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Recombinant BMP 4/7 Fusion Protein Induces Differentiation of Bone Marrow Stem Cells

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ABSTRACT

Bone morphogenetic proteins (BMPs) induce differentiation of mesenchymal cells to cartilage and bone. We cloned BMP4 and BMP7 cDNAs from human placenta and fetal cartilage cells, respectively, and used an *Escherichia coli* expression system to produce recombinant BMP4 and BMP4/7 proteins. Differentiation of primary cultures of bone marrow stem cells (BMSC) treated with BMP4 or BMP4/7 was evaluated by Von Kossa staining and by determining alkaline phosphatase activity and osteocalcin level. BMP4/7-induced BMSC differentiation more potently than BMP4. We showed that BMP4/7 fusion protein expressed in *E. coli* is biologically active and is a novel strategy to treat bone injury in a clinical setting. J. Cell. Biochem. 112: 3054–3060, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: BONE MARROW STEM CELLS; BONE MORPHOGENETIC PROTEIN; OSTEOGENESIS; PROKARYOTIC EXPRESSION; FUSION PROTEIN; *ESCHERICHIA COLI*

Bone marrow stem cells (BMSCs), which are mainly found in bone marrow, connective tissue, and in interstitial tissues of organs, are known for their multipotency and ability to differentiate to osteogenic cells, cartilage cells, muscle cells, tendon cells, fat cells, stromal cells, and other mesodermal cells [Ng et al., 2004]. The use of BMSCs is therefore regarded as a powerful new approach in the field of tissue engineering. The differentiation of BMSCs is characterized by the acquisition of phenotypic markers which are specific to the differentiated cell population. In the presence of bone morphogenetic proteins (BMPs), BMSCs can differentiate to osteoblasts which have high levels of alkaline phosphatase (ALP) activity and osteocalcin and other markers of osteoblast cells [Katagiri et al., 1990].

BMPs belong to the transforming growth factor β superfamily except BMP1. A number of studies have investigated the osteoinductive properties of BMPs. Among them, recombinant human BMP2 and BMP4 have been reported to induce differentiation of a murine mesenchymal cell line to an osteogenic lineage [Ahrens et al., 1993]. Transfection of rabbit bone marrow stromal cells with human BMP4 was reported to repair mandibular osseous defects [Jiang et al., 2006]. BMP7 is widely distributed in the embryonic heart, brain, kidney, bone, and other tissues, and plays an important regulatory role in embryogenesis [Vaccaro et al., 2002]. Recombinant BMP7 was found to retard the progression of cartilage degeneration in an osteoarthritic rabbit model [Hayashi et al., 2010]. Importantly, BMP2 and BMP7 have been used in human clinical trials to treat open tibial fractures and pseudoarthroses, respectively [Friedlaender et al., 2001; Govender et al., 2002; Desmyter et al., 2008]. In addition, BMP4 also inhibited proliferation, enhanced migration of breast cancer cells [Ketolainen et al., 2010], and induced differentiation and chemosensitization of colorectal cancer stem cells [Lombardo et al., 2011]. The importance of BMPs is underscored by the fact that mice deficient in *Bmp2* and *Bmp4* are non-viable, while mice deficient in *Bmp7* die shortly after birth [Luo et al., 1995; Winnier et al., 1995; Zhang and Bradley, 1996].

Mature BMPs are typically more than 100 amino acids in length and the peptides are generally connected by disulfide bonds to form the active dimer. Co-expression of BMP genes was recently shown in mouse embryos where BMP2 and BMP7 colocalized in developing limbs [Lyons et al., 1995]. However, BMP heterodimers have not been successfully isolated to date. Although BMP homodimers have been reported to promote bone regeneration [Vaccaro et al., 2002; Jiang et al., 2006; Hayashi et al., 2010], the clinical doses of homodimers required to achieve bone regeneration were extremely high [Boden et al., 2002] and carried a risk of side effects such as overstimulation of osteoclastic activity and ectopic bone formation [Kaneko et al., 2000]. In contrast, BMP heterodimers were found to more potently induce differentiation, ALP activity, and bone formation [Zhu et al., 2004; Koh et al., 2008; Zheng et al., 2010].

In order to advance the efforts to optimize protein therapy strategies for BMP-mediated bone regeneration, a number of groups have successfully used prokaryotic expression systems for large-

Abbreviations used: BMP, bone morphogenetic proteins; BMSC, bone marrow stem cells; ALP, alkaline phosphatase.

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scale production of biologically active, recombinant BMPs [Klosch et al., 2005; Ihm et al., 2008; Zhang et al., 2010]. In this study, we investigated the feasibility of using *Escherichia coli*-derived human recombinant BMP4/7 fusion protein to induce differentiation of BMSCs.

MATERIALS AND METHODS

AMPLIFICATION OF BMP4 cDNA

Fresh placental tissues were provided by the Gynecology Department, The First Affiliated Hospital of Harbin Medical University, China. Tissue was minced, homogenized, and RNA extraction was performed using Trizol reagent (Gibco Laboratories, Grand Island, NY) according to the manufacturer's instructions. BMP4 cDNA was synthesized using the One-Step RT-PCR kit (Clontech) according to the manufacturer's instructions. The BMP4 primers were: Primer I: 5′-CGGAATTCAACTTAATGAGGGAGG-3′; Primer II: 5′-TTGGATCCGCTGCGGGAAGC-3′.

CHONDROCYTE CULTURE AND AMPLIFICATION OF BMP7 cDNA

Cartilage tissue was obtained from aborted 5-month-old fetuses at The First Affiliated Hospital of Harbin Medical University. Tissue was minced, digested with trypsin and collagenase II at 37°C, and filtered through a 200 mesh nylon sieve. The cell suspension was centrifuged, washed, and evaluated for cell viability determined by trypan blue exclusion assay. Cell suspensions of >90% viability were cultured at 37°C in DMEM (10^5 cells/ml, Gibco Laboratories) supplemented with 10% fetal bovine serum (Harbin Veterinary Research Institute, Harbin, China). The culture medium was changed every 4 days. Cells that were 100% confluent were digested with 0.25% trypsin and the primary chondrocytes were subjected to type II collagen immunohistochemical staining. BMP7 cDNA was synthesized from 1×10^6 chondrocytes as described above. The BMP7 primers used were: Primer I: 5′-TAGGATCCGCCGCGGGAG-GATCCACG-3′; Primer II: 5′-AACTGCAGTAACTAGTGGCAG-3′.

CLONING AND EXPRESSION OF BMP4 AND BMP4/7

The BMP4 cDNA fragment, encoding the mature BMP4 peptide (374 bp) was cloned into the *Eco*R I–*Bam*H I site of the pBV222 prokaryotic expression vector (obtained from Harbin Veterinary Research Institute), to generate the BMP4 expression vector, pBV222–BMP4. The BMP7 cDNA, encoding the mature BMP7 peptide (450 bp), was then inserted in pBV222–BMP4 at the *Bam*H I–*Pst* I site to generate the BMP4/7 expression construct (pBV222–BMP4/7) in which BMP4 and BMP7 genes were fused head-to-tail in the correct reading frame (Fig. 1A).

E. coli DH5α cells were transformed with the BMP4 or the BMP4/7 expression vectors and cultured in LB medium in the presence of Ampicillin (100 μg/ml) at 37°C. until the OD value reached 0.6, IPTG (0.4 mM) was added and cells were quickly transferred to 42°C and cultured for 4–6 h.

PROTEIN ISOLATION AND RENATURATION

The expressed proteins were isolated, dissolved, denatured, and renatured as described [Wen et al., 2008]. First, the bacteria were harvested, washed, and digested with lysozyme (1 mg/ml), deoxy bile sodium (40 mg/ml), and DNase I (0.3 ng/ml). Then the lysate was centrifuged at 4°C at 12,000*g* for 15 min. The resulting pellet was resuspended and incubated in 9 volumes of lysis buffer (0.5% Triton X-100, 10 mmol/L EDTA Na_2, pH 8.0) for 5 min at room temperature. After centrifugation at 12,000*g* for 5 min at 4°C, the precipitates (inclusion bodies) were washed in the presence of Triton X-100 to remove cellular debris and proteins, resuspended in lysis buffer (0.1 mmol/L PMSF, and 8 mol/L urea) and incubated at room temperature for 1 h. Then 9 volumes of a solution containing 50 mmol/L NaCl, 50 mmol/L KH_2PO_4, and 1 mmol/L EDTA·Na_2 were added. The incubation continued for 30 min, during which the pH of the reaction was maintained at 10.7 using KOH. The pH of the mixture was then adjusted to 8.0 with HCl and the mixture was incubated for 30 min before centrifuging at 12,000*g* for 5 min. The supernatant was collected and renaturation was performed by

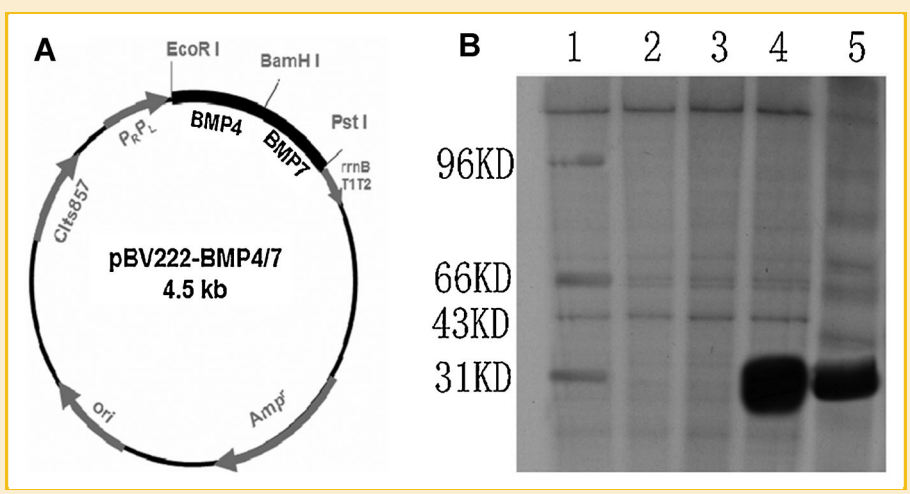

Fig. 1. Cloning and expression of BMP4/7. A: Plasmid map of prokaryotic expression vector pBV222–BMP4/7. B: expression of BMP4/7 fusion protein. 1, molecular standard; 2, *E. coli* lysate; 3, empty vector-transformed *E. coli*; 4, protein from purified inclusion bodies; 5, protein from pBV222–BMP4/7-transformed *E. coli*.

slowly adding 1 ml of this urea–protein solution to 9 ml of buffer containing 2 mmol/L reduced glutathione and 0.2 mmol/L oxidized glutathione. The mixture was incubated at room temperature for 3 h and then assayed for the presence of protein. Bacterial lysates were analyzed routinely on 15% sodium dodecyl sulfate–polyacrylamide gels and protein bands were visualized by staining with 0.25% Coomassie brilliant blue R-250.

ISOLATION, CULTURE, AND TREATMENT OF BMSCs

Low glucose DMEM (Gibco Laboratories) was used in BMSCs isolation and culture. Cells were harvested from the iliac bone marrow biopsy specimens obtained from healthy volunteers. They were diluted 1–2 times and centrifuged at 300*g* for 10 min. The precipitated cells were resuspended to 1×10^7 cells/ml. The cell suspension was then carefully layered on a gradient of Percoll (1.073 g/ml). After centrifugation at 900 rpm for 30 min, white flocculent cells were carefully harvested from the junction of the middle and the bottom layers. Cells were then diluted, washed, adjusted to a density of 2×10^5 cells/ml and cultured at 37°C in a 5% CO_2 humidified incubator. After 48 h, a small number of spindle-shaped cells adhered. Non-adherent cells were discarded. Cell clones were observed after about 7 days of cell culture and a dense adherent cell layer was observed after 14 days, when cell monolayers were digested with 0.05% trypsin and passaged at a density of 6,000 cells/cm^2.

The effects of BMP proteins on the differentiation of BMSC were evaluated by adding purified BMP4 or BMP4/7 (200 ng/ml) to the culture medium of 3rd–5th passage cells and evaluating cell growth and differentiation after 7 or 14 days of induction.

FLOW CYTOMETRY ANALYSIS

BMSCs were characterized by flow cytometric analysis. Third generation BMSCs were harvested and diluted to a density of 1×10^6 cells/ml. Cells were washed twice with PBS, resuspended in 1 ml pre-cooled PBS, and then fixed in 4 ml ethanol (−20°C) for 24 h. The cells were harvested by centrifugation, blocked with 0.1% bovine serum albumin solution at 4°C for 30 min, and then incubated in the dark at 4°C for 30 min with FITC-labeled monoclonal antibodies against CD34, CD44, CD45, and CD90 (1:30 dilution, Abcam, Cambridge, UK). The samples were then analyzed on a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA) with an excitation wavelength of 488 nm. Red blood cells were used to calibrate the cytometer with a coefficient of variation of within 5%. Ten thousand cells were determined for each treatment.

VON KOSSA STAINING

BMSCs were grown on coverslips, induced with recombinant BMP proteins and cell morphology was observed after 7 days. After 14 days of induction, coverslips were stained with the conventional Von Kossa stain. Briefly, the coverslips were incubated with 2% silver nitrate for 30 min under the UV lamp. The coverslips were washed with distilled water for 5 min, treated with 5% sodium thiosulfate solution for 2 min, and washed with water for 5 min. The cells were then stained with 0.1% nuclear fast red dye for 1–2 min, washed with distilled water for 5–10 s, conventionally dehydrated, and fixed with neutral resin. Uninduced cells were used as controls.

DETERMINATION OF ALP ACTIVITY AND OSTEOCALCIN LEVELS

BMSCs were cultured for 5, 10, 15, or 20 days, and then digested with 0.2% trypsin for 5–8 min. After centrifugation, cell pellets were washed and lysed with 1% Triton solution. The ALP activity was detected using a kit (Boster Bioengineering Limited Company, Wuhan, China). ALP activity was measured in terms of King-Armstrong (KA) units. A KA unit is defined as the quantity of phosphatase that acts on an excess of disodium phenyl-phosphate (pH 9 for 30 min.) to liberate 1 mg of phenol.

BMSCs were cultured for 14 days and osteocalcin levels were determined using a Radioimmunoassay kit (East Asia Institute of Immune Technology, Beijing, China) according to the manufacturer's instructions. Briefly, 100 μl cell culture medium was collected and incubated with 100 μl ^{125}I labeled osteocalcin antibody at 4°C for 24 h. Separation reagent was added and incubated at room temperature. The reaction was centrifuged at 4°C, supernatant was discarded and ^{125}I counts were determined.

STATISTICAL ANALYSES

Normally distributed continuous variables were compared by one-way analysis of variance (ANOVA). When a significant difference between groups was apparent, multiple comparisons of means were performed using the Bonferroni procedure with type-I error adjustment. Data are presented as means ± SD. All statistical assessments were two-sided and evaluated at the 0.05 level of significant difference. Statistical analyses were performed using SPSS 15.0 statistics software (SPSS, Inc., Chicago, IL).

RESULTS

CLONING AND EXPRESSION OF BMP GENES

We established a primary culture of fetal cartilage cells. Immunohistochemical characterization of these cells showed brown, granular, cytoplasmic type II collagen staining. Microscopic examination showed that the cartilage cells were polygonal or oval in shape and secreted a small amount of extracellular matrix. After passaging, the cells appeared polygonal in shape, with an increase in refraction and extracellular matrix content. We used toluidine blue staining to show that cell nuclei were purple and the extracellular matrix was light blue (data not shown).

We cloned BMP4 cDNA from fresh human placental tissue and BMP7 from the primary culture of cartilage cells. The BMP4/7 fusion construct was created. Figure 1A depicts the BMP4/7 prokaryotic expression vector. The sequences of the cloned genes were identical to the sequences corresponding to the BMP4 and BMP4/7 mature peptides as reported by Genbank (data not shown). *E. coli* DH5α cells were transformed with BMP4 or BMP4/7 fusion proteins and induced with IPTG. Inclusion bodies and protein lysates were analyzed by SDS–PAGE. The BMP4/7 fusion protein was visualized as a strong 31 kDa band present in the inclusion bodies (Fig. 1B, lanes 4 and 5).

PROPERTIES OF CULTURED BMSCs

Bone marrow stem cells were isolated and purified from iliac bone marrow specimens of healthy volunteers. Primary cultures of BMSCs showed the presence of spindly and adherent cells after 72 h of culture and cell colonies after about 7 days of culture. We observed an increase in cell density with time and the cells looked like grass bundles (Fig. 2A,B). We used flow cytometry to show the presence of BMSC markers. Our primary cultures of BMSCs showed positive biomarkers of BMSCs such as CD44 and CD90. The cells were negative for CD34 and CD45, which is characteristic of BMSCs. The BMSC concentration was greater than 90% and the purity of CD44, CD90, CD34, and CD45 was 92.78%, 94.26%, 1.41%, and 1.57%, respectively (Fig. 3).

BMP4/7 FUSION PROTEIN-INDUCED BMSC DIFFERENTIATION

Primary cultures of BMSCs were incubated in the presence of partially purified, recombinant BMP4/7 protein (200 ng/ml). Untreated cells showed uneven growth after 7 days in culture. In contrast, we showed that treatment of BMSCs for 7 days with BMP4/7 fusion protein caused differentiation of bone marrow stromal cells to osteoblasts, with a transition from spindle-shaped cells to cubic or cylindrical cells and congregation of the cells. Calcium nodules were apparent after 14 days of induction. Von Kossa staining showed that these were black nodules surrounded by bone cells. No obvious morphological changes were observed in uninduced cells (Fig. 2C–F).

We then determined the effect of BMP4 and BMP4/7 fusion protein on the differentiation of BMSCs by measuring bone-specific markers ALP and osteocalcin. We showed significant differences in ALP activity and osteocalcin levels in the BMP4 and BMP4/7 treated BMSCs when compared with untreated cells ($P < 0.001$). BMP4/7 fusion protein was significantly more efficient at stimulating ALP activity when compared with control cells or BMP4 treated cells (98.44 ± 11.96 KA unit vs. 35.93 ± 6.96 KA unit; $P < 0.001$; Fig. 4A). We used radioimmunoassay to show that levels of osteocalcin in the BMP4/7 group were also significantly higher than in the control group or BMP4 group (both $P < 0.001$; Fig. 4B).

DISCUSSION

In this study, we cloned human BMP4 from placental tissue and BMP7 from human embryonic cartilage cells. The expression of BMP7 in human fetal chondrocytes suggests that BMP7 is involved in human cartilage and bone formation during fetal development. We showed that BMP4 and BMP4/7 proteins expressed in a prokaryotic expression system effectively induced the osteogenic differentiation of BMSCs. The differentiation was evident by the significant osteoinductive changes and higher expression of ALP and osteocalcin.

Repair of skeletal defects continues to be a major challenge, making the osteoinductive role of BMPs potentially important in a clinical setting. However, the large doses of recombinant BMPs required to achieve clinically significant bone induction have been a major impediment to their widespread use [Friedlaender et al., 2001; Govender et al., 2002]. Prokaryotic expression systems offer advantages in terms of easy scale-up and economics of recombinant protein production, especially for proteins whose post-translational

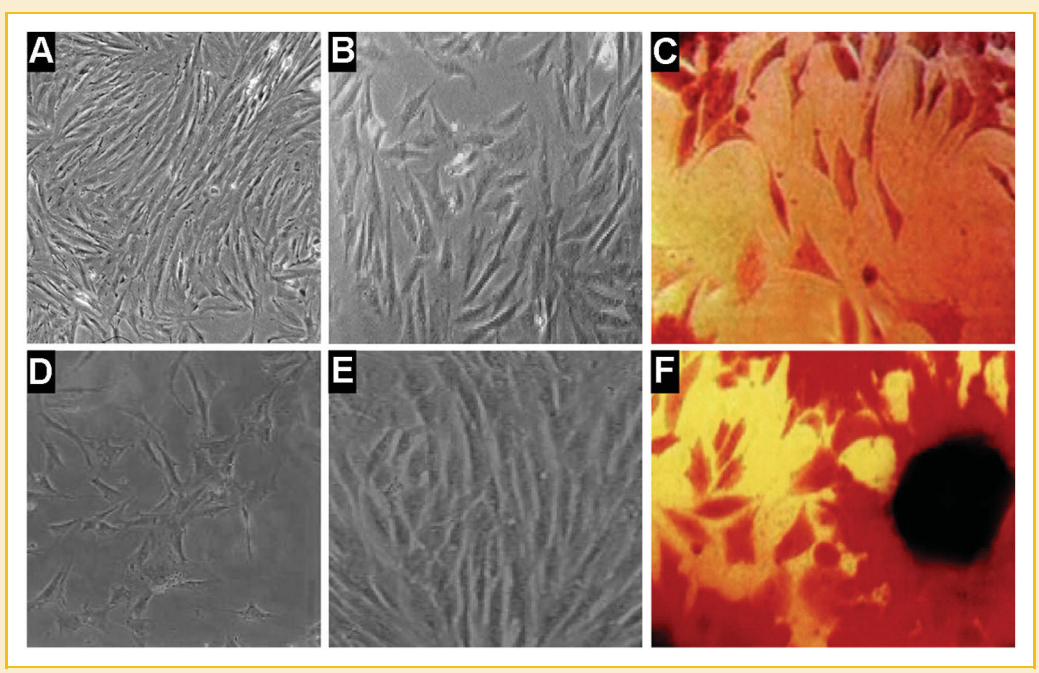

Fig. 2. Morphology of BMSCs. A: Primary cell culture (×100); B: primary cell culture (×200); C: VG stain of uninduced cell culture at 14 days (×200); D: cell culture induced with BMP4/7 for 7 days (×100); E: cell culture induced with BMP4/7 for 7 days (×200); F: VG stain of cell culture induced with BMP4/7 for 14 days, VG stain (×200). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

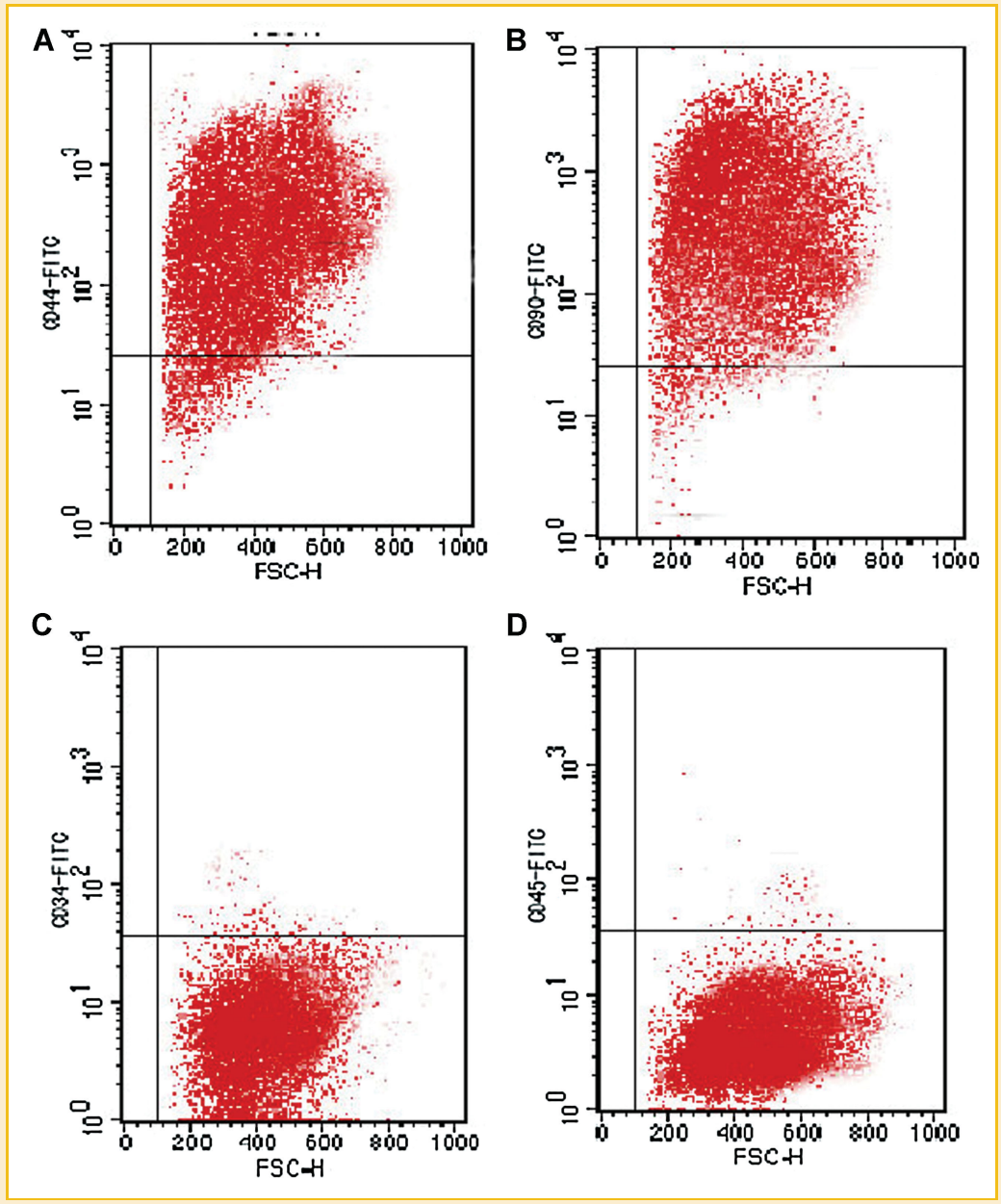

Fig. 3. Flow cytometry of BMSC stained with anti-CD44 (A), CD90 (B), CD34 (C), and CD45 (D). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

modifications do not affect biological activity. Although all BMPs are glycosylated in vivo, their osteoinductive activity is not dependent on glycosylation [Elima, 1993]. Therefore, prokaryotic expression systems have been extensively used for large-scale expression of BMP4 [Klosch et al., 2005] and BMP2 [Ihm et al., 2008; Zhang et al., 2010]. Recombinant BMP2 and BMP7 have been approved for human trials in a number of countries, making it imperative to optimize large-scale production of these proteins [Rutherford et al., 2003].

Recombinant Xenopus BMP4/7 has been shown to have a potent mesoderm inducting activity [Suzuki et al., 1997; Nishimatsu and Thomsen, 1998]. The cysteine residues play an important role in advanced protein structure formation, and those in the BMP4 and BMP7 peptides in our construct might promote the formation of a fusion protein that mimicked a BMP heterodimer. BMP heterodimers have previously been shown to be more effective than BMP homodimers in promoting cell differentiation [Suzuki et al., 1997; Valera et al., 2010]. We show here that recombinant BMP4/7 fusion protein, expressed in a prokaryotic expression system is biologically active and has a higher osteoinductive effect than BMP4 by itself. This suggests the feasibility of scaling up recombinant BMP4/7 fusion protein production in prokaryotic expression systems to overcome the limitations seen with using homodimers which are biologically

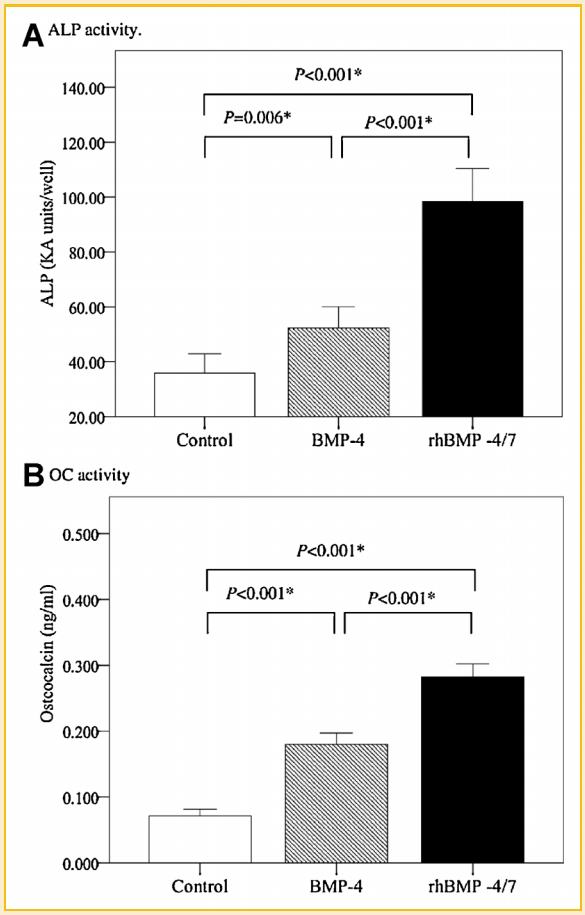

Fig. 4. Measurement of alkaline phosphatase activities (A) and osteocalcin levels (B) in BMSCs treated with BMP4 and BMP4/7. ALP activity was measured in terms of the amount of ALP required to act on an excess of disodium phenyl-phosphate to liberate 1 mg of phenol (KA units) in one well of microplate. Osteocalcin level was measured in terms of ng/ml.

active only at high doses [Friedlaender et al., 2001; Govender et al., 2002].

Our BMP4/7 fusion protein, which mimicked a heterodimer, efficiently promoted cell differentiation and these data agreed with a number of other studies showing that fusion proteins BMP2/7 and BMP2/6 were efficient at inducing osteoblastic differentiation [Zhu et al., 2004; Valera et al., 2010; Zheng et al., 2010]. However, the mechanisms behind the synergistic effect of BMP4 and BMP6 [Aoki et al., 2001] and the increased efficiency of heterodimeric or fusion BMPs over homodimeric BMPs on osteoinduction are still not clear. It has been speculated that (1) the fusion protein may have a higher combined affinity toward the receptors for each subunit or (2) the heterodimer may have a lower affinity toward Noggin, a BMP-induced competitive antagonist, when compared with the respective homodimers [Zhu et al., 2006; Zheng et al., 2010]. Another recent study speculated that BMP2/7 may activate BMP receptors more efficiently than the homodimers by inducing clustering of tetrameric receptor subunits with enhanced signaling properties [Koh et al., 2008]. Enhanced binding of heterodimeric BMPs to their receptors could lead to enhanced Smad-dependent and Smad-independent pathways resulting in more efficient osteogenic and chondrogenic activities [Isaacs et al., 2010; Valera et al., 2010].

Some important limitations of our study included that fact that we did not perform optimization studies for our protein expression experiments. We also did not determine protein stability or do a detailed protein structure analysis. We plan to address these questions in future studies. It will also be interesting to validate our data using an animal model in future experiments and to more closely examine molecular mechanisms underlying BMP4/7-mediated induction of BMSC differentiation.

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