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 wher 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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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 california* 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% CO₂ 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-Tag 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.

**Recombinant baculovirus production**

For the production of recombinant baculovirus, Sf9 cells were seeded in a 6-well tissue culture plate at 10⁵ 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 double 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).

**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
rhBMP-2/7 expressing recombinant baculovirus

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 virus. 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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