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Molecular Cloning of Bone Morphogenetic Protein-2 (BMP- 2) in pGEM-b1 Vector and Transformation into E.coli Rosetta Strain

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Abstract

Background: *Bone Morphogenetic Protein 2 (BMP- 2) belongs to the TGF-β superfamily of proteins and plays an important role in the development of bone and cartilage. BMP- 2 is also associated with maintenance and repair of damaged bone. Recombinant human bone morphogenetic protein 2 (rhBMP- 2) is now produced by genetic engineering techniques and used in treatment of thin bone fractures in the jaw and spine. In this study we aimed to extract and amplify the BMP- 2 gene from human osteoblast cell line MG-63, insert the amplified BMP- 2 gene into pGEM-b1 cloning vector, and then transform the recombinant vector into the E.coli strain of Rosetta. This technique can be used in future research and BMP- 2 expression.*

Methods: *After culturing MG-63 cells, approximately 5 million viable cells were used for extraction of Total RNA. The extracted RNA was used for cDNA synthesis in RT-PCR reaction. Then, the BMP- 2 gene was amplified by specific primers and the PCR product was cloned in the pGEM-b1 vector. Chemically competent E.coli cells were prepared using CaCl2 0.1 M and transformed with recombinant pGEM-b1 vector under heat shock. The transformed E.coli Rosetta bacteria were inoculated on LB agar medium containing Ampicillin. Bacterial colony containing recombinant vector was isolated and used for plasmid extraction. The extracted plasmid was used for specific PCR to confirm the presence of BMP- 2 gene in pGEM- b1 vector.*

Results: *After transformation, the E.coli Rosetta had the ability to become resistant to ampicillin and could grow on ampicillin-containing medium. While non-transformed E.coli Rosetta could not grow on LB agar containing ampicillin. The 1100 bp fragment was obtained from PCR amplification with specific primers, indicating that the BMP- 2 gene was inserted into pGEM-b1 vector.*

Conclusions: *The pGEM-b1 vector and E.coli Rosetta strain were not used for BMP- 2 cloning in previous investigations. Therefore, this method may be a useful approach to reduce the challenges ahead of the optimization of BMP- 2 production.*

Keywords: Bone morphogenetic protein 2, Molecular cloning, Genetic vectors, Bacterial transformation.
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Introduction

Ossification or osteogenesis is a process that begins with the migration and mitosis of mesenchymal stem cells (MSCs), continues with the differentiation of MSCs into osteoprogenitor cells, and completes with the maturation of bone progenitor cells into osteocytes. Many growth factors are involved in the generation and repair of bone and other connective tissues. These growth factors are classified according to their biological activity into several families: insulin-like growth factors, platelet-derived growth factor, fibroblast growth factors, epidermal growth factor (EGF), and transforming growth factor β (TGF-β).[1,2]

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily and are known as multifunctional cytokines. These proteins can induce the formation of new bone and cartilage and play a central role in the maintenance and repair of damaged bone. More than 20 different BMPs have been identified and sub-classified according to the similarities of their function and amino acid sequences.[3] Bone morphogenetic protein-2 (BMP-2), which is a member of the BMP subgroup of TGF-β superfamily, was demonstrated to play a key role in bone generation and is involved in the hedgehog pathway, TGF-β signaling pathway, cytokine–cytokine receptor interaction, cardiac muscle cells differentiation, and the epithelial-mesenchymal transition phenomenon.[4]

Currently, BMPs are produced by recombinant DNA technology and are used in dentistry, oral and maxillofacial surgery, and orthopedic applications. In fact, BMP molecules have been synthesized through recombinant DNA technology and have been imported into the desired location (e.g., into the broken bone). BMPs activate the cell signaling pathways in MSCs and cause stem cell migration toward the injured area, thereby allowing MSC differentiation into osteoblasts to participate in bone fracture healing. BMP-2 and BMP-7 are known as osteoinductive proteins and are capable of inducing the osteoblast differentiation. Therefore, the expression and purification of BMP-2 and BMP-7 in various expression systems are greatly regarded, and recombinant human BMP-2 and -7 (rhBMP-2 and rhBMP-7) have been approved by the FDA.[5]

BMPs have been expressed using various protein expression vectors and hosts, such as Escherichia coli, Pichia pastoris, Baculovirus/insect cells, and mammalian cell lines.[6-8] To develop research in the field of rhBMP-2 production for clinical use, we decided to use the pGEM-b1 vector and E. coli Rosetta strain as a new vector–host expression system. In this study, we aimed to

extract and amplify the BMP-2 gene from human osteosarcoma cell line MG-63, insert the amplified BMP-2 gene into the pGEM-b1 cloning vector, and then transform the recombinant vector into the E. coli Rosetta strain. This technique may improve the expression of recombinant BMP-2 in the future.

Materials and Methods

The human osteosarcoma cell line MG-63 was obtained from the Pasteur Institute of Iran. The MG-63 cells were cultured in Dulbecco's modified Eagle's medium-high glucose supplemented with 10% fetal bovine serum and 2% pen–strep (final concentrations: 10-units/ml penicillin G, 10-g/ml streptomycin). Cells were cultured in a humidified incubator at 37°C, using a standard mixture of 95% air and 5% CO2. For passaging, cells were detached with trypsin/EDTA and subsequently replated.

Total RNA was extracted from 5 million viable MG-63 cells using a GF-1 Total RNA Extraction Kit (Vivantis Technologies, Malaysia), according to the manufacturer's protocol. The ratio of absorbance at 260-nm and 280-nm wavelength was used to assess the purity of extracted RNA.

First-strand cDNA was synthesized using the Viva 2-steps RT-PCR kit (Vivantis Technologies, Malaysia). In the first step, a mixture of 10-µg RNA, 1-µl random hexamer primer (50 ng/µl), and 1-µl dNTP mix (10 mM) was prepared in 10-µl DEPC water and incubated at 65°C for 5 min and chilled on ice for 2 min. In the second step, a mixture of 2-µl 10× buffer M-MuLV and 100 units of M-MuLV reverse transcriptase was prepared in 10-µl DEPC water and added to the first mixture. This was then incubated at 42°C for 60 min. The reactions were further incubated at 85°C for 5 min to terminate the reaction.

Specific primers for the BMP-2 gene were designed using Gene Runner software, and the restriction sites of SalI and KpnI enzymes were embedded at the 5′ end of forward and reverse primers, respectively. The primer pairs were synthesized by SinaClon BioScience co., Iran. The BMP-2 primer sequences are shown in Table 1.

A PCR amplification mixture was prepared in a final volume of 30 µl containing 5-µl cDNA, 3-µl 10× buffer, 1.5 mM MgCl2, 0.8 mM dNTP, 0.5 µM of each primer, 1 U Taq DNA polymerase, and water up to 30 µl. The thermal cycler (primus, Germany) was programmed for incubation at 94°C for 2 min, 35 cycles with denaturation at 94°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 45 s, and final extension at 72°C for 7 min. To check the PCR reaction, 5-µl PCR product was mixed with 6× DNA loading dye and loaded onto 1.5% agarose gel. After the electrophoresis (100 V for 50 min), the gel was stained with ethidium bromide (0.5 µg/ml) and the amplicon was visualized using a UV trans-illuminator (Uvitec, UK). The remaining 25-µl PCR product was purified using the AmbiClean Kit (Vivantis Technologies, Malaysia) and subsequently digested using SalI and KpnI restriction enzymes (Thermo Scientific, USA): 3-µl Tango buffer 10× (Thermo Scientific, USA) and 1 µl of each restriction enzyme was added to purify the PCR product, and the mixture was incubated at 37°C for 3 h.

The pGEM-b1 vector was also double digested using SalI and KpnI restriction enzymes (Thermo Scientific, USA): 3-µl Tango buffer 10× (Thermo Scientific, USA) and 1 µl of each restriction enzyme was added to 200–500-ng plasmid, and the mixture was incubated at 37°C for 3 h. The digested pGEM-b1 vector was electrophoresed in 1% agarose gel. The target band was cut and linearized pGEM-b was extracted from agarose gel according to the instructions for the GF-1 Gel DNA Recovery Kit (Vivantis Technologies, Malaysia).

The recombinant pGEM-b1 vector containing the BMP-2 gene was generated by mixing 60-ng BMP-2 PCR product, 30-ng linearized pGEM-b1 vector, 1-µl 10× ligation buffer, 2 U of T4 DNA ligase (Thermo Scientific, USA), and water up to 10 µl. The ligation mixture was incubated overnight at 16°C.

E. coli Rosetta cells were grown overnight in 5-ml LB broth. The next day, 150-µl overnight culture (OD 600 = 1.2) was transferred to 10-ml LB broth and incubated at 37°C for 2 h with shaking (180 rpm). The bacterial cells (OD 600 = 0.6) were collected by centrifuging at 4500 rpm (4°C) for 15 min. The cell pellet was resuspended in 5-ml ice-cold 0.1 M CaCl2 and placed on ice for 30 min. The bacterial pellets were collected again by centrifugation at 4500 rpm for 15 min. The process was repeated once with 2.5-ml ice-cold 0.1 M CaCl2. Finally, the cell pellet was resuspended in 100-µl ice-cold 0.1 M CaCl2 and transferred to a 1.5-ml Eppendorf tube. Next, 6-µl ligation reaction was added to competent cells and placed on ice for 30 min. The recombinant pGEM-b1 vector was transformed into E. coli Rosetta by heat shock at 42°C for 2 min and immediately placed on ice for 5 min. Subsequently, 800-µl LB broth was added to an Eppendorf tube and incubated at 37°C for 1 h. Finally, the cells were spread on an LB agar plate with 100-µg ampicillin and incubated at 37°C.

Single colonies were picked from the LB agar plate containing 100-µg ampicillin after incubation at 37°C overnight. Colonies were then inoculated into 5–10-ml LB broth containing ampicillin and incubated at 37°C for 16–18 h with shaking. The pGEM-b1 vector was extracted from the bacterial culture using a GF-1 Plasmid Extraction Kit (Vivantis Technologies, Malaysia) according to the manufacturer's protocol and the presence of extracted plasmid was checked on a 1.5% agarose gel.

PCR amplification with specific primers (Table 1) was performed to confirm the presence of the BMP-2 gene in the pGEM-b1 vector. PCR mixture was prepared in a final volume of 30 µl containing 30-ng recombinant pGEM-b1 vector, 15-µl Taq DNA polymerase 2× Master Mix (Ampliqon, Denmark), 0.5 µM of each primer, and water up to 30 µl. The thermocycler program was the same as the BMP-2 amplification using cDNA. Agarose gel electrophoresis was performed by loading 5-µl PCR product into 1.5% agarose gel. The gel was run at 100 V for 50 min and stained with ethidium bromide solution. The amplicon was visualized using UV trans-illuminator (Uvitec, UK).

Results

In this study, we used the pGEM-b1 vector, which is a cloning vector with an ampicillin resistance marker. After transformation, the E. coli Rosetta has the ability to become resistant to ampicillin and can grow on an ampicillin-containing medium, whereas non-transformed E. coli Rosetta cannot grow on LB agar containing ampicillin.

Table 1. BMP-2 specific primers

BMP-2 primers	Restriction enzymes sites	Sequence	Tm	%GC
Forward	SalI	ATAAGCGACGCAGTTCCGCAGCTGG	64.8 °C	60 %
Reverse	KpnI	ATAAGGTACCGCGACACCCACAACC	62.7 °C	56 %

The recombinant pGEM-b1 vector containing the BMP-2 gene was extracted from an overnight culture of transformed E. coli Rosetta, and BMP-2 gene was amplified using specific primers. The 1100-bp fragment was obtained by PCR amplification with specific primers, indicating that the BMP- 2 gene is inserted into a pGEM-b1 vector (Figure 1).

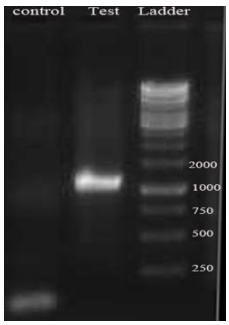

Figure 1. PCR product of BMP-2 gene amplification. Lane 1: negative control, Lane 2: Test, Lane 3: DNA ladder 1Kb (250- 10000 bp)

Discussion

Since the identification of BMPs in 1965, extensive studies have been performed on bone healing. Particularly, BMP-2, as a member of TGF-β superfamily and a regulator of bone regeneration, has been widely investigated. Kunikazu et al., (2006) showed that BMP-2 can act as an initiator of endogenous bone repair and that mice lacking the ability to produce BMP-2 in their bones have spontaneous fractures. The results of their study demonstrated that BMP-2 is a necessary component of the signaling cascade that governs fracture repair. Although other osteogenic stimuli are present in the limb skeleton of BMP-2 deficient mice, they are not able to repair damaged bone.[9]

Tian et al., (2009) designed a bicistronic vector containing BMP-2 and vascular endothelial growth factor (VEGF)-165 genes. VEGF is an important mediator of angiogenesis that plays a key role in bone growth through the endochondral ossification pathway. This bicistronic vector has been used to investigate the interaction between BMP-2 and VEGF in bone formation. The results of this study indicated that BMP-2 and VEGF have a synergistic effect on bone regeneration.[10]

A large amount of BMP-2 is needed for both bone tissue engineering research and clinical application. Thus, an effective way is necessary to produce a sufficient amount of the BMP-2 protein. With the advance in plant biotechnology, transgenic plants have been targeted as a bioreactor to produce desired recombinant proteins. Therefore, the expression of the recombinant human BMP-2 gene (rhBMP-2) was studied in tobacco plants using GUS as a reporter gene. The difference between expression levels in root, stem, and leaf tissues were analyzed by a GUS activity assay, semi-quantitative RT-PCR, and Western blotting. The results indicated that the expression levels of fusion protein in root and stem tissues were significantly higher than those in leaf tissues.[11]

Prokaryotic expression systems of rhBMP-2 have several advantages over other systems. The prokaryotic system allows one to obtain large quantities of recombinant proteins in a short time. A simple and inexpensive bacterial cell culture facilitates the use of these microorganisms.[12] Zhang et al., (2010) showed that BMP-2 genes can be cloned from MG-63 cells by RT-PCR and rhBMP-2 can be expressed in E. coli. In this study, BMP-2 genes were cloned into a pET30 (a) vector and transformed into E. coli BL21 and rhBMP-2, which was expressed as inclusion bodies. They tried to optimize the conditions for the prokaryotic expression system to improve the soluble expression of rhBMP-2, including the culture temperature, carbon source, IPTG concentration, and culture temperature. However, they failed to obtain any soluble rhBMP-2. Therefore, the inclusion bodies were harvested and solubilized in the solubilization buffer and the denatured rhBMP-2 was purified by Ni-NTA column.[13]

In general, the production of recombinant proteins for therapeutic purposes is greatly considered in recent years. Recombinant BMP-2 expression and purification from bacterial expression systems may be of great interest because of the osteoinductive properties of this protein.

However, because the production of recombinant proteins requires an expression system, the choice of the expression system becomes the determinant factor for efficiency and quality of the final BMP molecules produced. The pGEM-b1 vector and E. coli Rosetta strain were not employed for BMP-2 cloning in previous investigations. Therefore, this method may be a useful approach to reduce the challenges ahead of the optimization of BMP-2 production. It seems that E. coli Rosetta is a proper candidate for the expression of rhBMP-2 in a short time, using a simple and inexpensive method.

Conflict of Interest

The authors declare that they have no conflict of interest.

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