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Expression, purification and osteogenic bioactivity of recombinant human BMP-4, -9, -10, -11 and -14

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ABSTRACT

Bone morphogenetic proteins (BMPs) are cytokines from the TGF-β superfamily, with important roles during embryonic development and in the induction of bone and cartilage tissue differentiation in the adult body. In this contribution, we report the expression of recombinant human BMP-4, BMP-9, BMP-10, BMP-11 (or growth differentiation factor-11, GDF-11) and BMP-14 (GDF-5), using *Escherichia coli* pET-25b vector. BMPs were overexpressed, purified by affinity his-tag chromatography and shown to induce the expression of early markers of bone differentiation (e.g. smad-1, smad-5, runx2/cbfa1, dlx5, osterix, osteopontin, bone sialoprotein and alkaline phosphatase) in C2C12 cells and in human adipose stem cells. The described approach is a promising method for producing large amounts of different recombinant BMPs that show potential for novel biomedical applications.

Introduction

Discovered in 1965, bone morphogenetic proteins (BMPs) are a group of several cytokines belonging to the transforming growth factor-β (TGF-β)[1] superfamily. These proteins have strong inducing activity in bone and cartilage formation, and also have important roles during embryonic patterning and early skeletal development [1]. During the last decades, BMPs have been used as powerful osteoinductive components in several late-stage tissue engineering products for bone grafting [2]. Since 2002, BMP-2 and BMP-7 received approval for specific clinical uses [3].

Despite being extensively investigated, to date, most research has been focused only in a few BMPs, particularly in BMP-2 and BMP-7, whilst little is known about the physiological roles and the biomedical relevance for the remaining BMPs. Both, BMP-4 and BMP-9 have been shown clearly bone-inducing roles, as shown by their ability to induce osteogenic differentiation in C2C12 cell line [4] and orthotopic ossification in mice [5]. BMP-4 is also implicated in diverse roles during embryonic development, and as a differentiation factor to hematopoetic and nerve cells [6–8]. BMP-14 (or growth and differentiation factor-5) induces mainly tendon and cartilage formation [9,10] and has been also studied in healing of periodontal ligament [11]. BMP-11 and BMP-10 have not been identified for any bone-inducing potential. BMP-10 has expression restricted solely to the heart tissue and is essential to cardiac morphogenesis [12,13], while BMP-11 (or growth and differentiation factor-11) has roles in the establishment of axial patterning [14]. In former reports, human BMP-4 and BMP-14 have been expressed in *E. coli* and reported to induce alkaline phosphatase marker in murine cells but these were not tested in human cells [15,16]. In addition, the expression of human BMP-9, -10 and -11 in *E. coli* is not yet reported. In this work, we described the production, purification and bioactivity of recombinant human BMP-4, -9, -10, -11 and -14 by a novel method that was previously reported for the overexpression of human BMP-2 [17]. The different BMPs were analyzed by their ability to induce the mRNA expression of several markers of bone differentiation in C2C12 cells and in human adult

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[1] *Abbreviations used:* rhBMP, recombinant human bone morphogenetic protein; TGF-β, transforming growth factor β; CHO, Chinese Hamster Ovary; PCR, polymerase chain reaction; HSV, herpes simplex virus; *E. coli*, *Escherichia coli*; LB, Luria-Bertani broth; SDS–PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis; PBS, Phosphate buffer saline; DMEM, Dulbecco's modified Eagle's medium; ALP, alkaline phosphatase; MTS, (3-(4,5)-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); RT-PCR, reverse transcription polymerase chain reaction; BSP, bone sialoprotein; Runx2/Cbfa1, runt-related transcription factor 2/ core binding factor alpha-1; Dlx5, distal-less homeobox 5.

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stem cells from adipose tissue. Our results suggest a promising and straightforward way for the production of different BMPs, all capable of inducing cell differentiation in the aforementioned models.

Materials and methods

Cloning and expression of rhBMP-4, -9, -10, -11 and -14

The sequences coding the mature (bioactive) domains of human BMP-4, BMP-9, BMP-10, BMP-11/GDF-11 and BMP-14/GDF-5 were obtained from different bacterial clones (Sanger Institute, UK), which contained the full genes encoding the human BMPs: clone reference RP11-808F04 for BMP-4, RP11-463P17 for BMP-9, RP11-88N13 for BMP-10, RP11-55N12 for BMP-11, and RP11-173P06 for BMP-14. According to the data from the UniProtKB/Swiss-Prot database (http://www.expasy.org) and from the Human Genome Project (http://www.ensembl.org/homo_sapiens), the sequences coding for the bioactive domains of these BMPs, are located in one sole exon, thus allowing direct PCR cloning of these BMPs from the above mentioned clones, as was previously described [17].

DNA coding for the mature/bioactive protein domains was amplified by PCR using the primers indicated in Table 1. PCR-products were cloned in a pET-25b vector (Novagen, USA) via the two restriction sites, BamHI and XhoI, that were used to transform *E. coli* BL21(DE3) strain (Invitrogen, UK). The restriction sites for these enzymes were specific.

DNA cloning and manipulation were performed according to standard protocols [18]. The integrity of the cloned PCR products was verified by DNA sequencing [19] using a ABI PRISM310 Genetic Analyzer. Transformed *E. coli* BL21(DE3) strain with pET-25b/rhBMP were inoculated in 50 ml Luria-Bertani (LB) medium with 50 μg ampicillin/ml and incubated at 37 °C, until an OD_{600} of 0.8 was obtained. To induce recombinant protein expression IPTG 1 mM was then added to the culture media, and the temperature lowered to 25 °C during 24 h. Biomass was collected by centrifugation (4000*g*, 20 min, 4 °C), washed once with PBS and stored at −20 °C.

Purification of rhBMPs

Frozen bacteria were resuspended in lysis buffer (20 mM sodium phosphate buffer, 0.5 M NaCl, 1 mg/ml lysozyme), with a protease inhibitors (Complete Mini EDTA-free, Roche). Bacteria cells were ultrasonicated 6 times for 30 s with intervals of 5 min on ice and supernatant and pellet fractions collected by centrifugation (4000*g*, 30 min, 4 °C). The pellet was then incubated overnight in solubilization buffer (phosphate saline buffer, 30 mM imidazole, 0.7 M L-arginine, pH 10.0) at 18 °C with gentle stirring and the supernatant containing soluble rhBMP collected after centrifugation (4000*g*, 20 min, 4 °C). The pH of supernatant was adjusted to 7.5 with 0.5 M HCl and applied to a pre-equilibrated HisTrap chromatography column (Amersham). Briefly, the column was equilibrated with five column volumes of sodium phosphate buffer containing 30 mM imidazole, washed extensively with 100–120 ml of sodium phosphate buffer containing 60 mM imidazole and the sample eluted in 20 ml of phosphate buffer supplemented with 400 mM imidazole. A flow rate of 5 ml/min was kept during the whole procedure by the use of a peristaltic pump. Purified protein was desalted with the use of a HiTrap desalting column (Amersham) and freeze–dried. BMPs were resuspended in sterile phosphate buffer saline, prior to bioactivity tests. Protein concentration was measured by Bradford method [20] using bovine serum albumin standards of known concentration.

Protein purity was estimated using SDS–PAGE image analysis (GelPro Analyzer 4.0, Media Cybernetics).

Table 1
Oligonucleotide primers for cloning of human BMP-4, -9, -10, -11 and -14. The nucleotides for restriction by BamHI and XhoI are underlined in the forward and reverse primers, respectively. The codons representing the beginning and the end of BMP sequences are represented in bold.

Gene	Primer sequence: forward/reverse
BMP-4	5′ CGG GAT CCA **AGC** CCT AAG CAT CAC TCA C 3′
	5′ CCC TCG AG**G CG**G CAC CCA CAT CC 3′
BMP-9	5′ CGG GAT CCA **AGC** GCC GGG GCT GGC AG 3′
	5′ CCC TCG AG**C CT**G CAC CCA CAC TCT GCC AC 3′
BMP-10	5′ CGG GAT CCA **AAC** GCC AAA GGA AAC TAC 3′
	5′ CCC TCG AG**T CT**A CAG CCA CAT TCG GAG 3′
BMP-11	5′ CGG GAT CCA **AAC** CTG GGT CTG GAC TG 3′
	5′ CCC TCG AG**A GA**G CAG CCA CAG CGA TC 3′
BMP-14	5′ CGG GAT CCA **GCC** CCA CTG GCC ACT C 3′
	5′ CCC TCG AG**C CT**G CAG CCA CAC GAC TC 3′

Western-blot detection of rhBMPs

Protein samples were mixed with SDS–PAGE loading buffer and heated at 95 °C for 5 min. Samples were separated by using handcast 10–12% reduced SDS–PAGE gels and coomassie blue-R was employed for visualization and staining of gels. Samples were then electro-transferred (wet Western-blot transfer) to a nitrocellulose membrane at 100 V for 60 min. The membrane was blocked with 2% (w/v) BSA in PBS-T buffer (PBS, 0.05% Tween 20) and incubated with peroxidase conjugated anti-His antibody (Sigma) diluted 1:2000. Image detection was performed with ChemiDoc XRS and Quantity One software (BioRad).

Bioactivity tests

C2C12 cells were seeded at 2.5×10^4 cells/ml per well in a 24-well plate, attached in Dulbecco's modified Eagle's medium (DMEM) with 1% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37 °C with 5% CO_2 in a humidified environment. Cells were incubated for 5 days with BMP-4, -9, -10, -11 and -14, purified by the method described above (500 ng/ml) and a control with no cytokine. MTS cell viability assay was performed in C2C12 cells after 3 days of culture [21]. Primary cultures of human adult stem cells isolated from adipose tissue [22] were cultured under similar conditions and incubated with BMP-4, -9 and -14 (500 ng/ml) for 3 days of cell culture. For RT-PCR, the mRNA of cells was extracted with TriZol Reagent (Invitrogen, USA) from triplicate 24-well assays following the procedure provide by the supplier. Samples were quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA) and cDNA synthesis performed with iScript cDNA synthesis Kit (BioRad, USA). PCR was performed with specific primers for osteogenic markers. Agarose gels were visualized using Eagle Eye imaging system (Stratagene) and gene expression was analyzed with Gel-Pro Analyzer (Media Cybernetics), using polymerase II expression as a housekeeping marker.

Statistical analysis

Experiments were performed at least in triplicate and expressed as means ± standard deviations. Student's *t* test was used for statistical analysis using a two-tailed paired test and analyzed by a paired analysis of variance. Statistical significance was defined as $p < 0.05$ for a 95% confidence.

Results

Cloning, expression and purification of rhBMPs

The PCR originated single products for the coding regions of human BMP-4, -9, -10, -11 and -14 (data not shown). DNA sequencing

revealed a total correspondence to the encoded nucleotides and corresponding amino acids. *Escherichia coli* BL21(DE3) strain transformants were then cultivated and collected as described in Material and methods section. After cell lysis, rhBMPs were found mainly in the pellet (fraction corresponding to cell debris) (data not shown). Soluble protein was recovered using phosphate buffer containing 0.7 M L-arginine, pH 10.0, as indicated in Materials and methods. After purification by affinity chromatography, the rhBMPs were visible as monomer, dimer and polymer, with Western-blot, using a antibody against the six-histidine tag (Fig. 1). Estimating from coomassie blue-stained SDS–PAGE, the BMPs were pure up to 95% (Table 2).

Cytotoxicity of rhBMPs

Bioassays were performed in murine myoblast C2C12 cell line, by the administration of purified rhBMP-2. The different proteins did not show cytotoxicity in C2C12 cells after three days of cell culture at 50 and 500 ng/ml (Fig. 2). MTS is a viability/proliferation test and an inverse relationship of toxicity to cells can be assumed. BMP-4 (50 ng/ml), BMP-9 (50 ng/ml) and BMP-10 (500 ng/ml) significantly stimulated cell proliferation by approximately 10–25% ($p < 0.05$).

Expression of osteogenic markers induced by rhBMPs

Semi-quantitative RT-PCR revealed an increase in expression of several early osteoblast differentiation markers in C2C12 cell line after five days of cell culture with the different BMPs (Fig. 3). The increased mRNA levels were mean values of independent assays, and normalized against the expression of polymerase II. Expression of early osteochondral transcription factor runx2/cbfa1 was induced by 2- to 3-fold with BMP-4 and BMP-11, 4-fold with BMP-10 and BMP-14, and 6-fold with BMP-9 ($p < 0.05$ in all cases). Expression of smad-1 and smad-5, two signaling molecules for BMPs, were also induced but in a lower extent. The expression of smad-5 with induced 2-fold with BMP-4 and BMP-9 ($p < 0.05$). Expression of smad-1 was induced with BMP-9 (2-fold, $p < 0.05$). With BMP-10, BMP-11 and BMP-14, there was no significant increase in the expression of smad-1 or -5. Osterix, a transcription factor downstream of runx2/cbfa-1 and a marker of commitment to osteoblastic lineage, was induced both with BMP-4 (2-fold) and BMP-9 (2-fold, $p < 0.05$) and its expression maintained with the other BMPs. Osterix expression was slightly decreased by BMP-11 (not statistically significant). The expression of transcription factor dlx5 was nearly the same with all BMPs, with a slight increase with BMP-4 (1.5-fold) which is not significant. Alkaline phosphatase (ALP), bone sialoprotein and osteopontin, three markers of bone formation, were induced with some of these BMPs. ALP was induced with BMP-4, BMP-9 (2-fold, $p < 0.05$) and BMP-10 and -14 (1.5-fold), and reduced by half (0.5-fold) with BMP-11 ($p < 0.05$). Bone sialoprotein expression was up-regulated with BMP-14 (3-fold) and with BMP-4 (5-fold, $p < 0.05$). Osteopontin expression was up-regulated mainly with BMP-9, BMP-14 and less with BMP-4 and BMP-10 ($p < 0.05$), compared with basal expression in the control and with BMP-11. Osteocalcin, a marker specific of late-stages of bone mineralization, was not detected in any assay. The observations on cell morphology correlated well with the induction of the several osteogenic markers (Fig. 4). BMP-9, and to a less degree BMP-4 and BMP-14, induced an osteoblast-like morphology.

In the primary cultures of fat-derived human stem cells, BMP-4, BMP-9 and BMP-14 were also able to induce an increase in the expression of the different early markers of osteogenic differentiation (Fig. 5). BMP-4 induced the highest levels of osteogenic markers, particularly runx2 (7-fold, $p < 0.05$), bone sialoprotein (7-fold, $p < 0.05$) and osteopontin (8-fold, $p < 0.05$). BMP-9 induced runx2 (2-fold) and alkaline phosphatase (2-fold), while BMP-14 induced the expression of alkaline phosphatase (5-fold, $p < 0.05$) and bone sialoprotein (3-fold, $p < 0.05$). Bacteria transformed with blank vector (e.g. no BMP) did not induce any expression of osteogenic genes (data not shown).

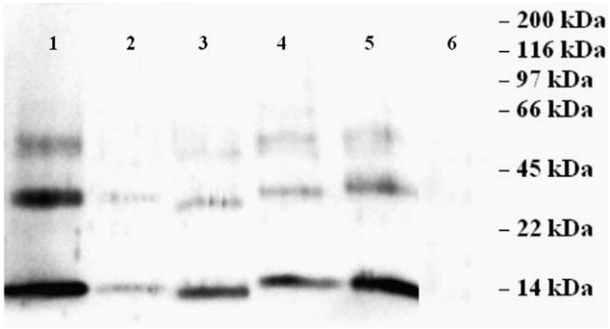

Fig. 1. Western-blot of purified rhBMP-4, -9, -10, -11 and -14 and a negative control (respectively, 1–6) using an anti 6-His tag antibody. The BMPs are visible in monomer, dimer and polymer.

Discussion

In this work we report a straightforward methodology for the expression of recombinant human BMP-4, BMP-9, BMP-10, BMP-11/GDF-11 and BMP-14/GDF-5 in *E. coli*, that showed bioactivity in both C2C12 cell line and human adult stem cells.

Although the concept of overexpressing BMPs in *E. coli* is not novel, we have shown, for the first time, the overexpression of functional BMP-9, -10 and -11 using *E. coli* and characterized the bioactivity of BMP-4, BMP-14 and BMP-9 using primary cultures of human adult stem cells, which has not been shown before. Using a single-step method, which was previously reported for the solubilization of rhBMP-2 [17], the recovery with 0.7 M L-arginine buffer resulted in monomer, dimer and polymer, in a similar way to former reports [23,24], with up to 50 mg purified BMPs per liter of culture broth.

Table 2
Purification of recombinant human BMP-4, BMP-9, BMP-10, BMP-11 (GDF-11) and BMP-14 (GDF-5).

Steps	Crude lysate[a]		L-Arginine solubilization		Ni-NTA chromatography	
Protein	Total protein (mg)[b]	Purity (%)[c]	Total protein (mg)	Purity (%)	Total protein (mg)	Purity (%)
hBMP-4	497.5 Yield 100%	24.4	203.6 Yield 70.5%	42.3	53.6 Yield 40.0%	91.0
hBMP-9	531.5 Yield 100%	17.2	220.9 Yield 66.5%	27.5	45.9 Yield 44.5%	88.7
hBMP-10	533.3 Yield 100%	14.9	261.3 Yield 77.4%	23.7	22.7 Yield 25.5%	92.4
hBMP-11	506.4 Yield 100%	13.5	278.5 Yield 80.1%	19.7	23.4 Yield 30.1%	87.7
hBMP-14	569.1 Yield 100%	19.7	252.5 Yield 73.4%	32.5	35.0 Yield 29.5%	95.1

[a] The recombinant hBMPs were purified from 1 L *E. coli* culture.
[b] Total protein quantification was determined using Bradford method.
[c] Estimated from SDS–PAGE image analysis.

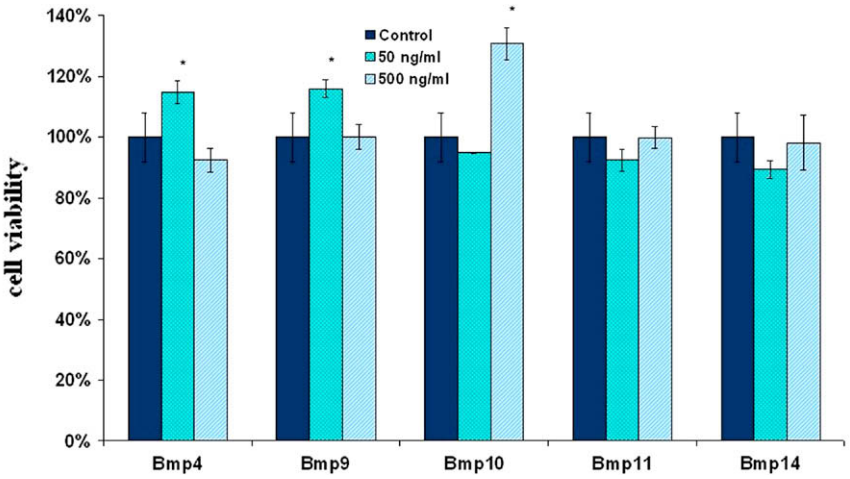

Fig. 2. Tetrazolium salt (MTS) test performed in C2C12 cells as a function of the concentration of the different rhBMPs, after three days of cell culture. Cell viability is expressed in percentage (mean ± S.D., $n = 3$, $^{*}p < 0.05$).

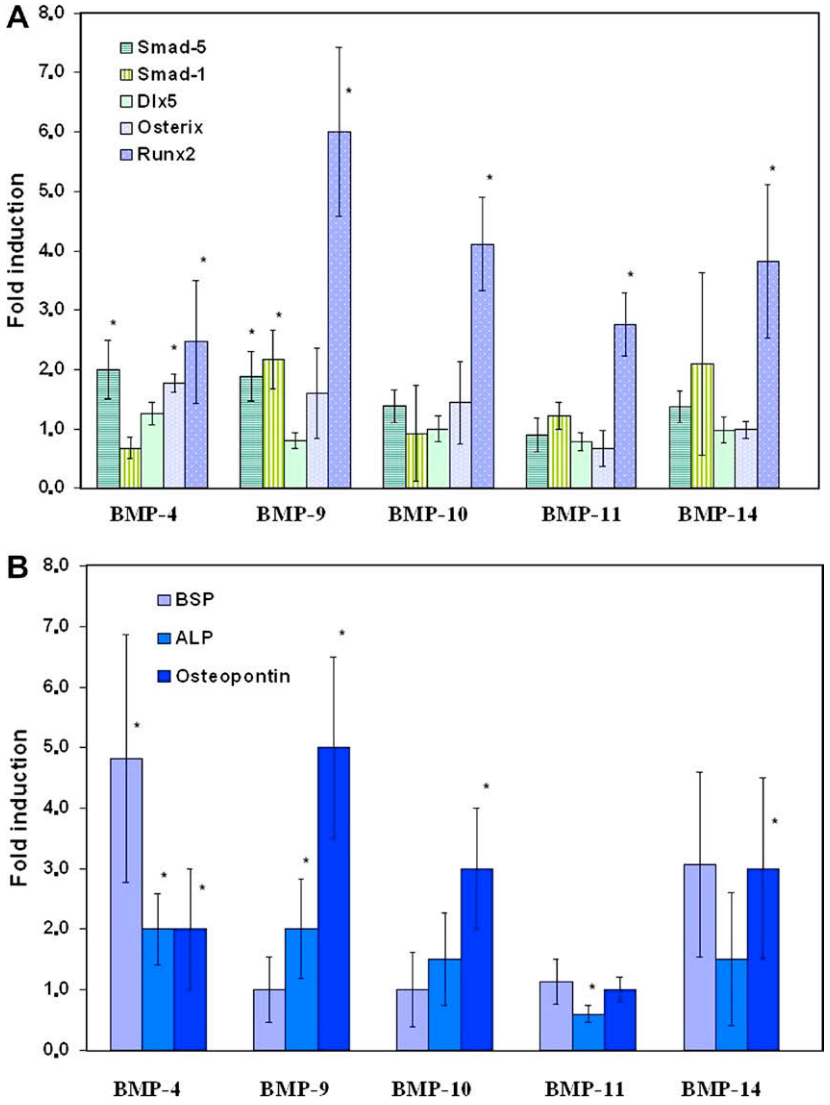

Fig. 3. mRNA expression of signaling molecules and transcription factors (A) and osteogenic-specific markers (B) induced by BMPs, measured by semi-quantitative RT-PCR after five days of cell culture in C2C12 cell line. Bars represent mean values ± SD of gene expression fold variation of at least three independent determinations ($^{*}p < 0.05$). Smad-1 and Smad-5 were induced mostly for BMP-4, -9 and -14, Osterix for BMP-4 and -9, Runx2 was induced for all BMPs but higher for BMP-9. Bone sialoprotein was induced mostly for BMP-4 and -11; ALP was induced 1.5- to 2-fold for all BMPs except BMP-11; osteopontin was induced mainly for BMP-9, BMP-10 and BMP-14.

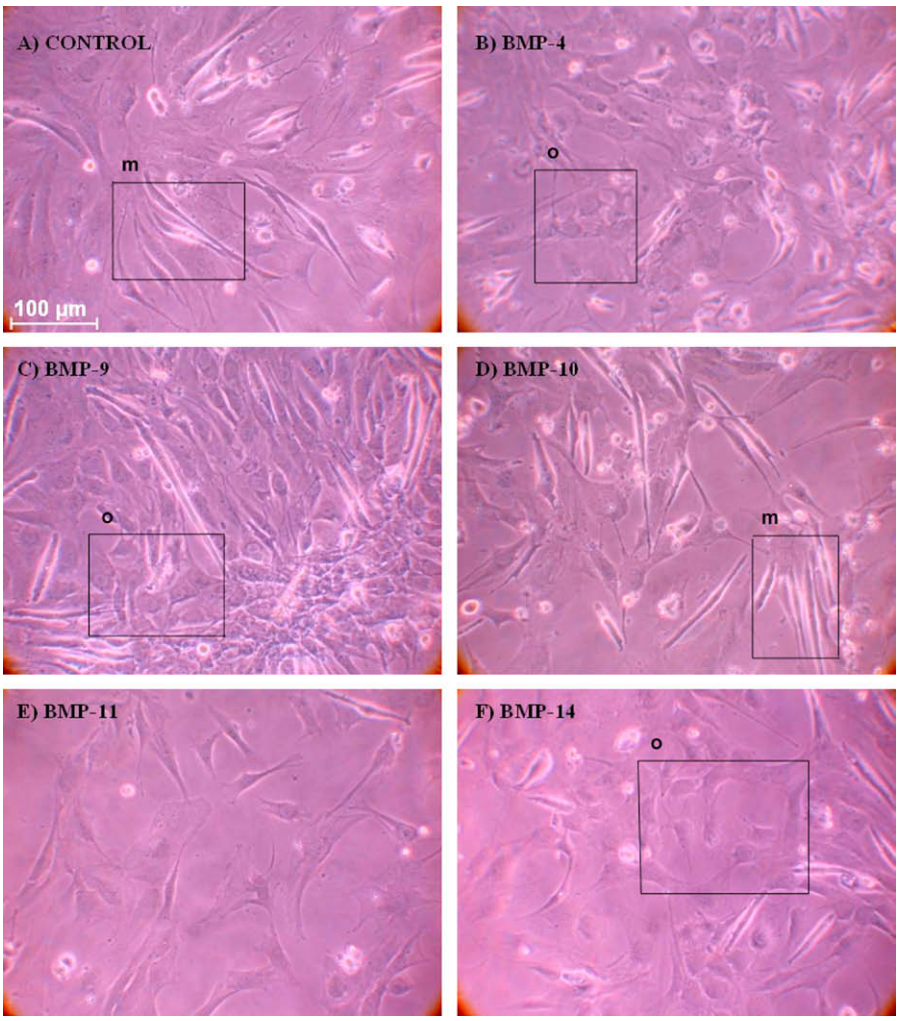

Fig. 4. Cell morphology of C2C12 cells after five days of cell culture with (A) no growth factor (control), (B) 500 ng/ml rhBMP-4, (C) 500 ng/ml rhBMP-9, (D) 500 ng/ml rhBMP-10, (E) 500 ng/ml rhBMP-11, and (F) 500 ng/ml rhBMP-14. Purified BMP was used ×400. Osteoblast-like morphology (o) was observed in C2C12 cells stimulated with BMP-4, BMP-9 and BMP-14 and myoblast-like morphology (m) in control and cells stimulated with BMP-10.

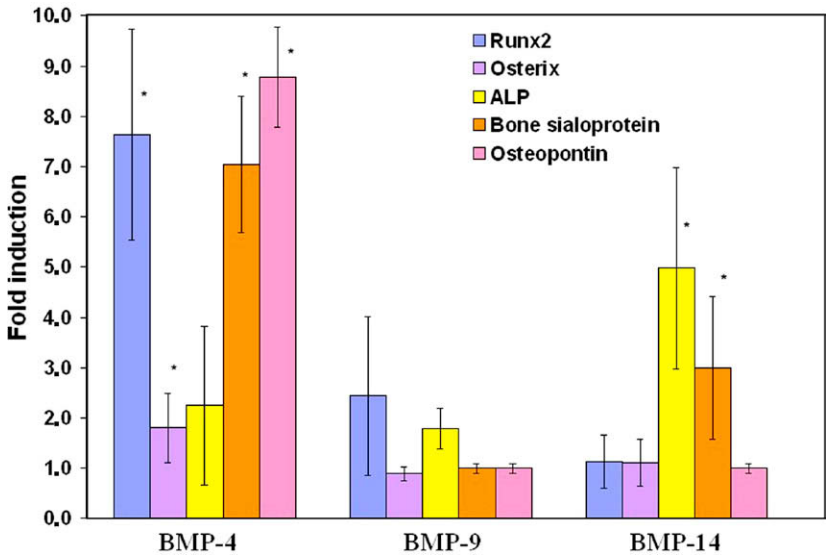

Fig. 5. mRNA expression of early markers of osteogenic differentiation induced by BMPs, measured by semi-quantitative RT-PCR after three days of human adult stem cell cultures. Bars represent mean values ± SD of gene expression fold variation of at least three independent determinations ($^{*}p < 0.05$). The expression of osteogenic markers was induced mainly with BMP-4. BMP-9 induced runx2 and ALP (2-fold) and BMP-14 induced solely ALP (5-fold) and bone sialoprotein (3-fold).

All tested BMPs have shown evidence of bioactivity in C2C12 cells. C2C12 cell line is a well-known model for studying the osteogenic differentiation induced by BMPs as has been reported by several investigators [17,24,25]. The different BMPs induced several early markers of BMP signaling (smad-1/-5, runx2/cbfa-1 or osterix) and of bone mineralization (bone sialoprotein, osteopontin or ALP). Semi-quantitative RT-PCR analysis of mRNA expression was selected since it is well established that upon binding of the BMPs to their cell receptors, the expression of these genes is highly induced, triggering osteogenic differentiation [1]. In C2C12 cells, BMP-4, -9 and -14 appeared to have had the most significant effects in the markers of bone formation. BMP-11 solely induced significantly the BMP-activated transcription factor runx2, showing that probably this BMP does not have any important role in bone formation and healing, besides those described in the literature during embryonic development [14]. In human stem cells, BMP-4, -9 and -14 also demonstrated bioactivity, despite the fact that the osteogenic potential was higher for BMP-4 than BMP-9 or -14, which could point for the stronger bone-inducing potential of this cytokine.

Despite the fact that these recombinant BMPs show bioactivity, further studies will be performed to assess the detailed response of cells to BMPs, including the induction of the different markers by RT-PCR, and immunostaining analysis.

In conclusion, the approach we detailed herein shows a promising route for producing large amounts of several different bioactive recombinant BMPs. The results in human adult stem cell cultures suggest that some of these BMPs could be used with success for future clinical applications.

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