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Development of Refolding Process to Obtain Active Recombinant Human Bone Morphogenetic Protein-2 and its Osteogenic Efficacy on Oral Stem Cells

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BMP-2 is a well-known TGF-beta related growth factor, having a significant role in bone and cartilage formation. It has been employed to promote bone formation in some clinical trials, and to differentiate mesenchymal stem cells into osteoblasts. However, it is difficult to obtain this protein in its soluble and active form. hBMP-2 is expressed as an inclusion body in the bacterial system. To continuously supply hBMP-2 for research, we optimized the refolding of recombinant hBMP-2 expressed in *E. coli*, and established an efficient method by using detergent and alkali. Using a heparin column, the recombinant hBMP-2 was purified with the correct refolding. Although combinatorial refolding remarkably enhanced the solubility of the inclusion body, a higher yield of active dimer form of hBMP-2 was obtained from one-step refolding with detergent. The refolded recombinant hBMP-2 induced alkaline phosphatase activity in mouse myoblasts, at ED_{50} of 300-480ng/ml. Furthermore, the expressions of osteogenic markers were upregulated in hPDLSCs and hDPSCs. Therefore, using the process described in this study, the refolded hBMP-2 might be cost-effectively useful for various differentiation experiments in a laboratory.

Key words: Recombinant human bone morphogenetic protein-2 (rhBMP-2), Refolding, Heparin column purification, Osteogenic induction

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Introduction

Bone morphogenetic proteins (BMPs) are signaling molecules belonging to transforming growth factor β (TGF-β) super family. Approximately 20 subtypes of BMPs have been identified so far [1]. Although BMPs are originally known to function in osteogenic regulation, they are also involved in pleiotropic effects for differentiation, development, and maintenance of various tissues such as neural crest cell differentiation [2], differentiation of chondroblasts to synthesize cartilage matrix [3, 4], and limb bud patterning [5, 6]. BMP-2 (or BMP2A) and BMP-4 (or BMP2B) are common BMPs that can induce bone regeneration and ectopic bone formation in vertebrates [1, 7, 8]. When BMP-2 is applied to mouse mesodermal progenitor cells, cells can be induced to chondrocytes and osteocytes [9]. BMP-2 can lead to osteoblastic lineage of myoblastic cells and inhibit myogenic differentiation by transcriptional repression of myoglobin D gene [10, 11]. BMP signaling cascade involves type I and type II receptors and transcription factors of Smad family. Binding of BMPs to type II can induce type I receptor phosphorylation, leading to

phosphorylations of Smad1, Smad5, and Smad8 for transcriptional regulation of BMP downstream target genes [12, 13]. BMP signaling can be regulated by various antagonists. These factors can bind to BMP receptors or BMPs directly, thus preventing BMP-receptor interaction. With its biological functions, BMP-2 has been evaluated for clinical use in regeneration of damaged tissues such as bone fractures [14, 15]. As the demands for BMP-2 has been so high in clinical and research use, many researches have attempted to carry out efficient purification of BMP-2. Although BMPs can be purified directly from bones, the process is complicated and protein yield is usually very low [16]. Production of BMPs from mammalian cell culture is also inefficient, although such expression system in eukaryotic cells does not require renaturation [17]. For this reason, expression of BMPs using prokaryotic system in a large scale has been attempted. However, BMPs are initially detected in insoluble pellets in *E. coli* expression, they have to be solubilized by using high concentration of salts followed by refolding [18]. So far, by modifying codon usage of human protein so that it is suitable for expression in *E. coli*, scaling-up soluble expression of synthetic hBMP-2 had been performed successfully [19]. The objective of this study was to provide an effective purification process for active form of recombinant hBMP-2 by optimizing the solubilizing-refolding process in bacterial system.

Materials and Methods

Construction of human BMP−2 expressing plasmid

To construct the recombinant hBMP-2 expressing plasmids, the cDNA of hBMP-2 (amino acids 283-396) were amplified with primers; forward, 5'-CAAGCCAAACACAAA CAG-3' and reverse, 5'-GCGACACCCACAACCCTC-3'. The PCR products were subcloned into pET6XHN vector (Figure 1) and transformed into chemically competent *E. coli* cells (DH5α). The DNA sequences of hBMP-2 expressing plasmid were confirmed by sequence analysis.

Protein expression and purification

For protein expression, hBMP-2 expression plasmid was transformed into *E. coli* BL21(DE3). Transformants were cultured in LB medium containing 100 μg/ml of ampicillin untill stationary phase. Then 1/100 of the culture was inoculated in fresh media. When optical density at 600nm (OD_{600}) reached 0.6, protein expression was induced by adding IPTG at final concentration of 100μM at 30°C for 24 hours with vigorous shaking. For preparation of inclusion body, cells were harvested and resuspended in PBS(pH7.4) and disrupted thoroughly by sonication on ice. After centrifugation at 15,000xg for 15min at 4°C, the pellet of inclusion body was resuspended and washed. For solubilization, the pellet was resuspended with solubilization buffer (4M Guanidine-HCl, 100mM Tris, pH8.5, 100mM DTT, and 1mM EDTA) and incubated at room temperature for 24 hours. To discard insoluble substances, the solution was centrifuged at 18000xg for 15min at 4°C. To refold the protein, 7 volumes of renaturation buffer (50mM Tris-HCl, pH8.5, 1M NaCl, 5mM EDTA, 5mM total Glutathione) containing 0.75M 2-(cyclohexylamino) sulfonic acid (CHES) was added with solubilized rhBMP-2 and incubated at room temperature for 48 hours. The refolded protein solution was dialyzed three times in PBS containing 0.3M guanidine (pH 7.4) for 2 hours. For combinatorial refolding, detergent and alkali were treated sequentially for the first and second refolding, respectively. For the second refolding by alkali, 9 volumes of alkali buffer (50mM KH_2PO_4, 50mM NaCl, 1mM EDTA, pH10.7) was added followed by incubation at room temperature for 30min. It was then neutralized. Soluble fractions from the refolding step were loaded into Heparin-Sepharose column® (BioVision, USA). Monomer and dimers of recombinant hBMP-2 were eluted with PBS containing indicated concentration of NaCl.

Cell culture

C2C12 mouse myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 20% fetal bovine serum and antibiotics in 5% CO_2 at 37°C. For osteogenic induction, the C2C12 cells were treated with commercial rhBMP-2 (cat.10426-HNAE, Sino Biological Inc., China) and purified rhBMP-2 for 5 days. Human dental pulp stem cells (hDPSCs) and human periodontal ligament stem cells (hDPLSCs) were cultured by following the previous procedure [20, 21]. In brief, human third molars were provided with adult patients (17-28 years of age) under the approval by the IRB of the Dankook Dental Hospital (DKUDH IRB 2016-12-005). Pulp and periodontal ligament tissues were obtained from tooth, chopped, and digested by 3mg/ml collagenase type I (Millipore) and 4 mg/ml dispase (Sigma) for 1 hour at 37°C. Cell suspension was incubated with α-Minimal Eagle's medium (α -MEM, Hyclone) containing 20% fetal bovine serum and

antibiotics in 5% CO_2 at 37°C. In order to osteogenic differentiation, cells were cultured with additive supplements consisted with 100μM ascorbic acid, 500nM dexamethasone, and 5mM β-glycerophosphate for 7-14 days.

Assay of Alkaline phosphatase (ALP) activity

Intracellular ALP activity was measured following the manufacturer's instructions (BioVision) with modification. In brief, washed C2C12 cells ($4x10^4$~$1x10^5$) were homogenized in assay buffer, following by centrifugation to remove insoluble material at 13,000 xg for 3 min. The total sample volume was adjusted to 80μl with Assay Buffer, and 50μl of the 5mM pNPP solution was added to each well, followed by incubating for 60 min at 25°C in the dark. 20μl of stop solution was added and mixed to terminate the reaction. The ALP activity was measured at 405nm in a micro plate reader.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The expression levels of osteogenic markers were analyzed by qRT-PCR. Briefly, total cellular RNA was isolated from cells, and used for cDNA synthesis by using ReverTra Ace® qPCR RT kit (Toyobo Corporation). The qPCR was performed by using iTaq™ Universal SYBR®Green Supermix (BioRad) system. Primers used are listed in Table1. The cycling parameters of qPCR were followed; 1 cycle for 1 min at 95°C, 40 cycles for 15 sec at 95°C and 1 min at 60°C. After PCR, a dissociation curve was constructed in the range of 65°C to 95°C. The GAPDH was used as an internal control to normalize the variability in target gene expression. Relative quantity (△Cq) for each gene is calculated with this formula: $\triangle Cq=2^{(Cq(Min)-Cq(Sample))}$. Cq(Min), average Cq for sample with the lowest average Cq for GOI; Cq(Sample), average Cq for sample; GOI, Gene of Interest. Normalized Expression (△△Cq) is the relative quantity of each gene normalized to the quantities of GAPDH gene. The calculation for normalized expression is described in the following formula, $\triangle Cq = \triangle Cq_{(Sample)}/\triangle Cq_{(Ref)}$ from three independent experiments.

Results

Construction of the expression plasmid of human BMP-2 fused to $(His-Asn)_6$tag

Mature peptide of hBMP-2 (amino acids 283-396) was introduced into pET6xHN-C (TaKaRa) expression vector containing poly-(His-Asn) tag in the carboxy-terminal of the expressed protein (Figure 1). Based on the subcloning strategy, the size of recombinant hBMP-2 was estimated at 13.9 kDa.

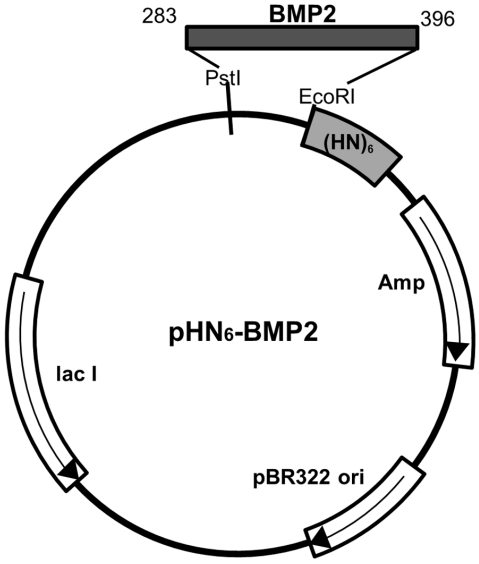

Figure 1. The construct of the recombinant hBMP-2 expression plasmid. The open reading frame of human BMP-2 (from amino acid 238 to 396) was inserted into the pET6XHN vector containing the hexa histidine-asparagine $(HN)_6$ tag. The restriction enzyme PstI and EcoRI were used and the tag is positioned at the C-terminus of hBMP-2.

Optimal refolding condition for purification of the active form of recombinant human BMP-2

To establish the optimal condition for refolding recombinant hBMP-2, two different strategies were tested and evaluated by comparing their purification yields. In these procedures, inclusion body of recombinant hBMP-2 was solubilized by treatment with guanidine and denatured proteins were refolded by treatment with detergent (Figure 2A, a) or combinatorial treatment with detergent and alkali (Figure 2A, b). After cultivation in LB medium supplemented with ampicillin, protein induction was performed for log phase cells at 30°C for 24 hours with 100μM of IPTG. As expected, although hBMP-2 with C-terminal $(His-Asn)_6$ tag was highly expressed in *E.coli*, the bulk of the protein partitioned to the insoluble fraction after cell lysis as inclusion body after centrifugation (Figure 2B, lane 4). Next, the inclusion body of recombinant hBMP-2 was denatured and solubilized by treatment with high concentration of guanidine hydrochloride. For refolding, CHES detergent or a combination of detergent and alkali were used for repetitive

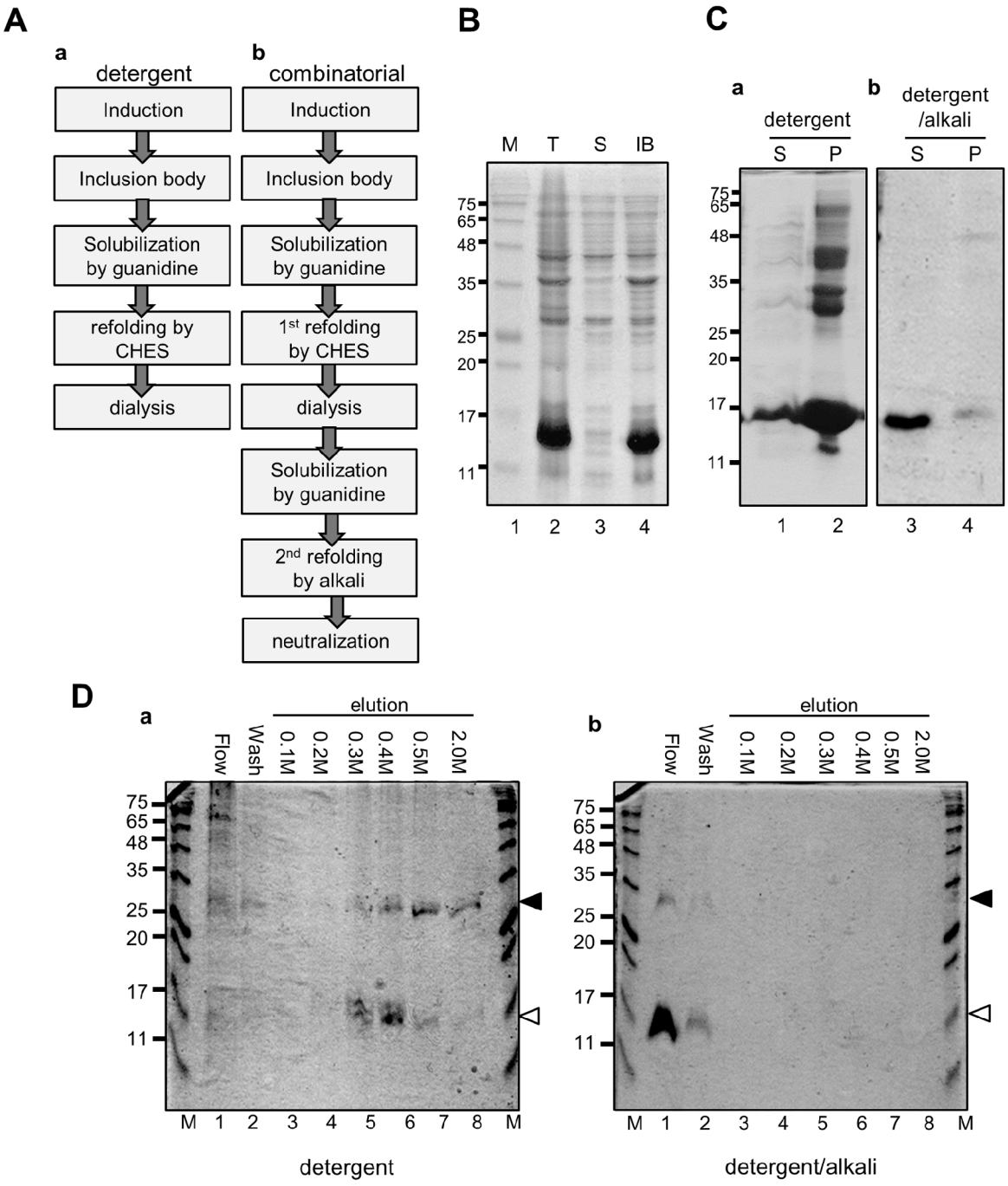

Figure 2. The expression and purification of active form of recombinant hBMP-2. (A) Flowcharts for purification of recombinant hBMP-2. Inclusion bodies solubilized by guanidine followed by refolding by detergent (a) or combinatorial refolding (b). (B) Preparation of inclusion body. Lane 1, protein markers; lane 2, total cell lysate (T); lane 3, soluble fractions (S); lane 4, inclusion body (IB). (C) Dissolution of hBMP-2 by refolding procedures. The soluble (S) and insoluble (P) fraction after refolding were analyzed by SDS-PAGE. a, refolding by detergent CHES; b, combinatorial refolding. Lanes 1 & 3, supernatants after centrifugation of refolding samples; lanes 2 & 4, pellets after centrifugation of refolding samples. (D) Recombinant hBMP-2 in heparin column fractions during purification. The recombinant hBMP-2 solutions after refolding by detergent (a) and combinatorial refolding by detergent and alkali (b) were loaded into heparin column, and each fraction was analyzed by non-reducing condition of SDS-PAGE. The proteins were detected by Coomassie Brilliant Blue staining. 1, flow through; 2, washing fraction; 3, elution fraction in 0.1 M NaCl; 4, elution fraction in 0.2 M NaCl; 5, elution fraction in 0.3 M NaCl; 6, elution fraction in 0.4 M NaCl; 7, elution fraction in 0.5 M NaCl; 8, elution fraction in 2 M NaCl. Monomer and dimer forms of hBMP-2 were indicated as open (▷) and filled (▶) arrowhead, respectively.

refolding. This combinatorial refolding procedure was performed by using detergent for the 1st refolding and alkali for the 2nd refolding. After removing detergent by dialysis and neutralizing pH, soluble and insoluble fractions were analyzed on SDS-PAGE. When detergent was used, denatured protein was not actively refolded and much of the proteins were partitioned in insoluble pellet (Figure 2C, lane2 in a). To increase the refolding efficiency, we performed the 1st and 2nd refolding by combinatorial treatment with detergent and alkali. Interestingly, most proteins were successfully refolded and partitioned in soluble fraction (Figure 2C, lane3 in b). When the soluble fraction of recombinant hBMP-2 refolded by detergent was loaded into the heparin column, the active BMP-2 was eluted by high concentration of NaCl as dimeric form (Figure 2D, lanes 7&8 in a),whereas monomeric protein was eluted by lower concentration of NaCl (Figure 2D, lanes 5&6 in a). Although there was a considerable amount of soluble protein refolded by the combinatorial refolding procedure using

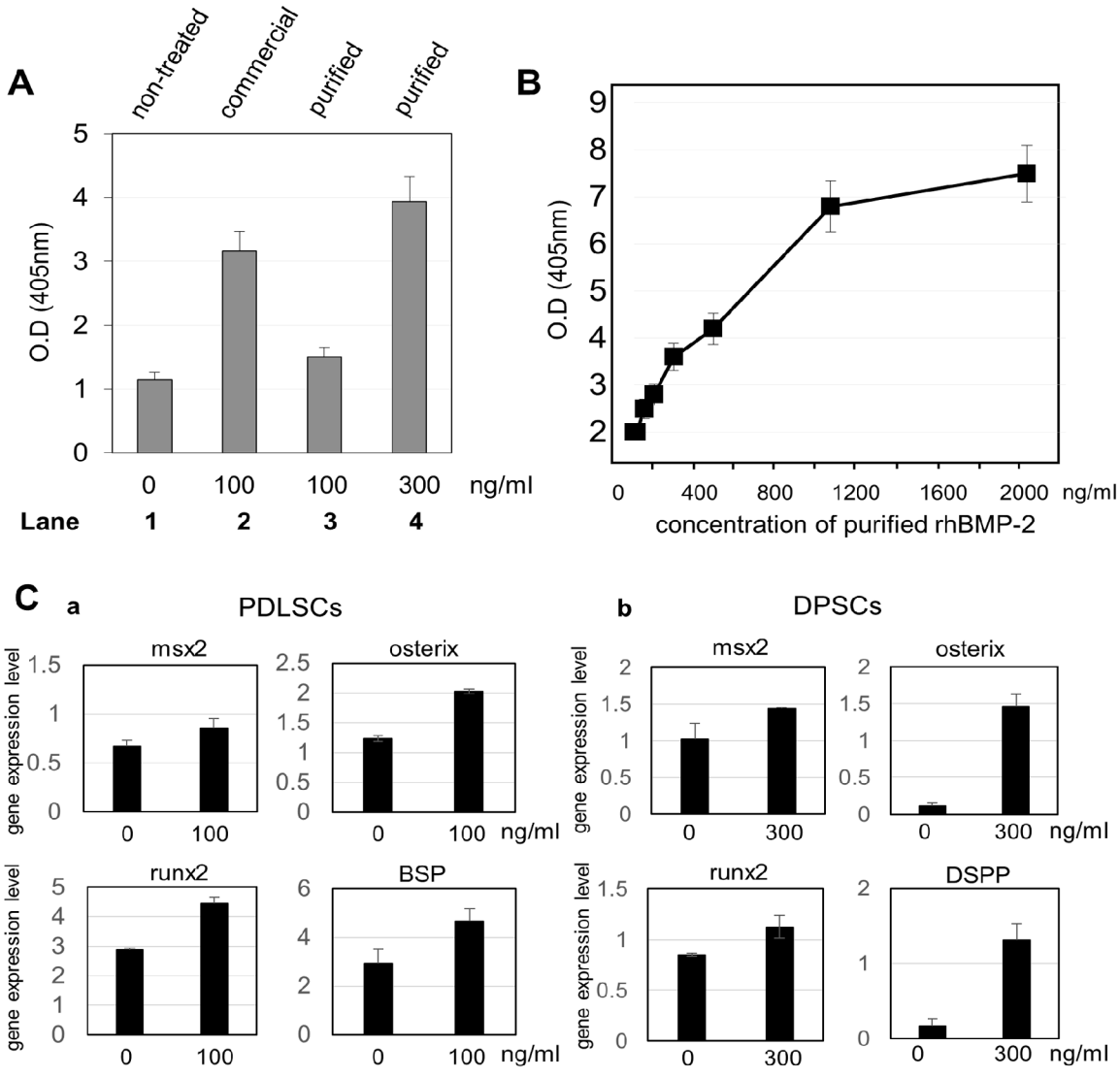

Figure 3. Activity of alkaline phosphatase and osteogenic gene expression were induced by the purified recombinant hBMP-2. (A) Alkaline phosphatase activity induced by commercial and the purified recombinant hBMP-2 (purified) in C2C12 cells. 1, non-treated control; 2, 100ng/ml of a commercial recombinant hBMP-2; 3, 100 ng/ml of the purified recombinant hBMP-2; 4, 300ng/ml of the purified recombinant hBMP-2. (B) Induction of alkaline phosphatase activity by the purified recombinant hBMP-2 in dose dependent manner. (C) mRNA expression of osteogenic and dentinogenic markers in oral cells. (a) The hPDLSCs were treated with 100ng/ml of the purified hBMP-2 for 7 days. The expression of msx2, osterix, runx2, and bone sialoprotein was analyzed by qRT-PCR. (b) The hDPSCs were treated with 300ng/ml of the purified hBMP-2 for 7 days. The expression of msx2, osterix, runx2, and dentin sialophosphoprotein was analyzed by qRT-PCR.

both detergent and alkali (Figure 2C, lane 3 in b), these proteins could not interact with heparin at all. Most of them were collected in flow-through fraction (Figure 2D, lane 1 in b).

Purified recombinant hBMP−2 induces the activity of alkaline phosphatase and osteogenic gene expression

The biological activity of the purified fraction of BMP-2 was determined by measuring its induction of alkaline phosphatase in C2C12 myoblasts at different doses (Figure 3A). For this purpose, we assayed alkaline phosphatase (ALP) activity, a marker of the osteoblastic phenotype. Whereas ALP activity was increased 2.8 times by treatment with 100ng/ml of a commercial recombinant hBMP-2 (Figure 3A, 1 & 2), it was increased 1.3 times by the purified hBMP-2 in this study (Figure 3A, 1 & 3). When 300ng/ml of the purified protein was used for treatment, ALP activity was increased 3.4 times (Figure 3A, 1 & 4), indicating that the purified recombinant hBMP-2 in this study contained relatively lower activity compared to the commercial one. In dose-dependent response, approximate ED_{50} of the purified recombinant hBMP-2 on ALP activity in C2C12 cells was 100-480ng/ml (Figure 3B). The average ED_{50} value of ALP activity of the commercial BMP-2 was 80-200ng/ml. Therefore, this purified recombinant hBMP-2 might be useful for further studies.

To investigate the induction potential for osteogenic differentiation of the recombinant hBMP-2, the purified hBMP-2 was used to treat human dental tissue-derived stem cells. Expression levels of osteogenic and dentinogenic markers were then examined. After 7 days of treatment with 100ng/ml of the purified hBMP-2 in human periodontal ligament cells (hPDLSCs), mRNA expressions of early osteogenic makers such as msx2, osterix, runx2, and bone sialoprotein were increased by 1.3, 1.6, 1.5, and 1.6 times, respectively, compared to their respective controls (Figure 3C, a). Additionally, treatment with 300 ng/ml of hBMP-2 in human dental pulp stem cells (hDPSCs) for 7 days increased mRNA expression levels of osteo/dentinogenic makers such as msx2, osterix, runx2, and dentin sialoprotein by 1.4, 13.3, 1.3, and 8.2 times, respectively, over their respective controls (Figure 3C, b).

Discussion

BMP-2 has been used frequently in laboratory and clinical application for osteogenic induction, bone regeneration, and ectopic bone formation. However, due to the limitations of research costs, we can not use this protein and other cytokines anytime in preliminary experiments. Our goal of this work was to set up an effective method for laboratory level production of an active human BMP-2. Although there is a common disadvantage of inclusion body formation, we used *E.coli* culture system because of simplicity, convenience, and high yield of protein. Inclusion body formation is not always undesirable; very high levels of expression in cells, easy isolation of protein from some contamination, and resistance to proteolysis. For making dissolve lump of the rhBMP-2 protein, we focused on protein refolding. Generally, recovery and refolding of the bioactive proteins from inclusion body have been performed using alkaline pH [22, 23], high pressure[24], detergents [25, 26], organic solvents [27] and low concentration of chaotropes [28, 29]. To increase the recovery efficiency, the denatured proteins were refolded by co-treated with both detergent and alkali (Figure 2A). Although most proteins were successfully refolded and partitioned into soluble fraction (Figure2C), they were not active: these proteins could not interact with heparin at all (Figure 2D). It has been reported that TGFβ family proteins such as BMPs can interact with each other to form active dimers and the dimeric form has heparin-binding potential [17, 30]. When we used detergent only for milder refolding, dimeric rhBMP-2 proteins were obtained, even solubility efficiency was much lower. These data suggested that rhBMP-2 retaining dimerization potential might be successfully purified from inclusion body by using the detergent refolding method. Moreover, although solubilization efficiency of inclusion body was extremely increased by using the combinatorial refolding procedure, this soluble fraction totally lost its dimerization potential. We pooled dimeric fractions from the heparin column, concentrated, and used for further functional assay. In dose-dependent response, approximate ED_{50} of the purified rhBMP-2 on ALP activity in C2C12 cells was similar with that of the commercial BMP-2 (Figure 3B). In addition, the purified hBMP-2 induced osteogenic differentiation in human dental tissue-derived cells (Figure 3C). Finally, we optimized the proper refolding procedure to purify the active recombinant hBMP-2 protein from inclusion body in this study. Refolding under detergent condition was the best way to solubilize the inclusion body while maintaining their dimer formation potential. Although repetitive refolding had a significantly

higher efficiency in solubilizing the inclusion body, the dimeric potential required for active BMP-2 was totally lost by the repetitive folding process. This purified rhBMP-2 might be useful for laboratory level studies.

Conflict of interest

The authors declare that they have no conflicts of interest.

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