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Expression of Recombinant Human Bone morphogenetic protein 2 (hBMP2) in Insect cells

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Abstract

Bone morphogenetic protein 2 (BMP2) plays an important role in the development of bone and cartilage. It is involved in the hedgehog pathway, TGF beta signaling pathway, and in cytokine-cytokine receptor interaction. It is involved also in cardiac cell differentiation and epithelial to mesenchymal transition. In this study, We expressed human BMP2 (hBMP2) recombinant protein using Baculovirus Expression Vector System (BEVS) in Sf9 insect cells. The hBMP2 cDNA was cloned into baculovirus transfer vector, pBacgus-4x-1 and recombinant baculovirus was screened out through X-gal and GUS-fusions assay. Western blot analysis shown that molecular weight of hBMP2 recombinant protein was about 44.71 kDa.

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Introduction

Bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to the transforming growth factor ß (TGF ß) superfamily (Park *et al*., 2013). The roles of BMPs in embryonic development and cellular functions in postnatal and adult animals have been extensively studied in recent years. BMP forms a complex with extracellular matrix proteins; for this reason its biological activity is confined to local niche. Furthermore, BMP signaling is antagonized by endogenous extracellular proteins such as follistatin and noggin which block the BMP receptors (Varga and Wrana, 2005). BMP binds to Type-I (BMPR1A,BMPR1B) and Type-II (BMPRII) serine-threonine kinase transmembrane receptors and triggers a signal transduction cascade initiated via Smad family proteins. Signaling through smads takes place via three smad family proteins: Receptor-mediated smads (smad1, 5 and 8), co-smad/smad4 (common mediator of smad) and inhibitory smads (smad 6 & 7, negative regulators of smad) (Beck *et al*., 2006). The BMP-smad pathway activates direct or indirectly BMP target genes in the nucleus via co-transcriptional partners (Varga and Wrana, 2005). The activity of BMPs was first identified in the 1960s, but the proteins responsible for bone induction remained unknown until the purification and sequence of bovine BMP-3 (osteogenin) and cloning of human BMP-2 and 4 in the late 1980s (Wozney *et al*., 1988). To date, around 20 BMP family members have been identified and characterized.

BMP2 is one of the well-characterized molecules in the BMP family and identified as factors extractable from bone and triggering

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ectopic bone formation at non-skeletal sites in vivo (Urist *et al*., 1997). Also, BMP2 can accelerate the ossification of extensive bone lesion and play an important role in bone repair (Yasko *et al*., 1992). The mature BMP-2 protein is closely related to the Drosophila dpp protein and the Xenopus BMP-2. The human BMP-2 can substitute the Drosophila homologue dpp during dorsalventral patterning, and, vice versa, a recombinant dpp protein induces ectopic bone formation in rats. The mature BMP-2 is a homodimer of two 114-residue subunits representing the C-terminal sequence of a long precursor protein of 396 amino acids. Each of the BMP2 monomers (114 residues) contains a cystine knot which is necessary to stabilize the entire structure since this globular protein lacks the common hydrophobic core. A hydrophobic core between the monomers is created during dimerization and this result in low solubility of BMP2 in aqueous solutions (Scheufler *et al*., 1999). Only one of the four N-glycosylation sites present in the proprotein is retained and used in the mature BMP-2 polypeptide chains (Israel *et al*., 1992). Great efforts have been made to obtain adequate amount of BMP2 protein. It has been shown that it is very difficult to extract BMP2 directly from the human or animal's bones. With the help of molecular biotechnology, the recombinant BMP2 has been expressed in Escherichia coli, Chinese hamster ovary (CHO), mammalian cells and silkworm larvae (Wozney *et al*. 1988; Hammonds *et al*. 1991; Ishida *et al*. 1994). BMP2 has also been expressed in adenovirus. This adenovirus can efficiently transduce human bone marrow mesenchymal stem cells leading to enhanced bone formation in vivo (Olmsted-Davis *et al*. 2002).

Recombinant baculoviruses are widely used to express heterologous genes in cultured insect cells and insect larvae. Since 1985, when the first protein (IL-2) was produced in large scale from a recombinant baculovirues, the baculovires system has become one of the most versatile and powerful eukaryotic vector systems for recombinant protein expression. The baculovirus expression vector system (BEVS) is particularly advantages over other expression systems: safety, ease of scale up, high levels of recombinant gene expression and use of cell lines ideal for suspension culture.

In this study, to produce the human bone morphogenetic proteins 2 (hBMP2) recombinant protein, we used the Baculovirus Expression Vector System (BEVS) and found to be expressed using by western blot analysis.

Materials and Methods

Cell culture

Sf9 cells derived from the pupal ovarian tissue of *Spodoptera frugiperda* was cultured at 27°C in TC-100 medium (Sigma) containing 10% (v/v) heat-inactivated fetal bovine serum (Gibco, USA), as descrigbed previously (Yun *et al*., 2005).

Construction of baculovirus transfer plasmid

The cDNA of human BMP2 gene served as template for the PCR with the primers containing sense primer (5′-GCGGCC GCAGACGGACTGCGGTCTCCTAAAGGTCGACCATGG TGGCCGGGACCCGCTGT-3′) and an anti-sense primer (5′-CTCGAGGTGGTGGTGGTGGTGGCGACACCCACA ACCCTCCAC-3′). It was appended with a His6-taq site in the C-terminus. At the same time, two restriction sites *Not* I and *Xho* I were introduced to N-terminus and C-terminus, respectively. The hBMP2 fragment was subcloned into pGEM-T-easy vector (Promega, USA), double digested with *Not* I and *Xho* I, purified by agarose gel electrophoresis, and subcloned into the baculovirus transfer vector pBACgus-4x-1 (Novagen, Germany). The resulting experimental vector was named pBACgus-4x-hBMP2 (Fig. 1).

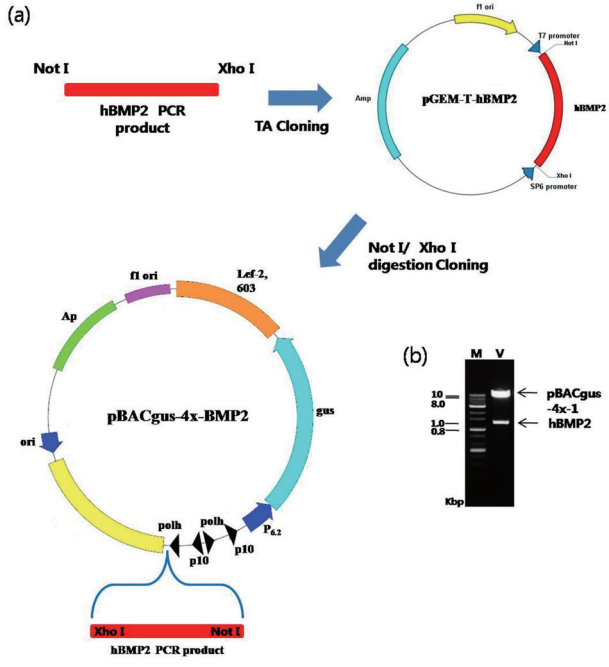

Fig. 1. Construction of the baculovirus transfer vector. The PCR product for ORF flanked by *Not* I/*Xho* I stie was inserted into pGEM-T vector. The pGEM-T-hBMP2 were digested with *Not* I/*Xho* I and then DNA fragment was subcloned into the *Not* I/*Xho* I site on pBACgus-4x-1 (a). The ppBACgus-4x-hBMP2 was digested with *Not* I/*Xho* I (lane V). M, 1kb DNA marker (b).

Construction of the recombinant baculovirus

Recombinant baculovirus was prepared by the transposition of expression cassettes from recombinant plasmid according to the instruction manual of the BacVector baculovirus expression system (Novagen, Germany). The BacVector baculovirus expression system was used to generate the recombinant AcNPV. In brief, an purified transfer plasmid, pBACgus-4x-hBMP2 was co-transfected with BacVector Triple Cut Virus DNA (Novagen, Germany) using FuGENE HD Transfection Reagent (Roche, Germany) into Sf9 insect cells. Cells were incubated at 27°C for 4 days, after the medium was collected and screened a recombinant AcNPV with methods of limited dilution. The resulting recombinant virus was named Ac-hBMP2.

Production of recombinant protein

In order to produce recombinant protein hBMP2, seeded a T75 flask with 2×10^7 Sf9 cell in 10ml medium and infected the Ac-hBMP2 with an MOI of at least 5pfu per cells. Infected cells were cultured Sf-900 III SFM (GIBCO, USA) at 27°C and monitored by cell counting and examination of their size distribution using a cell counter. Media were removed 72 h after infection and cells were harvested by scraping

Expression assay

The cells were lysed by the addition of I-PER Insect Cell Protein Extraction Reagent (Pierce, USA) as described under material and methods. The cell lysate was mixed with SDS sample buffer (62.5 mM Tris-HCl pH6.8, 6% (w/v) SDS, 30% glycerol, 125 mM DTT, 0.03 (w/v) bromphenol blue) and separated by 12% (v/v) SDS-PAGE. The separated gel was fixed and stained with 0.1% Coomassie Brilliant Blue R-250. For Western blot analysis, SDS-PAGE was performed as described above, and the proteins were blotted onto a nitrocellulose membrane in transfer buffer (25 mM Tris/HCl, pH 7.6 and 192 mM glycine in 20% methanol) at 30 V overnight at 4◦C (Towbin *et al*., 1979). The membrane was then blocked by incubation in a 1% BSA solution for 2 h at room temperature (RT). The blocked membrane was incubated with the mouse anti-BMP2 monoclonal antibody (Abnova, USA). The membrane was washed in buffer containing 10mM Tris/HCl (pH 8.0), 100mM NaCl, and 0.05% Tween 20 (TBST) and incubated with HRP-conjugated goat anti-Mouse IgG (abcam, UK) for 1 h at RT. After washing with TBST, the membrane was detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA). Densitometry of Western blot signal was obtained using the Image J program (NIH Image, USA).

Results and Discussion

There is tremendous demand for BMP2 protein due to its great potential for bone injury repair. The object of this research is to construct a high level human BMP2 (hBMP2) expression. In this study, to produce the human bone morphogenetic protein 2 (hBMP2) recombinant protein, we used the Baculovirus Expression Vector System (BEVS) (Fig. 1). Baculovirus transfer plasmid used a pBACgus-4x-1 (Novagen) designed for cloning and co-expression of up to four target genes or multiple copies of the same gene in insect cells. The plasmid contained *gus* gene encoding ß-glucuronidase, which was used as a reporter to verify recombinant viruses by staining with X-gluc. DNA sequence revealed a 1191 bp DNA fragment encoding C-terminal 397 amino acid of hBMP2 protein. In the recombinant plasmid, pBACgus-4x-hBMP2, BMP-2 expression was under the direct control of a polh promoter. Recombinant baculovirus, *vAc*-hBMP2, was selected by plaque assay. To analysis whether the hBMP2 gene was correctly introduced in wild type AcNPV viral genome, *vAc*-hBMP2 viral DNA was extracted from Sf9 cells infected with *vAc*-hBMP2. The extracted *vAc*-hBMP2 viral DNA used as template for PCR screening. PCR product was amplified with sense and antisense primer used in construction of the baculovirus expression transfer vector, pBACgus-4x-hBMP2. The DNA fragment of 1191 bp was correctly amplified on a 1% agarose gel. (Data not shown). The result suggests that the polyhedrin gene of *AcNVP* be replaced correctly with the hBMP2 gene. Also, to confirm whether hBMP2 mRNA was expressed, the present of its transcript was examined by RT-PCR. RT-PCR analysis was done using mRNA extracted from Sf9 cells infected with *vAc*-hBMP2. The PCR product of 1191 bp was correctly amplified on a 1% agarose gel, suggesting that hBMP2 was expressed in Sf9 cells (Fig. 2). Western blot analysis was used for confirmation of hBMP2 recombinant protein expression.

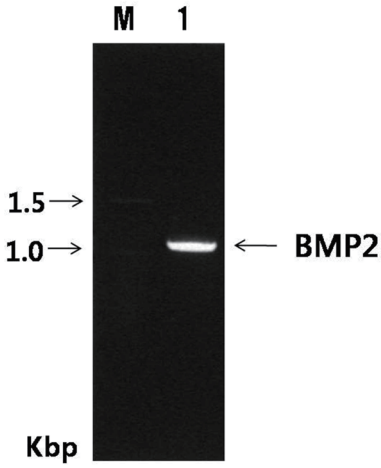

Fig. 2. Analysis of hBMP2 recombinant protein by RT-PCR. Total RNA was prepared from infected Sf9 cells with recombinant vac-BMP2 using by TRI REAGENT according to the manufacture's protocol. Total RNA (2μg) was amplified by a three-step protocol using High-Capacity cDNA Reverse Transcription kit. RT-PCR was performed with sense and antisense primer used in construction of the pBACgus-4X-hBMP2 transer vector. Lane M, 1 kb ladder DNA marker; lane 1, infected with recombinant *vac*-BMP2.

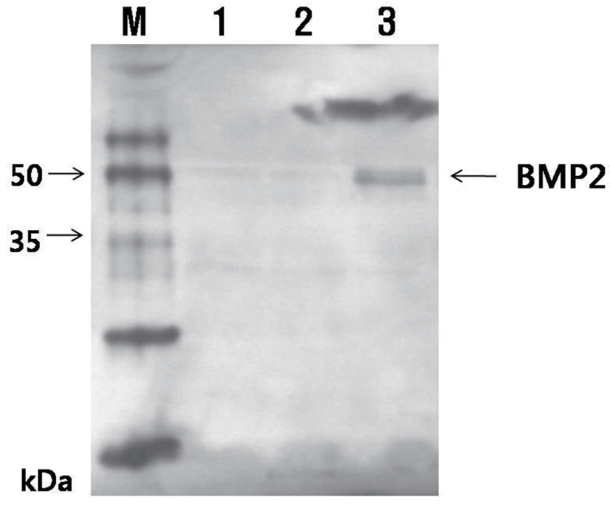

Fig. 3. Western blot analysis of hBMP recombinant protein. The cells with infected *AcNPV* or recombinant *vac*-BMP2 were incubated at 27°C for 3 days, harvested, and lysed with SDS sample buffer. The protein samples were electrophoresed on a 12% SDS-PAGE and transfer to a PVDF membrane. Western blot analysis used mouse anti-His antibody. Lane M, molecular mass standard; lane 1, Sf9 cells were mock-infected; lane 2, infected with the wild-type *AcNPV*; lane 3, infected with recombinant *vAc*-hBMP2.

The result was showed that band of approximately 44.71 kDa was detected (Fig. 3). As these results, we confirmed that the hBMP2 recombinant protein was expressed in Sf9 cells.

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