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Recombinant human BMP-2/-7 heterodimer protein expression for bone tissue engineering using recombinant baculovirus expression system

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Abstract

Bone morphogenetic proteins (BMPs) are essential growth factors for bone formation, skeletal development and bone regeneration. The BMP-2/7 heterodimer is known to have remarkable effects on osteogenic induction that are even stronger than the BMP-2 or BMP-7 homodimers. We designed a recombinant human BMP-2/7 (rhBMP-2/7) heterodimer protein with four glycine residues between BMP-2 and BMP-7 protein to facilitate free bond rotation of domains. The Baculovirus Expression Vector System (BEVS) is routinely used to produce recombinant proteins in the milligram scale. In this study, the BEVS was used to express the rhBMP-2/7 protein whrer the recombinant baculovirus was recovered in the host Sf9 cells. To confirm the biological activity of rhBMP-2/7 protein secreted from the BEVS as an osteogenic differentiation and induction factor, we measured the BMP-induced ALP activity. rhBMP-2/7 could be used as an alternative to BMPs to overcome limitations like short half-life and requirement for high concentrations. Furthermore, rhBMP-2/7 may be an efficient tool for various application studies such as bone regeneration and skeletal development.

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Introduction

The Baculovirus Expression Vector System (BEVS) is used extensively for the expression of a variety of recombinant proteins in insect cells (1-3). The BEVS has advantages such as a high capacity for large insert genes and similarity to mammalian cell's with respect to post-translational modification compared to prokaryotic cells and yeast (4-6). In addition, BEVS can produce large amounts of recombinant proteins and has been used in production at the industrial scale, for applications like gene therapy (4-6).

Osteoblastic differentiation is regulated by bone morphogenetic proteins (BMPs). BMPs belong to the transforming growth factor-beta (TGF-β) superfamily and are essential for osteoblastic differentiation. Recombinant human BMP-2(rhBMP-2) and BMP-7(rhBMP-7) are widely known to affect bone formation, remodeling, growth, proliferation and differentiation of osteoprogenitor cells (7-9). In previous studies,

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rhBMP-2 or the rhBMP-7 homodimers have been successfully demonstrated to possess biological activities such as promotion of healing bone defects in many animal experiments (9, 10). Interestingly, BMPs heterodimer forms were more potent and biologically active than the homodimer forms in bone induction and osteoblastic differentiation effects (5, 9, 11).

To use the BEVS for producing rhBMP-2/7 protein, the rhBMP-2/7 gene was inserted into the pBAC-gus4x-egfp shuttle vector in direct orientation with respect to the *Autographa califonia* nuclear polyhedrovirus polyhedron (*AcNPV polh*) promoter to generate the pBAC-gus-rhBMP-2/7-egfp shuttle vector (4, 5). Additionally, we tagged the rhBMP-2/7 protein with an additional 6× His-tag to facilitate collection of the recombinant baculovirus overexpressing human BMP-2/7 protein from the host cells. Consequently, rhBMP-2/7 was efficiently expressed and obtained from Sf9 cells. To confirm its biological activity in osteoblastic differentiation, we infected the C2C12 cells (murine osteoblastic cell line) with rhBMP-2/7 expressing recombinant baculovirus. Our results show that rhBMP-2/7 can be used to replace other bone formation and differentiation factors. Further studies need to be performed to determine the mechanisms involved in BMP heterodimers activities.

Materials and Methods

Cell culture

Sf9 cells were grown in Grace's insect medium (Gibco, CA, USA) containing 10 % heat-inactivated fetal bovine serum (FBS) (v/v) (Gibco) and 1 % gentamycin (w/v) in an incubator at 27°C. The mouse osteoblast cell line C2C12 was grown in Dulbecco's Modified Eagle's Medium (Gibco, CA, USA) supplemented with 10 % fetal bovine serum (FBS) (v/v) (Gibco), 1 % gentamycin (w/v) and was maintained in a humidified incubator with 95 % air and 5 % CO_2 at a temperature of 37°C.

Plasmid DNA construction

The fragments sizes of mature forms of rhBMP-2 and rhBMP-7 are 345-bp and 417-bp, respectively (9). We used specific primer pairs and *Top-taq* Premix polymerase (CoreBio systems, Seoul, Korea) to amplify the mature hBMP-2 and hBMP-7 fragments (primer pairs are shown in Table 1). To construct the rhBMP-2/7 expression vector, the rhBMP-2 gene from the PCR amplified product was excised by *Bam*HI/*Xho*I double digestion. This fragment (367-bp) was inserted into the *Bam*HI/*Xho*I restriction site of the pBAC-gus4X-egfp shuttle vector (12). The rhBMP-7 gene fragment (434-bp) was excised following *Xho*I/*Not*I double digestion and was inserted into the *Xho*I/*Not*I restriction site of the pBAC-gus-rhBMP-2-egfp shuttle vector in direct orientation with respect to the *polh* promoter in order to generate the pBAC-gus-rhBMP-2/7-egfp shuttle vector.

Table 1. PCR primer sequences

Name	Sequences (5' to 3')
Human BMP-2 (F)	AGATCTTCAGCTAGCATGCAAGCCAAAC ACAAACAGC
Human BMP-2 (R)	ATCGCTCGAGCCCTCCGCCACCGCGAC ACCCACAACCCTC
Human BMP-7 (F)	AGATCAGCTAGCAGATCTCGCTCGAGTC CACGGGGAGCAAACAGC
Human BMP-7 (R)	AGCTAAGATCTCCCTCCGCCACCGTGGC AGCCACAGGCC
EGFP (F)	GGTGAGCAAGGGCGAGGAGCT
EGFP (R)	TCTTGAAGTTCACCTTGATGCCG

Recombinant baculovirus production

For the production of recombinant baculovirus, Sf9 cells were seeded in a 6-well tissue culture plate at 10^5 cells/well with 2 mL of TNM-FH insect Medium (Welgene, Korea) containing serum and antibiotics. The cells were incubated overnight at 27°C to allow the cells to attain 70~80 % of morphology and attach to the bottom of the plate. On the next day, the baculovirus genomic DNA and the recombinant pBAC-gus-rhBMP-2/7-egfp shuttle vector were transfected into Sf9 cells using Cellfection®II Reagent (Invitrogen) according to the manufacturer's instruction. The transfected cells were incubated for at 27°C until cells showed cytopathic effect (CPE) by 72 h. Subsequently, 2 mL of culture medium from each well was collected and centrifuged to remove the cells and debris.

Western blotting analysis

Samples were prepared for western blot analysis in the following manner. Sf9 cells were infected with the pBAC-gus-

rhBMP-2/7-egfp recombinant baculovirus in 6-well plates. After 72 h, cells were collected and lysed in 1× Laemmli buffer [2 % sodium dodecyl sulfate (SDS), 5 % 2-mercaptoethanol, 10 % glycerol, 125 mM Tris, 0.001 % bromophenol blue, pH 6.8]. The 6× His monoclonal antibody was used to detect rhBMP-2/7 protein (Clontech Co., Japan). The protein levels were detected using the ECL western blotting analysis system (Amersham Pharmacia Biotech. Ltd., UK).

ALP staining

To demonstrate the biological effect of rhBMP-2/7, C2C12 cells were treated rhBMP-2/7 in a 96-well white-tissue culture plate. Untreated C2C12 cells were used as control. Cells were first washed thrice with phosphate-buffered saline (PBS) and fixed using absolute ethanol for one min. The fixed cells were then washed thrice with double distilled, stained using crystal violet solution (Sigma-Aldrich) for 5 min and washed five times with doube distilled water to remove the residual dye. ALP activity was visualized using the Sigma Fast BCIP/NBT substrate (Sigma-Aldrich). We observed the cells using a fluorescence stereomicroscope (Leica MZ16FA, Leica) to examine osteogenic differentiation and recorded our findings using the Leica camera (Leica DFC490, Leica).

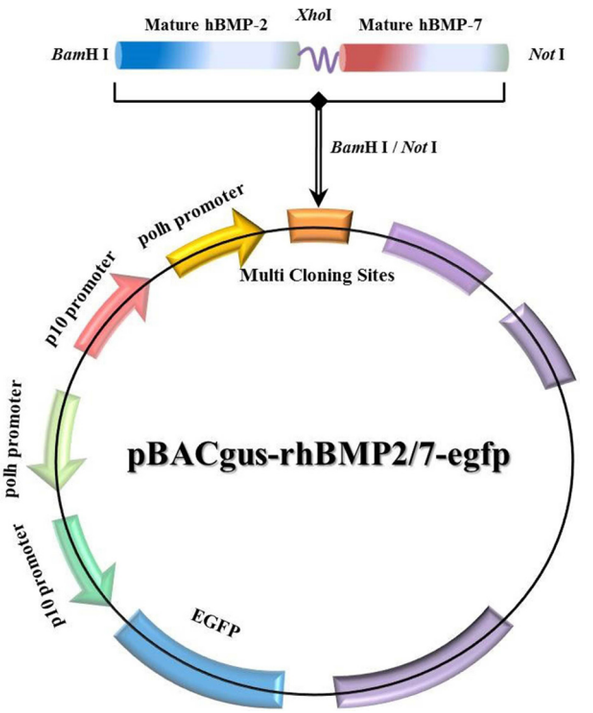

Fig. 1. Construction of baculovirus transfer vector. We inserted the four glycine sequence between the mature forms of rhBMP-2 and rhBMP-7 genes to allow free bond rotation. The 762-bp full-length cDNA was inserted into the pBAC-gus-rhBMP-2/7-egfp plasmid DNA using *Bam*HI and *Xho*I restriction enzyme sites. The rhBMP-2/7 gene of the cloned plasmid DNA was under the control of the *polh* promoter. Moreover, the secreted rhBMP-2/7 protein was tagged with 6× His amino acids for easy detection of the recombinant protein. The resulting plasmid is termed as pBAC-gus-rhBMP2/7-egfp.

Results and Discussions

To generate the rhBMP-2/7 expressing plasmid DNA for induction of osteoblastic differentiation, we designed the rhBMP-2/7 heterodimer protein with four glycine residues between rhBMP-2 and rhBMP-7 proteins to allow free bond rotation of domains. The rhBMP-2/7 gene was amplified and cloned into the pGEM-T vector and cloning was confirmed by sequencing analysis. As a result, the full-length 762-bp cDNA of rhBMP-2/7 was demonstrated. Subsequently, the rhBMP-2/7 and the *egfp* reporter gene expression cassettes were inserted under the *polh* and *p10* promoters of the standard baculovirus transfer vector pBAC-gus4x-egfp, respectively (Fig. 1), for high-level expression in infected insect cells. The recombinant baculovirus derived from *AcNPV*, which is widely used for large-scale expression of proteins in insect cells. This virus has potent advantages such as lack of toxicity in mammalian and infected insect cells. In addition, it allows high-level expression of recombinant proteins, easy manipulation, and insertion of large DNA sequences. In this study, we inserted the rhBMP-2/7 gene into the viral genome of *AcNPV* and transfected it in Sf9 cells to confirm it biological activity in the context of osteoblastic differentiation from osteoprogenitor cells.

Recombinant baculovirus was obtained by a homologous recombination strategy. Herein, we infected Sf9 cells with rBV-egfp as the wild-type control or with rBV-rhBMP-2/7-egfp as the recombinant virus. After 72 h, the genomic DNAs from baculovirus infected Sf9 cells was amplified using specific primers (BMP-2 F, BMP-7 R, EGFP F/R) to demonstrate the specific viral genome expression pattern of the recombinant baculovirus. The *egfp* gene was amplified from all the samples. However, the *rhBMP-2/7* gene was amplified only from the rBV-rhBMP-2/7-egfp recombinant virus infected cells. These results indicate that

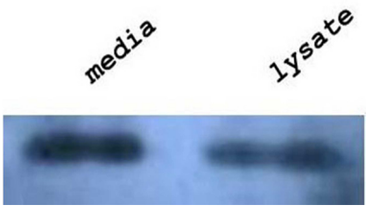

Fig. 2. Western blot analysis of rhBMP-2/7 fusion 6× His protein. Sf9 cells were infected with the recombinant baculovirus, harvested at 72 h postinfection, and then examined for rhBMP-2/7 heterodimer protein expression using 6× His monoclonal antibody (see Materials and Methods).

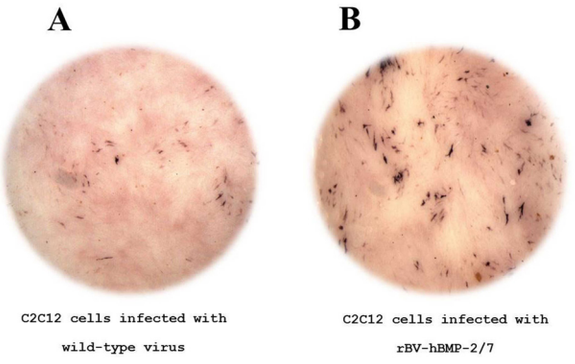

Fig. 3. Analysis of osteoblastic differentiation from C2C12 cells by ALP staining. To compare the osteoblastic differentiation, we infected the C2C12 cells with recombinant baculovirus containing rhBMP-2/7 or wild-type virus as a control. The ALP staining shows increased osteoblastic differentiation in (B) than in (A).

the recombinant baculovirus containing *rhBMP-2/7* and *egfp* genes replicated in the Sf9 cells (data not shown).

To confirm the expression of rhBMP-2/7 protein from the recombinant virus genome incorporated into the virus particles, we infected Sf9 cells with recombinant baculovirus for 72 h. Subsequently, we separated the media, infected cell lysate, and pellet from recombinant baculovirus infected cells. Two parts of the samples were analyzed by western blotting using 6× His monoclonal antibody (Fig. 2). Our western blot results show that rhBMP-2/7 was expressed in all parts of the infected cells. Moreover, rhBMP-2/7 was especially expressed in the pellet sample. Next, we investigated the biological activity of the rhBMP-2/7 heterodimer by measuring the effect of secreted rhBMP-2/7 protein as an osteogenic factor on differentiation of C2C12 cells. To confirm the biological role of the recombinant proteins, we infected C2C12 cells with rhBMP-2/7 expressing virus or with wild-type virus as a control. We then used the bone-specific marker, ALP, to evaluate bone differentiation. After 3 d, ALP staining in cells was visualized using Sigma Fast BCIP/NBT. As shown in Fig. 3, we confirmed that osteoblastic differentiation potential of the recombinant baculovirus expressing the rhBMP-2/7 was higher than that of the control wirus. These results suggest that rhBMP-2/7 demonstrates the proper effect on osteoblastic differentiation in C2C12 cells.

Our results demonstrate the role of the potential and biological function of the rhBMP-2/7 heterodimer protein using BEVS to increase the expression of osteoblast-specific genes in mouse pluripotent stem cells during osteogenic differentiation. BMPs are growth factors that have been used in various bone-related studies and clinical applications (7-9). However, several limitations are encountered in the therapeutic application of BMPs, such as requirement of relatively high concentrations, short half-lives, and high cost (9). To overcome these problems, we developed the rhBMP-2/7 heterodimer protein with four glycine residues between rhBMP-2 and rhBMP-7 proteins to facilitate free bond rotation of domains. Previous studies have reported that heterodimer forms of BMPs are more efficient than BMP homodimer forms in bone induction and osteoblastic differentiation effects (5, 9, 11). Therefore, we generated a recombinant baculovirus expressing the human BMP-2/7 protein. Mouse myoblastic C2C12 cells were infected with rhBMP-2/7 to confirm the biological activity of rhBMP2/7. The production of rhBMP-2/7 heterodimer protein using BEVS has more advantages and is distinct from other previous studies. Our results indicate that rhBMP-2/7 shows its biological activity as an osteoblastic growth factor. The development of recombinant baculovirus expressing the rhBMP-2/7 heterodimer by BEVS could be a useful research tool with significant clinical potential and applications for bone regeneration and skeletal development. Further studies examining recombinant rhBMP-2/7 protein production by BEVS will be provide enhanced functionality and efficiency for tissue engineering of bone tissue substitutes.

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