Construction and Identification of PIRES-BMP2-TGFβ3 Bicistronic Eukayotic Expression Vector*

MA Xiao-song, WANG Ying-zhen△, WANG Chang-yao, LIU Jin-zhao

(Department of Articular Surgery, The Affiliated Hospital of Medical College, Qingdao University, Qingdao 266003, China)

ABSTRACT Objective: To construct a bicistronic eukayotic expression vector pIRES-BMP2-TGF. Methods: The BMP2 gene was obtained from pGEMT/BMP2 plasmid by PCR. And it was inserted into bicistronic eukaryotic expression plasmid vector pIRES. The TGFβ 3 was extracted from human embryonal tissue by RT-PCR, then the gene was inserted into the plasmid pIRES-BMP2. The inserted target genes in the plasmid were detected by restriction enzyme digestion and nucleotide sequencing. Results: The direction and sequences of the new bicistronic eukaryotic expression vector pIRES-BMP2-TGFβ3 were correct. Conclusion: The bicistronic eukaryotic expression vector was successfully constructed.

Key words: BMP2; TGFβ3; Bicistronic eukaryotic expression vector

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Introduction

Although arthroplasty is a standard surgical technique, there are several complications associated with this procedure, including dislocation, infection, and loosening. Loosening has emerged as one of the most frequent long-term complications of arthroplasty and is the most common indication for revision [1]. Many methods have been used to enhance osseointegration to decrease the occurrence of loosening, including surface coatings of implants and the use of growth factors[2-4]. Bone tissue engineering and gene therapy might be a way to solve these problems. Mesenchymal stem cells (MSCs), for both self renewal and being multipotent, have been regarded as the most hopeful cell sources for bone tissue engineering and regenerative medicine [5]. To enhance the proliferation and orientational differentiation ability of stem cells by gene modification is hot in bone tissue engineering. 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. This study was to construct a bicistronic eukayotic expression vector pIRES-BMP2-TGF.

1 Materials and Methods

1.1 Materials

pGEMT/BMP2(The central laboratory of the affiliated Hospital of Qingdao university medical college), human embryonal tissue(The department of gynaecology and obstetrics, the affilicated hospital of Qingdao University Medical College), T4 DNA ligase, PrimerStar HS DNA polymerase, pIRES, Nhe Ⅰ,EcoR Ⅰ,Not Ⅰ, Sal Ⅰ restriction enzyme, Total RNA extraction kit, Reverse transcription kit, High-purity gel extraction kit, DNA marker(Takara Biotechnology(Dalian)CO.,LTD.)

1.2 Design of the primers

BMP2 gene sequenc(NM-001200) and TGFβ3(NM_003239) in GeneBank served as the template was used to design the primers. *Nhe* Ⅰ restriction enzyme cut site and protective base was added in the upstream primer, and *EcoR* Ⅰ restriction enzyme cut site and protective base was added in the downstream primer, the length of amplified fragment was 1191bp, *Sal* Ⅰ restriction enzyme cut site and protective base was added in the upstream primer, *Not* Ⅰ restriction enzyme cut site and protective base was added in the downstream primer(TGFβ3), the length of amplified fragment was 1239bp.

Table 1 The Primers of BMP2 and TGFβ3

Gene	Primer
BMP2	5'-CTAGCTAGCATGGTGGCCGGGACCCGCTG-3'(forward)
	5'-CGGAATTCCTAGCGACACCCACAACCCT-3'(reverse)
TGFβ3	5'-GCGTCGACATGAAGATGCACTTGCAAAG-3'(forward)
	5'-ATTTGCGGCCGCTCAGCTACATTTACAAGACT;3'(reverse)

1.3 Construction of pIRES-BMP2

The pGEMT/BMP2 severed as the template and the primer of BMP2 was used to amplify BMP2 gene. A total of 50μL PCR reaction system was added, including pGEMT/BMP2 1μL (10 ng),

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Author: MA Xiao-song(1983-),Male, in the Master, the main research directions: Sport injury and repair, E-mail:mxs_520@163.com

△Corresponding author: WANG Ying-zhen, Professor, Master Instructor, E-mail: wangyingzhenqd@163.com

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5× PrimeStar Buffer 10μL, dNTPs (2.5mmol/L) 4μL, PrimerStar HS DNA Polymerase 0.5μL, upstream primer and downstream primer quaque 1mM, H_2O 32.5μL. The reaction conditions were : 94℃ for 30 seconds, 98℃ for 10 seconds, 55℃ for 15 seconds, and 72℃ for 90 seconds,38cycles, finally, 72℃ maintained for 10 minutes. PCR product was purified by the PCR Purifiction Kit, then the PCR product and plasmid pIRES was cut by *Nhe* Ⅰnd *EcoRI*.. After digested, PCR product was purified by the PCR Purifiction Kit. The DNA fragment was inserted into the plasmid by T4 DNA ligase, 10μL system was added, including pIRES 1μL, cDNA (BMP2)4μL, T4 link 5μL was added at 16℃ for 2 hours. The DNA fragment was translated into *E. coli* DH5α and placed on LB plate at 37℃ in incubator for 16h. Monoclonal colony was picked up and shaken for 12-16 hours at 37℃ with a speed of 225 r/minutes. The plasmid was extracted and identified by using *Nhe* Ⅰ and *EcoR* Ⅰ double enzyme digestion. The recombinant plasmid pIRES-BMP2 was obtained.

1.4 Construction of pIRES/BMP2−TGFβ3

The total RNA was extracted by Trizol regent from human embryonal tissue, then was qualified and synthesized into cDNA according to reverse transcription kit instructions. The rection system contained 5× buffer 4 μL, ET enzyme 1μL, Oligo dT 1μL, Random6 1μL, Total RNA 1μg, H_2O 12μL. The reaction conditions were: 37℃ for 15 minutes, 85℃ for 5 seconds. The cDNA obtained by reverse transcription was served as the template and TGFβ3 gene was amplified. The reaction system and conditions were same as the BMP2 gene. The agarose gel electrophoresis was performed. PCR product was retrieved from gel electrophoresis strap based on high-purity gel extraction kit. PCR product and plasmid pIRES-BMP2 were cut by *Sal* Ⅰ and *Not.* After enzyme digestion, PCR product was purified by the PCR Purifiction Kit. The DNA fragment was inserted into the plasmid pIRES-BMP2 and transformed into *E. coli* DH5α. The reaction system and conditions were same as the BMP2 gene. The plasmid was extracted and identified by using *EcoR* Ⅰ enzyme digestion. The plasmid pIRES-BMP2-TGF β3 was obtained and sequenced by Sangon Biotech(Shanghai)Co.,Ltd.

2 Results

2.1 Amplification of BMP2 and TGFβ3 genes

BMP2 gene was amplified from pGEMT/BMP2, TGFβ3 gene was amplified from retrieve total followed by retrieve transcription and PCR amplification. The size of BMP2 gene was 1191bp, the size of TGFβ3 gene was 1239bp.

2.2 Identification of plasmid pIRES−BMP2

The plasmid pIRES-BMP2 was cut by *Nhe* Ⅰ and *EcoR* Ⅰ double enzyme. A gene fragment with 1191bp was obtained, which was in full agreed with BMP2 gene.

2.3 Identification of plasmid pIRES−BMP2−TGFβ3

The plasmid was cut by *EcoR* Ⅰ and a fragment about 1000bp would be obtained, which indicated TGFβ3 gene inserted into the plasmid pIRES-BMP2-TGFβ3.The sequence of the plasmid pIRES-BMP2-TGF β3 was in accordance with gene sequence in GeneBank.

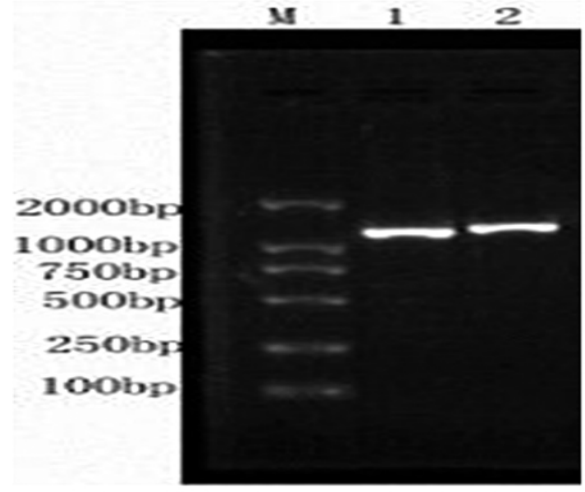

Fig. 1 The amplification of BMP2 and TGFβ3
M is Mark, Number 1 is BMP2 gene, Number 2 is TGFβ3 gene 1

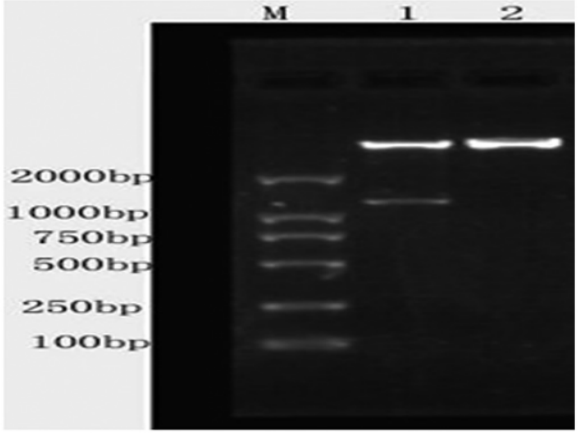

Fig.2 The identification of plasmid pIRES-BMP2
M is Mark, Number 1 is pIRES-BMP2 cut by *Nhe* Ⅰ and *EcoR* Ⅰ double enzyme, Number 2 is pIRES cut by *Nhe* Ⅰ and *EcoR* Ⅰ double enzyme

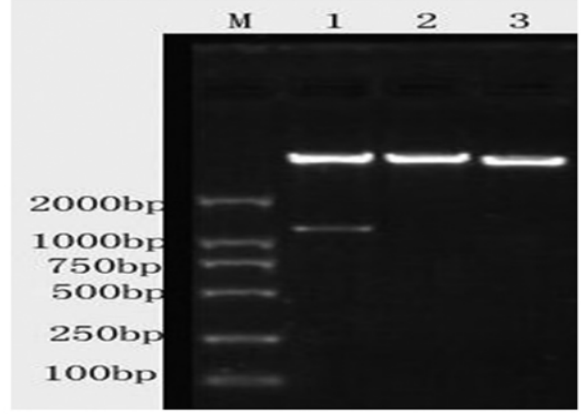

Fig.3 The identification of plasmid pIRES-BMP2-TGFβ3
M is Mark, Number 1 is pIRES-BMP2-TGFβ3 cut by *EcoR* Ⅰ, Number 2 is pIRES-BMP2 cut by *EcoR* Ⅰ, Number 3 is pIRES cut by EcoR Ⅰ

3 Discussion

Mesenchymal stem cells (MSCs) are a population of self-renewing, multipotent cells that are able to differentiate along several committed phenotypes including osteogenic, chrondogenic, adipogenic lineages in response to stimulation by multiple environmental factor [9]. Many cytokines and transcription factors play an important role in this procedure.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) super family of proteins that have diverse effects on cells of the mesenchymal lineage. Several BMPs could promote chondrogenic and osteogenic differentiation of mesenchymal stem cells in vitro, and bone formation and repair in vivo[10]. BMP-2 is a factor that has highest osteogenic potential to cause cartilage and bone formation[11]. It had been indentified that BMP-2 plays a significant role in the process of pyramid fusion, bone healing, as well as ectopic osteogenesis [12], which affects the biological behavior of tumor and generative cells [13], BMP2 has been used to increase the osseointegration in the experiment[14].

Transforming growth factor betas (TGFβs) are also the members of the transforming growth factor-β (TGF-β)super family and have three types in mammals(TGF-β1,TGF-β2,TGF-β3). They are known to have an integral function in the coupling of bone formation and resorption[15], and can also elicit increased bone formation both in vivo and in vitro. Since both BMP2 and TGF-β are involved in bone formation through different ways, it is possible that combined gene therapy of both genes might have more significant effects on bone regeneration than single gene alone. For example, recombinant human TGF-β2 (rhTGF-β2) induced the proliferation of human bone mesenchymal stem cells and increased their collagen I production in vitro, whereas BMP-2 promoted their difierentiation into an osteoblastic phenotype. At the same time, increased concentrations of rhTGF-β2 decreased alkaline phosphatase activity induced by rhBMP-2, which suggests their intertwined effects during bone formation [16].

In this study, the BMP2 and TGF-β3 genes were inserted into a bicistronic eukayotic expression vector. The plasmid pIRES-BMP2-TGFβ3 was constructed successfully. The plasmid would be transfected into Mesenchymal stem cells in the next experiment and the BMP2 and TGF-β3 genes would express in the cells, it is possible that combined gene therapy of both genes might have more significant effects on bone regeneration than single gene alone.

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双基因真核表达载体 pIRES-BMP2-TGFβ3 的构建与鉴定 *

马小松 王英振△ 王昌耀 刘金钊

(青岛大学医学院附属医院关节外科 山东 青岛 266003)

摘要 目的:构建与鉴定骨形态发生蛋白 BMP2 和转化生长因子 TGFβ3 双基因真核表达载体 pIRES-BMP2-TGFβ3。方法:首先,用 PCR 方法从质粒 pGEMT/BMP2 中扩增出 BMP2 基因全长,并将其连入双基因真核表达载体 pIRES,得到质粒 pIRES-BMP2,其次,从人胚胎组织提取总 RNA,反转录成 cDNA,以反转录的 cDNA 为模板,PCR 扩增出 TGFβ3 基因全长,将 TGFβ 3 基因连入质粒 pIRES-BMP2;用酶切的方法筛选出阳性重组质粒,并进行测序鉴定。结果:酶切鉴定证明已将 BMP2 和 TGFβ3 两个基因连入载体中,测序结果完全正确。结论:成功构建 PIRES-BMP2/TGFβ3 双基因真核表达载体。

关键词:骨形态发生蛋白;转化生长因子;双基因真核表达载体

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作者简介:马小松(1983-),男,硕士研究生,主要研究方向:运动损伤与修复,E-mail:mxs_520@163.com

△通讯作者:王英振,教授,硕士生导师,E-mail:wangyingzhenqd@163.com

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