation and bone formation. J Pharmacol Sci. 2008;108(1):18-31.
[22] Wang H, Yang ZH, Liu DM, et al. Association between two polymorphisms of the bone morpho-genetic protein-2 gene with genetic susceptibility to ossification of the posterior longitudinal ligament of the cervical spine and its severity. Chin Med J (Engl). 2008;121(18):1806-1810.
[23] Mukherjee A, Rotwein P. Akt promotes BMP2-mediated osteoblast differentiation and bone development. J Cell Sci 2009;122(5):716-26.
[24] Liu F, Bloch N, Bhushan KR, et al. Humoral bone morphogenetic protein 2 is sufficient for inducing breast cancer microcalcification. Mol Imaging. 2008;7(4):175-186.
[25] Kayani AR, Glister C, Knight PG. Evidence for an inhibitory role of bone morphogenetic protein(s) in the follicular-luteal transition in cattle. Reproduction. 2009;137(1):67-78.
[26] Smadja DM, Bièche I, Silvestre JS, et al. Bone morphogenetic proteins 2 and 4 are selectively expressed by late outgrowth endothelial progenitor cells and promote neoangiogenesis. Arterioscler Thromb Vasc Biol. 2008;28(12):2137-2143.
[27] Mukhopadhyay P, Webb CL, Warner DR, et al. BMP signaling dynamics in embryonic orofacial tissue. Cell Physiol. 2008;216(3):771-779.
[28] Zhou W, Edelman GM, Mauro VP. Transcript leader regions of two Saccharomyces cerevisiae mRNAs contain internal ribosome entry sites that function in living cells. Proc Natl Acad Sci U S A. 2001;98(4):1531-1536.
[29] Susa T, Kato T, Kato Y. Reproducible transfection in the presence of carrier DNA using FuGENE6 and Lipofectamine2000. Mol Biol Rep. 2008;35(3):313-319.
[30] Li GB, Lu GX. Gene delivery efficiency in bone marrow-derived dendritic cells: comparison of four methods and optimization for lentivirus transduction. Mol Biotechnol. 2009;43(3):250-256.
[31] Bergen JM, Park IK, Horner PJ, et al. Nonviral approaches for neuronal delivery of nucleic acids. Pharm Res. 2008;25(5): 983-998.
[32] Gao X, Kim KS, Liu D. Nonviral gene delivery: what we know and what is next. AAPS J. 2007;9(1):E92-104.
[33] Zhan YL, Bai JP, Ku RX, et al. Construction of the pIRES2-EGFP-BMP-2 plasmid and study on its transcriptional activity. Xinjiang Yike Daxue Xuebao. 2008;31(5):524-527.
[34] Zou DB, Zhou DS, Wang YH, et al. Expression and mechanism of cDNA of human bone morphogenetic protein 2 via eukaryotic expression vector in rabbit mesenchymal stem cells. Zhonghua Chuangshang Guke Zazhi. 2006;8(2):148-152.

# 双顺反子真核表达载体 pIRES2-EGFP-hBMP-2 的构建及鉴定*★

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**摘要**

**背景：**骨形态发生蛋白等因子及以其为中心构建的支架或载体已经广泛应用于实验研究中，并逐渐应用于临床，然而，治疗效果的示踪方法为实验提出了挑战。增强型绿色荧光蛋白基因的发现和应用解决了这一难题。

**目的：**构建双顺反子真核表达载体 PIRES2-增强型绿色荧光蛋白-人骨形态发生蛋白 2，检测其表达情况。

**方法：**利用 RT-PCR 方法从人骨肉瘤组织中提取人骨形态发生蛋白 2 基因，使之与 PMD18-T 载体连接，经酶切回收后，与目的载体 pIRES2-增强型绿色荧光蛋白连接。用 PCR 技术及基因测序鉴定构建结果。然后将构建好的载体转染人胚肾 293 细胞，通过荧光显微镜及 Western blot 技术观测其表达。

**结果与结论：**实验成功扩增了人骨形态发生蛋白 2 基因并构建了 pIRES2-增强型绿色荧光蛋白-人骨形态发生蛋白 2 载体，将 pIRES2-EGFP-hBMP-2 用脂质体转染入人胚肾 293 细胞后 48 h，荧光显微镜观察约 30%细胞发出绿色荧光，Western blot 结果发现在 $M_r$46×10$^6$ 处有一特异性骨形态发生蛋白条带，表明所构建载体中靶基因能成功表达。

**关键词：**骨形态发生蛋白；增强绿色荧光蛋白；转染；基因；软骨

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