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Osteogenic Differentiation of Human Mesenchymal Stem Cells Is Regulated by Bone Morphogenetic Protein-6

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Abstract Bone marrow-derived mesenchymal stem cells (MSC) are multipotent, self-renewing, mesodermal-origin stem cells that are sequestered in the endosteal compartment. MSC are maintained in a relative state of quiescence in vivo but in response to a variety of physiological and pathological stimuli, proliferate and differentiate into osteoblasts, chondrocytes, adipocytes, or hematopoiesis-supporting stromal cells. Little is understood regarding the cellular or molecular events underlying MSC fate decisions. We report that human MSC (hMSC) cultured in defined, serum-free conditions respond to a narrow spectrum of growth factors with osteogenic commitment, differentiation, and hydroxyapatite deposition. Of the osteogenic factors we examined, only treatment with bone morphogenetic protein (BMP) results in osteoinduction under defined serum-free conditions. Among BMP-2, 4, 6, and 7, BMP-6 is the most consistent and potent regulator of osteoblast differentiation and, of these BMPs, only BMP-6 gene expression is detected prior to hMSC osteoblast differentiation. Addition of exogenous BMP-6 to hMSC induces the expression or upregulation of a repertoire of osteoblast-related genes including type I collagen, osteocalcin, bone sialoprotein, and their regulatory transcription factors Cbfa1/Runx2, and Osterix. This translates into increased production of osteogenic extracellular matrix (ECM) with subsequent hydroxyapatite deposition. J. Cell. Biochem. 98: 538–554, 2006.

Key words: mesenchymal stem cell; bone morphogenetic protein; BMP-6; osterix; human

Bone formation is a complex process involving the recruitment of osteoblast progenitor cells to the bone surface with subsequent proliferation and differentiation into mature osteoblasts that mineralize the extracellular matrix (ECM). Little is understood concerning the nature of stem cells capable of generating osteoblast progenitor cells. Mesenchymal stem cells (MSC), a heterogeneous stem cell population within the bone marrow, can undergo differentiation into multiple mature cell types, including osteoblasts [Jaiswal et al., 1997, 2000; Pittenger et al., 1999]; however, microenvironmental cues that regulate MSC commitment to the osteoblast lineage remain unclear.

Several growth factor families regulate bone homeostasis as well as fracture healing in vivo. Fibroblast growth factors (FGFs) are mitogenic for primary murine osteoblasts in culture but fail to increase ECM [Kuznetsov et al., 1997; Kalajzic et al., 2003; Lane et al., 2003]. In vivo administration of FGF increases bone formation, while in vivo administration of Vascular endothelial growth factor (VEGF) at fracture sites causes increased angiogenesis, bone formation, and callus mineralization. Antibody neutralization of VEGF inhibits fracture healing suggestive of a role in osteoblast differentiation [Street et al., 2002]. Insulin-like growth

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factor (IGF-1) fulfills multiple functions, regulating the bone anabolic effects of PTH as well as osteoprogenitor proliferation [Bikle et al., 2002; Kasukawa et al., 2002]. Sonic hedgehog enhances the differentiation of murine preosteoblasts through cooperation with BMP-2 and by increasing PTHrP expression and secretion [Yuasa et al., 2002; Jemtland et al., 2003]. Platelet-derived growth factor (PDGF) stimulates proliferation and migration of murine preosteoblasts and calvarial osteoblasts [Mehrotra et al., 2004]. The contextual role of these factors in osteogenic fate determination of MSC is not well defined.

Bone morphogenetic proteins (BMPs) are members of the TFG-β superfamily, which regulate embryonic development and also induce ectopic bone formation in developed tissues [Urist, 1965; Hogan, 1996; Holleville et al., 2003]. In cells of the osteoblast lineage, BMPs 2, 4, and 7 induce expression of alkaline phosphatase, type I collagen, and other non-collagenous bone proteins found in osteoid; a phenotype consistent with differentiated osteoblasts [Cheifetz et al., 1996; Li et al., 1996; Lecanda et al., 1997; Cheng et al., 2003a]. While pre-osteoblasts demonstrate BMP responsiveness, contradictory results have been observed in more primitive MSC. In both mice and rats, BMPs promote osteoblast differentiation [Rickard et al., 1994; Puleo, 1997; Abe et al., 2000; Lou et al., 2000; Cheng et al., 2003a], whereas several studies determined that BMPs fail to induce osteoblast differentiation of human MSC [Diefenderfer et al., 2003; Gruber et al., 2003; Osyczka et al., 2004]. Clinical studies with BMP have produced mixed results depending on the site and BMP examined. Recent studies demonstrate that BMP-2 treatment enhances lumbar fusion and healing of open tibial fractures [Boden et al., 2002; Govender et al., 2002], while studies with BMP-7 have been more disappointing [Friedlaender et al., 2001].

Recent murine studies have elucidated molecular components of BMP signaling pathways. BMPs induce expression of the bone associated transcription factors, osterix (SP7) and DLX-5, that are critical for both skeletogenesis and osteoblast differentiation [Miyama et al., 1999; Levi et al., 2002; Holleville et al., 2003; Lee et al., 2003a,b]. Variations in the downstream components of BMP signaling may account for the observed differences in osteogenic induction between human and murine cells. For example, while Cbfa1/Runx-2 expression is regulated by BMPs in murine cells, in human cells Cbfa1/Runx-2 is expressed at relatively high basal levels [Tsuji et al., 1998; Lee et al., 1999; Diefenderfer et al., 2003]. Species divergence may indicate fundamental differences in the regulation of BMP signaling or the basal differentiation state between murine MSC and hMSC populations. Alternatively, differential regulation of BMP signaling between species may be attributed to variable responses to BMP family members. While most studies assume functional redundancy between the osteogenic BMPs (2, 4, 6, and 7), it is possible that the effects of BMPs on MSC differentiation are functionally discrete. Within the BMP family, these effects may be differentially controlled at the level of signaling and/or in the regulation of endogenous BMP production. As an example, in hMSC, BMP-6 signaling more so than BMP-4, was shown to augment the effects of other osteogenic factors [Sammons et al., 2004]. The selective induction of endogenous BMP-6 expression by human osteoblastic cells in response to dexamethasone and 17-β-estradiol [Rickard et al., 1994, 1998] indicates that the effects of this growth factor may be unique among the BMP family in mediating the cellular response to other families of osteogenic growth factors.

We hypothesized that hMSC would show different patterns of responsiveness to osteogenic BMPs. We report that under defined serum-free conditions, BMP-6, and to a lesser extent, BMP-7, induce osteoblastic differentiation of hMSC at a molecular and phenotypic level, while BMP-2 and -4 demonstrate modest osteogenic activity. These differences in BMP responsiveness may be explained in part by the expression of selective BMP-2 and BMP-4 antagonists and differential expression of type I BMP receptor.

MATERIALS AND METHODS

Isolation of Human Mesenchymal Stem Cells

Primary hMSC were isolated from vertebral bodies obtained through the National Disease Research Interchange (NDRI, Philadelphia, PA). Bone marrow extracted from vertebral bodies was placed in RPMI 1640 medium (Invitrogen, Carlsbad CA) supplemented with 2% FBS. The bone marrow suspension was layered over histopaque 1077 (Sigma, St. Louis,

MO) and centrifuged at 400*g* for 30 min to obtain mononuclear cells. Approximately 2×10^8 low density cells were placed in McCoy's 5a medium (Invitrogen) supplemented to additionally contain 20 μg/ml asparagine, 10 μg/ml serine, 0.75× MEM vitamins, 0.38× MEM amino acids, 75 μm non-essential amino acids, 100 U/ml penicillin / streptomycin, 2.3 mM L-Glutamine, 1.3 mM Sodium pyruvate, 0.06% Sodium bicarbonate, 50 μM BME (Invitrogen), and 20% defined FBS (Hyclone, Logan, Utah). The cells were subsequently cultured under multipotent hMSC conditions as previously described [Pittenger et al., 1999].

Induction of hMSC Osteogenic Differentiation

For cytokine induction studies, Passage 5–6 human MSC were placed in 12-well tissue culture plates at a density of 1.3 x 10^5 cells/well in serum-free supplemented McCoy's medium containing 1% ITS+ (BD Bioscience) and relevant cytokines. Where indicated, growth medium was supplemented with ascorbic acid (25 μg/ml), β-glycerol phosphate (5 mM), and dexamethasone (100 nM) (referred to as osteogenic media or OS) [Pittenger et al., 1999] or, β-glycerolphosphate and ascorbate (without dexamethasone) and the following growth factors: 25 ng/ml bFGF (Peprotech), 50 ng/ml FGF-8, 50 ng/ml FGF-10, IGF-1 (20 ng/ml, Peprotech), VEGF (50 ng/ml, Peprotech), 50 ng/ml SCF, 200 pM TGF-β (BD Biosciences, Bedford, MA), 500 ng/ml SHH, and 50 ng/ml PTH. As well, cells were treated with 20 nM (based on BMP dimer; 20 nM is equivalent to 800 ng/ml of glycosylated BMP) BMP-2, BMP-4, BMP-6, BMP-7 (R&D Systems), or BMP-14 (Peprotech) plus ascorbic acid and β-glycerol phosphate (5mM) as indicated in the Results. In some cases, BMP treatments were used in combination at 12 nM or in combination with dexamethasone. Unless otherwise indicated, BMP treatment was for 6 days, and then media was aspirated and exchanged with fresh media supplemented with ascorbate and β-glycerol phosphate.

Alkaline Phosphatase Colorimetric Assay

Approximately 1×10^4 MSC (passage 5–6) were plated in each well of a flat-bottom 96-well plate in 200 μl serum-free Assay McCoy's with 1% (v/v) ITS+ and the respective growth factors (BD Biosciences). After culturing for 12 days at 37°C, 7% CO_2, the media was aspirated and alkaline phosphatase activity was measured using a pNPP based assay kit (Sigma). Briefly, 50 μl of alkaline reaction buffer was added to each well followed by 50 μl of 2 × pNPP substrate solution. The plates were subsequently incubated for 1–20 min at 37°C, 7% CO_2. pNP production was measured using a Molecular Devices Spectramax microplate reader at 405 nm and compared to a standard curve. pNP production was normalized to cell number using the Rapid Cell Proliferation Kit (Calbiochem, catalog # QIA127).

Alkaline Phosphatase Cytochemistry

hMSC were plated and cultured under conditions identical to those above (alkaline phosphatase colorimetric assay). After culturing for 12 days, the media was aspirated and the cells washed twice in 1 × PBS. 1 NBT/BCIP tablet (nitro blue tetrazolium, Sigma, B5655-25TAB) was dissolved in 10 ml of ddH_2O. The NBT/BCIP solution was then added to the hMSC and incubated until a blue color was apparent.

Immunocytochemistry

All immunocytochemistry was performed with the Pierce (Rockford, IL) Immunopure ABC phosphatase staining kit per manufacturer's directions. Briefly, the cells were incubated in primary antibody (1:100–1:400 dilution, depending on the antibody) with 1.5% blocking serum for 30 min at room temperature. Each well was rinsed for 20 min in TBS and incubated in a biotinylated secondary antibody in 1.5% serum for 30 min. The Pierce AB reagent was added to each well (0.5 ml) and incubated for 30 min at room temperature. This solution was aspirated and each well washed several times with TBS for 10 min. Fast red TR/napthol AS/MX substrate (Sigma-Aldrich) was added to each well until the color developed.

Calcium Phosphate Cytochemistry

At day 21 of the cytokine induction, the cells were washed in 1 × PBS followed by fixation in paraformaldehyde (2%) in 1 × PBS (30 min, 4°C). Each well was washed several times in ddH_2O. The Von Kossa stain was performed as follows: silver nitrate solution (1%) was added to the cells and the plates were placed on a transluminator and irradiated with UV light for 1–2 h. Each well was washed with ddH_2O and then treated with 5% sodium thiosulfate for 1 min, followed by several rinses in ddH_2O. For Alizarin red S staining, cells were harvested at

day 21 (unless otherwise indicated), washed once in PBS, and then with ethanol (50%) for 5 min. The ethanol solution was removed and the Alizarin red S (1%) was added for 5 min. Each well was rinsed twice with PBS and then once with ddH_2O.

Raman Spectrography

Day 21 induced MSC were fixed in 70% methanol and allowed to air dry. The plates were then trypsinzed to remove all remaining cells and rinsed in tap water several times. The mineralized ECM was subsequently transferred to a quartz slide using a cell scraper. The samples were submitted for Raman spectroscopic analysis through the University of Michigan Core Center for Musculoskeletal Disorders.

Quantitative RT-PCR

Approximately 2.6×10^5 cells were placed into each well of a six-well plate and cytokine induction studies were performed (as described above). Total RNA was isolated at various time points using TRIzol (Invitrogen). RNA was dissolved in DEPC ddH_2O and stored at $-80^\circ C$. All primers were designed using the Primer3 program (Whitehead Institute, Cambridge, MA) and sequences are available upon request from the corresponding author. cDNAs were synthesized using Superscript II reverse transcriptase (Invitrogen). The cDNAs were subsequently diluted (approximately 1:100) in Bio-Rad IQ SYBR Green Supermix or Stratagene SYBR green 2× supermix for the quantitative PCR assay (per the manufacturer's instructions). All PCR samples were prepared in duplicate wells of a 96-well plate. Quantitative PCR was performed on a Bio-Rad I-cycler IQ quantitative thermalcycler or MJ Research Opticon 2. To ensure primer specificity, melt curves were performed after 45 cycles of PCR. Agarose gel electrophoresis was performed after melt curve analysis to further validate each primer set. Fold differences in gene expression were calculated using the ΔΔCt method [Pfaffl, 2001; Tichopad et al., 2003], comparing untreated controls to BMP-treated samples and normalizing to β-actin.

Statistical Analysis

Student's *t*-tests were used to determine whether treated groups, within donor, were significantly different from untreated controls at $P < 0.05$.

RESULTS

Bone Morphogenetic Proteins Regulate hMSC Osteoblast Differentiation

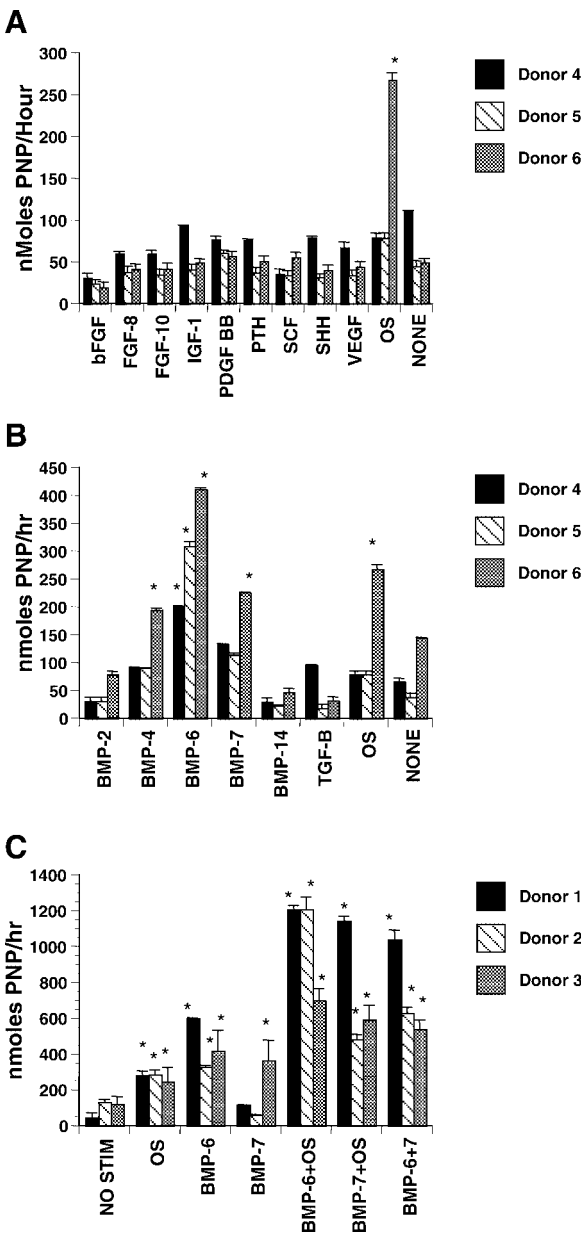
Previous studies demonstrated that VEGF, bFGF, IGF-1, TGF-β, and sonic hedgehog (SHH), play critical roles in osteogenesis [Kuznetsov et al., 1997; Kasukawa et al., 2002; Street et al., 2002; Yuasa et al., 2002; Kalajzic et al., 2003], but their role in the differentiation of hMSC to the osteoblast lineage is not clearly defined. We examined whether these factors could induce osteoblast differentiation of hMSC. These investigations were performed under defined serum-free conditions. Under these conditions, hMSC show little to no proliferation, while cells grown in 20% serum double approximately every 1.5–2 days (results not shown).

MSC treated with bFGF, FGF-8, or FGF-10 showed statistically significant decreases in alkaline phosphatase (AP) activity (relative to no growth factor controls), whereas SHH, PDGF-ββ, PTH, SCF, IGF-1, and VEGF had no significant effects on AP (Fig. 1A). MSC cultured in dexamethasone showed a variable increase in AP activity (Fig. 1A–C). Members of the TGF-β superfamily were potent inducers of bone AP enzymatic activity in hMSC (Fig. 1B). BMP-4 and BMP-7 significantly increase AP activity in hMSC in a single donor, while BMP-6-treated cells demonstrate a consistent high level of AP activity across all donors (Fig. 1B). Surprisingly, under serum-free conditions, hMSC treated with BMP-2, a powerful regulator of osteogenesis in other systems, showed no significant increase in AP activity in the donors we examined.

Since dexamethasone had only mildly osteoinductive effects under serum-free conditions, we evaluated whether it would act synergistically with suboptimal BMP levels to increase AP activity. A 2.5-fold increase in alkaline phosphatase enzymatic activity is seen in the presence of dexamethasone alone (Fig. 1C). Suboptimal BMP-6 or BMP-7 levels increased the induction of alkaline phosphatase activity an additional 2.5 to 4-fold depending on the donor. Similarly, BMP-6 and BMP-7 used in combination at 10 nM for each resulted in a higher level of alkaline phosphatase activity

than either 20 nM of BMP-6 or BMP-7 used alone. Taken together, these results support the concept that osteogenic factors used in concert are capable of acting synergistically.

The increased alkaline phosphatase activity observed when dexamethasone is combined with BMP may be due to the effects of dexamethasone-induced, endogenous BMP-6 production [Yamamoto et al., 2002]. We confirmed this dexamethasone effect using quantitative RT-PCR analysis. hMSC treated with dexamethasone for 6 days demonstrate a three-fold increase in BMP-6 gene expression, while BMP-2, BMP-4, and BMP-7 transcripts are below the threshold of detection ($Ct > 45$) (results not shown). However, expression of the BMP responsive transcription factors osterix, Dlx-5, or Cbfa1/Runx-2, showed no increase in the presence of dexamethasone alone (results not shown). In subsequent experiments, dexamethasone was not added in combination with BMPs.



BMP Treatment Results in Uniform Differentiation of hMSC

To examine the frequency of MSC undergoing osteoblast differentiation at the single cell level, alkaline phosphatase cytochemistry was performed with a nitro blue tetrazolium substrate (Fig. 2). Control hMSCs (no BMP treatment) were elongated and demonstrated no detectable alkaline phosphatase activity. MSC cultured for 12 days in dexamethasone show a low percentage (<10%) of alkaline phosphatase positive cells, consistent with its effects on alkaline phosphatase enzymatic activity. In sharp contrast, BMP-6-treated MSC showed significant changes in morphology, with cells becoming flattened in appearance, cuboidal in shape, and clustering in discrete areas of the well. All BMP-6-treated MSC demonstrate high levels of alkaline phosphatase activity (Fig. 2).

BMPs Activate an Osteoblast Transcriptional Program Under Serum-Free Conditions

To evaluate the effects of BMPs on MSC differentiation at the molecular level, a detailed analysis of gene expression was performed using quantitative RT-PCR. We examined the

Fig. 1. Differential induction of alkaline phosphatase activity by osteogenic growth factors. **A**: MSC were trypsinized, counted, adhered overnight in serum-containing media (1×10^4 cells/well in a 96-well plate), and subsequently cultured in serum-free conditions with ITS + supplement (1%, BD Biosciences) with relevant growth factors or dexamethasone (OS media). Alkaline phosphatase activity was measured 12 days post induction and normalized to cell number (Rapid Cell Proliferation Kit). **B**: MSC were treated with BMP-2, BMP-4, BMP-6, and BMP-7 (20 nM) for 6 days, subsequently cultured in the absence of BMP for 6 additional days, and alkaline phosphatase activity measured. **C**: MSC were treated with OS media, BMP-6 (20 nM), BMP-7 (20 nM), or suboptimal concentrations of BMP-6 or -7 in the following combinations: BMP-6 + BMP-7 (10 nM each), BMP-6 (10 nM) + OS, and BMP-7 (10 nM) + OS. Columns represent the average alkaline phosphatase activity of three independent experiments with triplicate wells per experiment. Error bars represent standard deviation of the mean. * indicates treatments that are significantly increased relative to untreated controls (None) ($P < 0.01$).

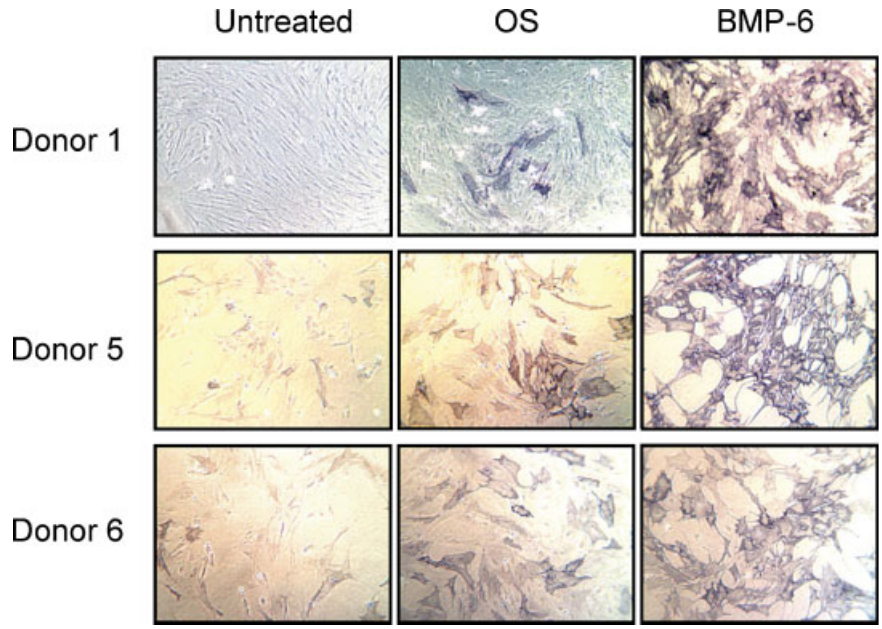


Fig. 2. MSC show differential alkaline phosphatase expression. Passage 4–6 MSC were cultured for 12 days with no treatment, treated with dexamethasone, or with 20 nM BMP-6. After rinsing once in PBS, nitro blue tetrazolium substrate was added to the cells for approximately 10–15 min. The substrate was removed with several washes and the cells were subsequently visualized with an Olympus IX70 inverted microscope connected to a SPOT RT digital camera. Purple staining indicates alkaline phosphatase activity. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

expression of a panel of osteoblast associated transcription factors and ECM proteins (Fig. 3A,B). Preliminary differentiation studies indicated that upon osteoinduction, donor line 5 was mildly mineralizing, donor lines 1 and 4 were moderately mineralizing, while donor line 6 was highly mineralizing, as determined by Alizarin red S staining. All other lines fell within this range. We, therefore, focused our gene expression studies on these donors.

Importantly, Cbfa1/Runx-2, the earliest and most specific marker of osteoblast differentiation [Ducy et al., 1997, 1999; Lee et al., 1997] is expressed at moderate to low levels prior to BMP treatment (threshold cycle (Ct) value 29–35, variability is donor dependent) and demonstrates relatively minor changes in the levels of gene expression 12 h or 4 days after BMP treatment. Krox-20, a zinc-finger transcription factor associated with endochondral ossification and biglycan expression [Levi et al., 1996; Heegaard et al., 1997; Widom et al., 2001], and a member of a family (Krox) shown to negatively regulate expression of alpha1(I), and alpha2(I) collagen, fibronectin, and elastin [Widom et al., 2001], showed decreased expression 12 h and 4 days after treatment with BMP. BMP-6 and -7 treatments resulted in the greatest decreases in Krox-20 gene expression. Osterix, a transcription factor required for endochondral bone formation that regulates transcription down-stream of Cbfa1/Runx-2 during skeletal development [Nakashima et al., 2002; Lee et al., 2003b] shows a dramatic and sustained increase in expression after BMP treatment (20–500 fold). Consistent with the alkaline phosphatase data (Fig. 1), BMP-4, -6, and -7 are more potent inducers of osterix expression than BMP-2. *Dlx-5*, a homeobox gene expressed in all skeletal structures following cartilage formation [Miyama et al., 1999; Holleville et al., 2003], shows a similar but more modest pattern of expression, with approximate 8–20-fold increases in expression levels at 12 h and day 4, with BMP-2 showing the smallest increase in gene expression. Alpha1(I) collagen showed slightly increased gene expression after 12 h of BMP treatment that returned to basal levels at 4 days. Bone sialoprotein II (BSP) levels were little changed after 12 h of BMP treatment, but were induced by BMP-4, -6, and -7 after 4 days. MSC treated with BMP-2 showed no increase in BSP expression at the time points we examined. Osteocalcin expression levels remained relatively unchanged after 12 h and 4 days of BMP treatment.

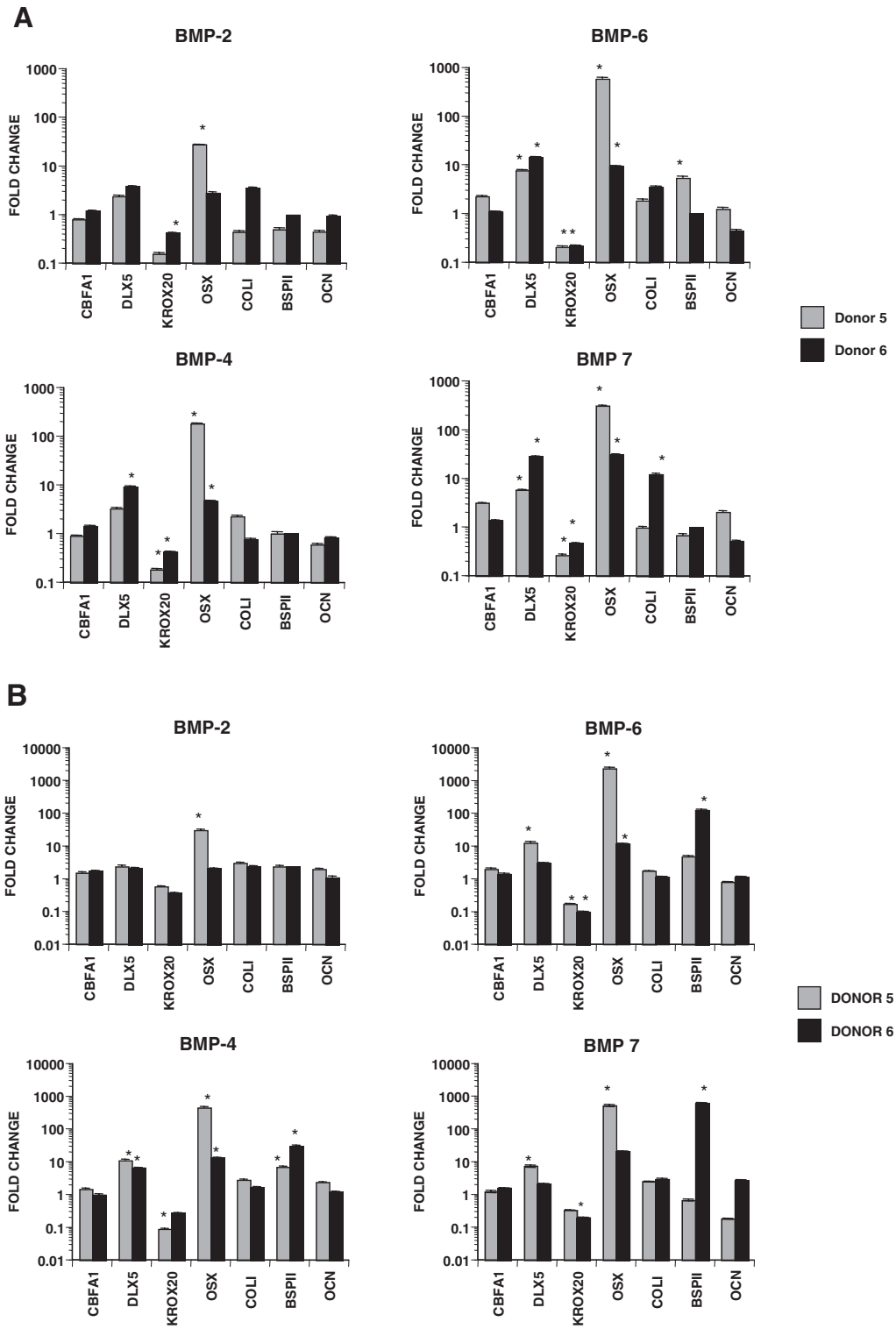


Fig. 3. Effects of osteogenic BMPs on MSC gene expression. Cells plated in 12-well plates were treated as previously described and harvested (**A**) 12 h and (**B**) 4 days after various BMP treatments. Results are reported as fold-change in gene expression relative to untreated controls and normalized to beta actin (ΔΔCt method). Columns reflect the mean value of at least three independent experiments. Error bars represent the accumulated standard deviation associated with the ΔΔCt value. * indicates that treatments are statistically different from basal cultures (time = 0) at $P < 0.05$. All experiments were done with hMSC from three different donors.

hMSC Show Variable BMP Receptor and Antagonist Expression

Of the osteogenic BMPs we evaluated, BMP-6 and BMP-7 had the most potent osteoinductive effects on hMSC. The effects of BMP-4 were markedly less than BMP-6 or BMP-7, and BMP-2 treatment yielded the lowest levels of osteoinduction as determined by alkaline phosphatase activity and gene expression patterns. To determine possible mechanisms for the differential BMP responsiveness, we evaluated the expression levels of type I BMP receptor.

BMPs signal through heterodimeric type I/type II BMP receptors. BMP-2 is thought to bind with highest affinity to BMP receptor type IB/II (ALK-6), and with lower affinity to receptor type IA/II (ALK-3), while the converse is true for BMP-4. BMP-6 and BMP-7 both bind with high affinity to activin receptor 1/BMP-RII (AR1 or ALK-2) [Aoki et al., 2001]. Differences in the levels of type I receptor expression could lead to varying degrees of responsiveness to specific BMPs. By quantitative PCR, basal expression levels of both ALK-2 and ALK-3 are approximately 16–100 fold higher than the BMP2 type I receptor, ALK-6 (Table I). None of the type I BMP receptors showed changes in expression after BMP treatment of hMSC.

Cellular responses to BMPs are modulated by a variety of secreted antagonists including chordin, dan, follistatin, and noggin, which bind with high specificity and affinity to BMPs, thereby blocking receptor–ligand interactions. Expression of these antagonists may offer a further explanation for the more consistent and robust response to BMP-6 relative to BMP-2 and BMP-4, since the affinity of these inhibitors for BMP-6 is significantly less than that for BMP-2 or BMP-4 [Piccolo et al., 1996; Glister et al., 2004]. Quantitative RT-PCR evaluation of BMP antagonist expression in hMSC demonstrated that noggin expression was very low (>40 CT) but BMP-6-inducible (Table II), while chordin expression was undetectable. Expression of dan and follistatin were more robust and donor-dependent, but showed no discernable pattern of BMP regulation.

TABLE I. Magnitude of BMP Receptor Expression as Indicated by Cycle Threshold (Ct)

Donor	ALK-2	ALK-3	ALK-6
1	32.8 (1.0)	31.1 (1.0)	35.5 (2.0)
5	31.9 (1.1)	30.6 (0.6)	38.1 (0.0)
6	31.5 (0.5)	30.6 (0.1)	38.3 (2.1)

n = 3 independent experiments for each donor Mean (SD). All values represent expression in untreated hMSC. No changes were observed in BMP receptor expression after BMP treatment.

BMP-6 Induces Temporal Changes in Gene Expression Consistent With Osteoblast Differentiation

Given its osteoinductive effects, we next evaluated temporal patterns of BMP-6-induced gene expression. As in the previous experiment, osterix expression was strongly induced at 1 day and was maintained for 18 days, 12 days after withdraw of BMP-6 (Fig. 4). Thus, continuous BMP treatment and signaling was not required to maintain osterix expression. Dlx-5, as well as Msx-2 [Cheng et al., 2003b], a transcription factor involved in skull development and osteoblast differentiation [Wilkie et al., 2000; Cheng et al., 2003b], also showed modestly increased expression at all time points examined. Twist-1 and Twist-2, transcription factors that negatively regulate Cbfa1/Runx-2 transcriptional activity during mouse skeletal development [Bialek et al., 2004], were expressed at moderate levels (average Ct values of 30 and 32, respectively), but show no change in expression after BMP treatment (results not shown). Lef-1, a transcription factor associated with the wnt signaling pathway [Labbe et al., 2000; Letamendia et al., 2001], shown to inhibit Cbfa1/Runx-2 activation of osteocalcin gene expression [Kahler and Westendorf, 2003], exhibited slightly increased expression at day 1, day 4, and day 18, indicating that wnt signaling may be activated by BMP (Fig. 4). Prior to BMP treatment, BSP and osteopontin expression was very low to undetectable (Ct value 37–>43). After BMP treatment, expression of these genes was modestly increased at day 4, with high expression by day 18. Alpha1(I) collagen showed little increase in expression at the time points examined, as did osteocalcin, with the exception of donor 1 at day 18 showing a large increase in expression (Fig. 4). Interestingly, Alpha1(I) collagen was expressed at relatively high levels without BMP treatment (Ct value 23–28), while osteocalcin was expressed at moderate levels without BMP treatment.

We also examined the expression of hedgehog family members SHH and IHH, as well as their associated transcription factors Gli-1 and Gli-2. Expression of SHH and IHH as well as the

TABLE II. Expression of BMP Anatagonists as Indicated by Cycle Threshold (Ct)

	Chordin		Dan		Follistatin		Noggin	
Donor	NT	BMP-6	NT	BMP-6	NT	BMP-6	NT	BMP-6
1	ND	ND	37.8 (0.8)	39.0 (0.7)	38.5 (0.9)	36.7 (1.6)	ND	ND
5	ND	ND	32.4 (0.7)	31.3	26.7 ()	26.6 (0.4)	41.6 (2.4)	35.8
6	ND	ND	30.9 (0.5)	32.9 (0.4)	26.8 (0.5)	30.1 (0.6)	41 (1.1)	38.8 (0.5)

n = 3 independent experiments performed in duplicate for each donor Mean (SD). ND, not detected.

transcription factors Gli-1 and Gli-2 were absent during the time course of BMP induction (results not shown). Although the wnt-associated transcription factor, Lef-1 showed increased levels of expression, we were unable to detect expression of the wnt ligands wnt-1, wnt-3a, wnt-5a, and wnt-7a at any timepoints, with or without BMP treatment. Wnt-5b was expressed at moderate to low levels (Ct value approximately 30–35), while wnt-8b, wnt-10b, and wnt-11 were expressed at low to undetectable levels (low Ct value = 35–40, undetectable $\geq$43) at day 1 of BMP treatment (results not shown). In general, expression of all wnts we examined decreased with time regardless of treatment. Of the wnt ligands we examined, only wnt-5b was BMP responsive, with decreased expression at days 1, 4, and 18 (Fig. 4). While wnt signaling may be active in BMP-6-induced hMSC, wnt ligands involved in such an autocrine loop remain unknown, but likely differ from those described in previous work performed in a murine system [Rawadi et al., 2003].

MSC can differentiate along multiple mesenchymal lineages, including the adipocyte and chondrocyte lineages [Pittenger et al., 1999]. To verify that BMP-treated hMSC were not differentiating to adipocytes, we examined expression of the adipocyte transcription factors PPARγ and SREBP-1, and of lipoprotein lipase (LPL), an enzyme involved in lipid metabolism. PPARγ and SREBP-1 expression were not detectable in BMP-treated or control hMSC, while LPL expression was low to undetectable and not BMP inducible (results not shown). hMSC only undergo chondrogenic differentiation when cultured as a micromass [Pittenger et al., 1999] and treated with TGFβ. To verify that the hMSC in our monolayer cultures were not undergoing chondrocyte differentiation, we examined expression of the chondrocyte transcription factor Sox-9 and type II and X collagen. Expression of these genes was very low and not BMP inducible (results not shown).

hMSC-Derived Osteoblasts Secrete Bone ECM Proteins and Deposit Hydroxyapatite Into the ECM

The presence of bone-associated matrix proteins was evaluated by immunocytochemistry. Untreated hMSC showed low amounts of type I collagen and virtually undetectable amounts of osteopontin (OPN), and bone sialoprotein II (BSP) in the ECM (Fig. 5). MSC cultured in dexamethasone, ascorbate, and BGP show increased deposition of type I collagen, but no increases in osteopontin and BSP. Importantly, MSC treated with BMP-6 showed significantly higher levels of type I collagen, OPN, and BSP. These matrix proteins were the most abundant in what appeared to be mineralized foci (data not shown).

ECM mineralization is characteristic of cells undergoing osteoblast differentiation. Calcium phosphate deposition was detected using both Von Kossa and Alizarin Red S staining. To determine if the differential effects of BMP-2, -4, -6, and -7 on hMSC gene expression were functionally significant, we performed side-by-side mineralization studies on BMP-treated MSC. Surprisingly, given our gene expression and AP data, BMP-2-treated hMSC show low-level mineralization at the highest BMP dose, while BMP-6-treated MSC show greater mineralization at all doses examined. BMP-4 yielded inconsistent results between donors, while mineralization induced by BMP-7 was barely above the threshold of detection (Fig. 6A). BMPs 2 and 6, 2 and 7, and 6 and 7 (10 nM each BMP) were also used in combination to evaluate synergistic or additive effects. Only combinations including BMP-6 resulted in robust mineralization (Fig. 6B).

The effects of BMP-6 on mineralization were examined further in subsequent experiments.

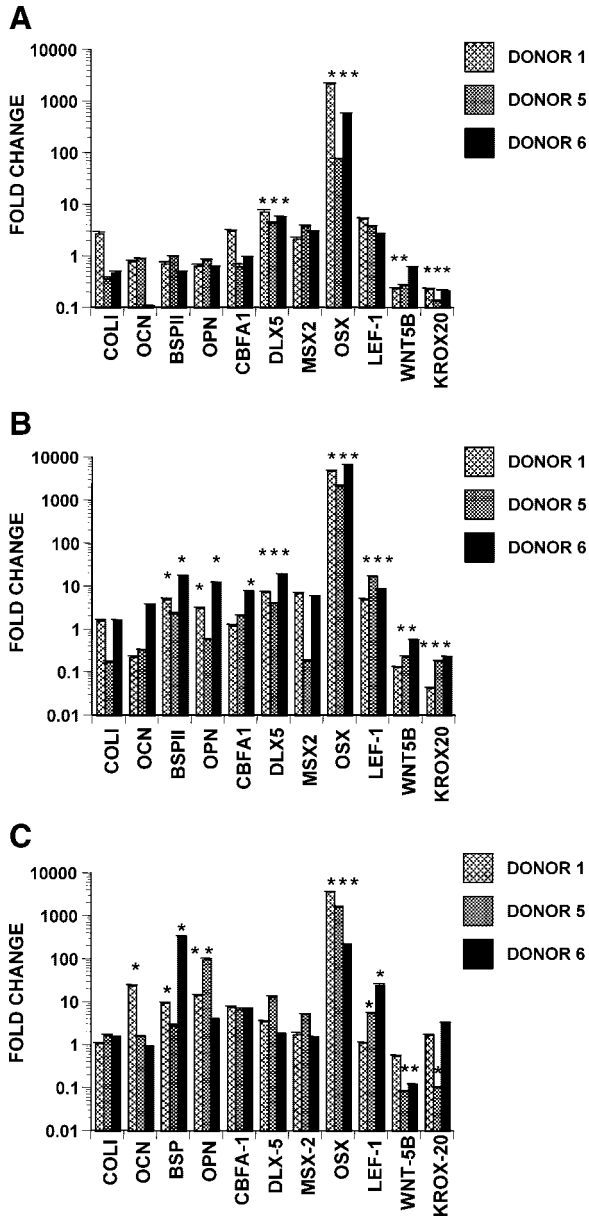


Fig. 4. Effects of BMP-6 on MSC gene expression. Results reflect fold-change in gene expression at (**A**) day 1, (**B**) day 4, or (**C**) day 18 after BMP-6 treatment relative to untreated controls and normalized to beta actin (ΔΔCt method). Columns reflect the mean value of at least three independent experiments. Error bars represent the accumulated standard deviation associated with the ΔΔCt value. * indicates that treatments are statistically different from basal cultures (time = 0) at $P < 0.05$. All experiments were done with hMSC from three different donors.

MSC treated with BMP-6 showed cell-associated foci of intense Alizarin Red S stain (Fig. 6C), while untreated MSC or cells treated with dexamethasone (latter not shown) showed little to no mineralization. The presence of calcium phosphate mineral was confirmed using Von Kossa staining (Fig. 6D). Mineralized ECM was first detectable between days 12–18, with peak mineralization occurring after day 18 of BMP induction (Fig. 6E). The most robust mineralization was observed with 3–6 days of BMP treatment followed by 12–15 days of culture without BMP (Fig. 6F).

To evaluate if higher serum concentrations during BMP-6 induction would result in more robust mineralization, inductions were performed in the presence and absence of serum (Fig. 6F). Cells treated with BMP-6 in the presence of serum demonstrated the highest levels of mineralization at day 21 post induction, but as expected, serum-adhered cultures (our standard conditions) also showed a significant degree of mineralization. Interestingly, initial adherence in serum appeared necessary for mineralization because cells adhered in the absence of serum did not show mineralization. The increased mineralization seen in the presence of serum was partly attributable to the higher cell density in these cultures.

A limitation of both Von Kossa and Alizarin Red S staining is that they detect calcium phosphate, but do not reveal any information about the physiochemical nature of the mineral. Thus, Raman spectroscopic analysis was performed to provide more detailed chemical analysis (Fig. 7). Raman spectrograph analysis demonstrates the presence of phosphate and carbonate substituted hydroxyapatite. The ν_1 phosphate band at 959/cm, indicative of apatitic calcium phosphate, was the strongest peak. The frequency was similar to that of mature cortical bone. Principle Component analysis also resolved spectral peaks at 1065–1070/cm indicating the presence of type B carbonate derivatized apatite, in which carbonate replaces phosphate in the apatite crystal lattice. The less prominent peaks at 1110 and 1150/cm were indicative of type A substituted carbonate, in which carbonate substitutes for hydroxyl groups within the crystal lattice. The higher ratio of type B to type A carbonate is indicative of more mature bone [Timlin et al., 1999]. Thus, the chemical characteristics of hMSC-derived osteoblast hydroxyapatite were similar to hydroxyapatite produced in vivo.

DISCUSSION

We report that hMSC are capable of undergoing marked osteogenic differentiation, activating bone-associated gene and protein

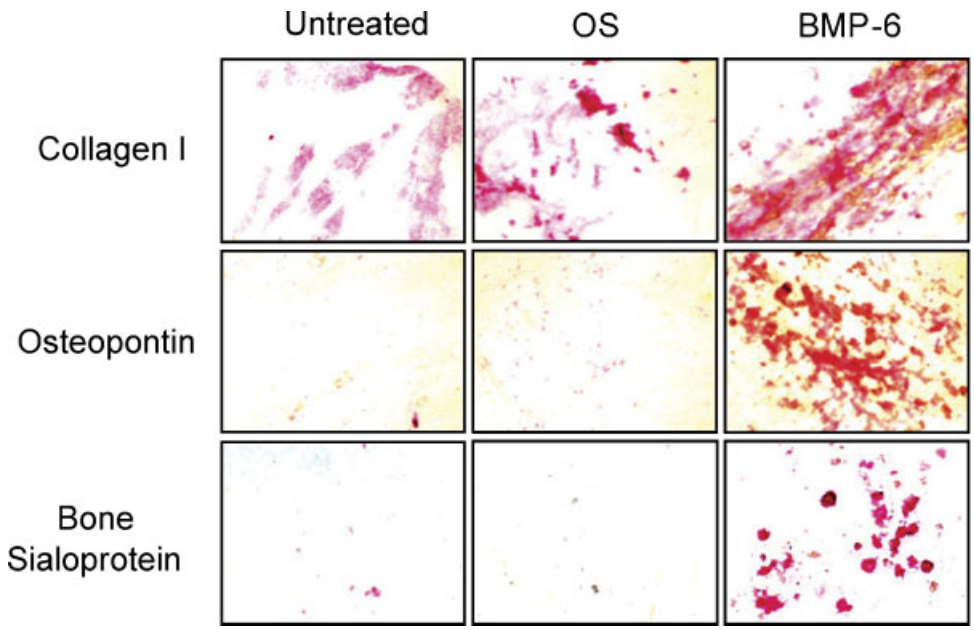


Fig. 5. BMP-6 regulates deposition of bone associated ECM proteins. Cells were plated in 12-well plates, treated as previously described, and harvested at d 21 for immunodetection of ECM proteins as described in the Materials and Methods. Red color indicates positive staining. OPN and BSP were virtually undetectable in untreated or OS-treated cultures, but show significantly increased deposition into the ECM following BMP-6 treatment. Untreated MSC demonstrate diffuse Collagen I-α1 expression, while BMP-6-treated cells show the highest levels of Collagen I. 10× magnification. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

expression, leading to ECM mineralization and hydroxyapatite formation. hMSC show differential responsiveness to known osteogenic growth factors, with BMP-6 demonstrating the most potent, donor-independent osteoinductive effects.

A primary strength of this study is that MSC were grown in chemically defined, serum-free culture media. This eliminates the confounding effects of serum growth factors and allows for a direct evaluation of the effects of individual growth factors. We observed that serum enhances the osteogenic capacity of hMSC, thus it is likely that factors present in serum act in concert with growth factors and obscure the specific effects of exogenous growth factors. We have found that the osteogenic growth factors IGF-1, bFGF, and VEGF have no direct osteoinductive effects on hMSC under serum-free culture conditions. Previous work has shown that IFG-1 expression is required for PTH responsiveness in bone [Bikle et al., 2002] and regulates osteoblast progenitor number in vivo [Kasukawa et al., 2002], VEGF promotes vascularization during fracture healing, and bFGF promotes vascularization and increases trabecular number and connectivity [Lane et al., 2003]. Our data strongly suggest that while these factors may play a role in the development of the skeleton or in mature osteoblast or preosteoblast function, they are not sufficient for hMSC differentiation into osteoblasts.

In our experimental system, among the osteogenic BMPs, BMP-6 is the most potent inducer of hMSC differentiation in vitro. Only BMP-6 expression is upregulated by dexamethasone treatment, while addition of exogenous BMP-6 consistently yields an osteoblast phenotype. Similarly, when BMPs are used in combination, only combinations containing BMP-6 promote robust mineralization. These results are consistent with those described by Boden et al. [1996] in rat calvarial cultures. Rat calvarial cells are more responsive to BMP6-induced differentiation and do not require glucocorticoid potentiation. Similar to our findings with hMSC, BMP6 production is also upregulated in rat calvarial cells treated with glucocorticoid [Boden et al., 1997]. Considered together, these results suggest that BMP6 may act at an earlier stage of osteoblast differentiation in multiple species and may be one of the factors mediating glucocorticoid-induced osteogenesis.

Our results are consistent with previous studies which have shown that hMSC are either

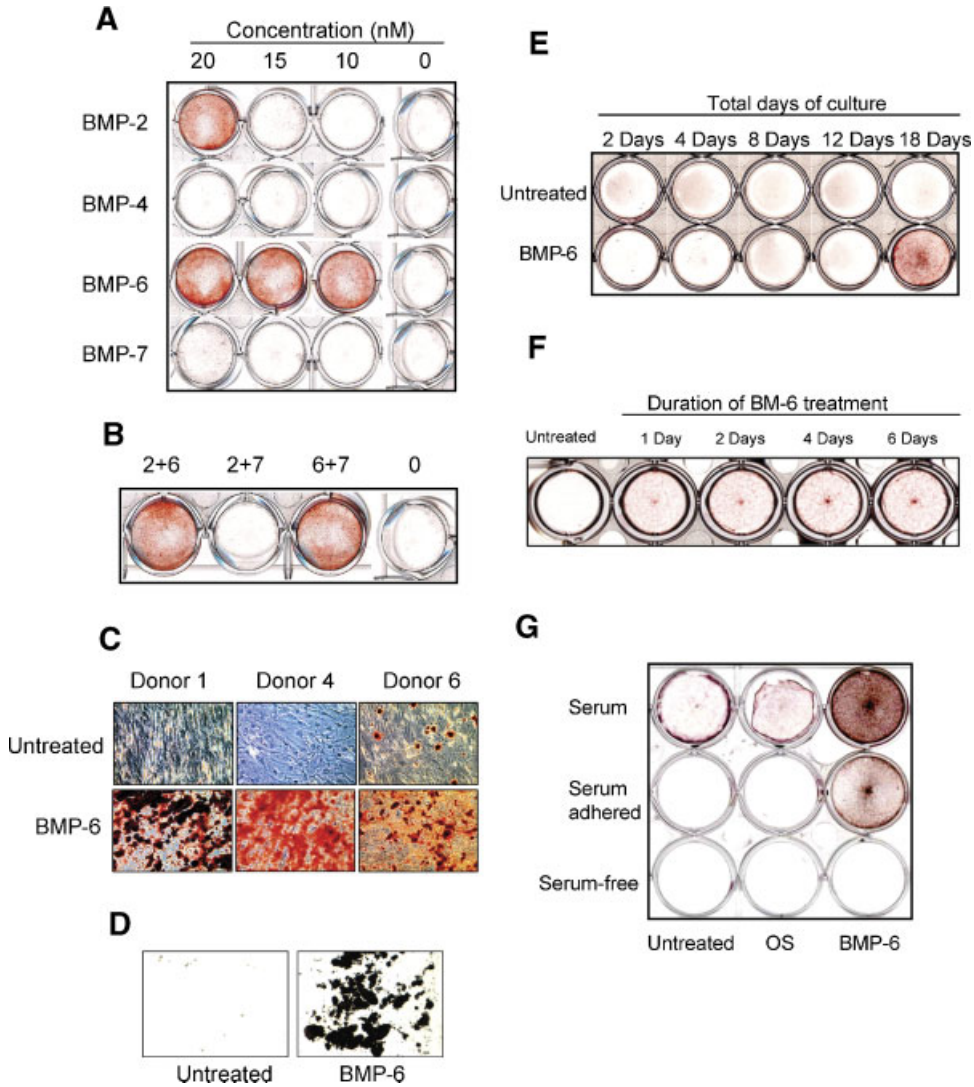


Fig. 6. BMP-6 induced mineralization of the ECM. (**A**–**B**) hMSC were treated with BMP-2, 4, 6, or 7 for 6 days at 20, 15, or 10nM, or added in combination where indicated (10nM each) and stained with Alizarin Red S to evaluate calcium phosphate mineral. **C**: BMP-6-treated hMSC were stained with Alizarin Red S to evaluate $CaPO_4$ mineral and visualized using bright-field microscopy. **D**: Von Kossa staining was performed to detect $CaPO_4$ mineral. Mineralized nodules were absent in untreated cells and dexamethasone-treated cells, while BMP-6 treament results in extensive $CaPO_4$ deposition and ECM mineralization, as determined by bright-field microscopy. **E**: A time course study was performed to determine when the average onset of mineralization occurred, as determined by Alizarin staining. **F**: To determine the duration of BMP-6 treatment sufficient for mineralization, cultures were treated for varying times and then treatement media was removed and replaced with basal media until harvest and Alizarin red S staining at day 18. **G**: To determine optimal mineralization conditions, we compared three different treatments: (1) plated immediately in serum-free conditions and BMP-6 treated 24 h later in serum-free (No serum adhesion), (2) adhered 24 h in serum and then placed in serum-free conditions with BMP (serum adhered), or (3) induced in serum conditions (serum cultured). All mineralization studies were performed using cells from at least four donors (primarily donors 1, 4, 5, 6), and repeated at least three times with each donor ($n \geq 3$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

not responsive to BMP-2 and BMP-4, or they require higher doses of BMP to induce differentiation [Diefenderfer et al., 2003]. The relative lack of responsiveness of hMSC to BMP-2 and BMP-4 might be partly explained by the expression of multiple families of secreted BMP antagonists. These antagonists have a high affinity for BMP-2 and BMP-4 and may selectively interfere with BMP-2 and BMP-4 signaling. BMP-6 and BMP-7 have much lower affinity interactions with these antagonists and would be relatively resistant to these effects.

Surprisingly, BMP-7 was unable to drive terminal differentiation of hMSC to functional,

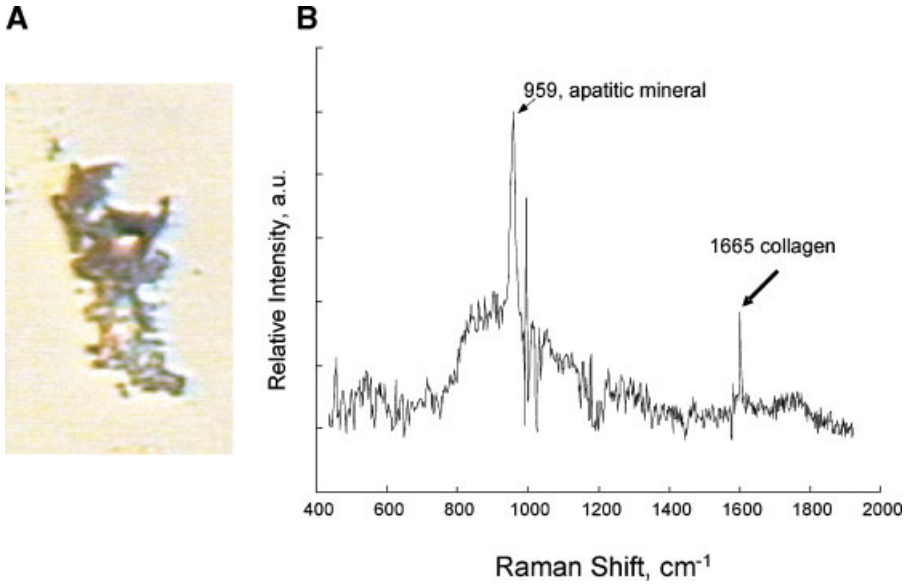


Fig. 7. BMP-6-treated MSC produce hydroxyapatite. Raman spectral analysis was performed to verify the presence of higher-order $CaPO_4$ mineral (hydroxyapatite) detected in more mature mineralized ECM. **A**: Representative image of mineral analyzed using Raman spectroscopy. **B**: Representative Raman spectrogram. Arrows indicate the 959 and 1665 peaks. Hydroxyapatite was not detected in untreated cultures (not shown). Experiments were conducted at least three times with at least three donors. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mineralizing osteoblasts despite the fact that it induced alkaline phosphatase production and changes in gene expression similar to BMP-6. BMP-7 and BMP-6 have a high degree of homology at the amino acid sequence level and both bind to the type I BMP receptors ALK-2 (and to a lesser degree ALK-6) [Ebisawa et al., 1999; Sebald et al., 2004], while BMP-2 interacts primarily with ALK-6 and BMP-4 interacts most highly with ALK-3 [Aoki et al., 2001]. Since both BMP-6 and BMP-7 have similar affinities for various antagonists and their receptors (very low nanomolar) [Sebald et al., 2004] and have been shown to induce osteoblast differentiation at similar doses in previous studies [Zhao et al., 2002; Gruber et al., 2004], it is unlikely that the differences in BMP responsiveness in hMSC are simply related to dose-dependency or receptor affinity. Thus, it is possible that down-stream signaling in hMSC by BMP-6 is different than that of other BMPs. Although BMP-6 signaling has not been well-characterized, expression of BMP signaling enhancers [Lin et al., 2005] and BMP co-receptors [Samad et al., 2005] may create an environment that is more permissive for BMP-6 osteoinduction.

Previous studies have demonstrated that BMP-2/BMP-7 heterodimers are more potent inducers of osteoblast differentiation than homodimers [Zhao et al., 2005]; thus, we anticipated that co-administration of recombinant BMP-2 with BMP-7 could yield a similar result with hMSC. However, co-administration of BMP-7 with BMP-2 failed to induce terminal differentiation of hMSC. Dimeric BMP ligands transduce signals to the cytoplasm through the formation of ternary complexes with two type I and two type II BMP or activin receptors [Sebald et al., 2004]. As stated above, type I and II BMP and activin receptors have unique affinities for different BMP family members. It is possible that BMP heterodimers recruit a greater diversity of receptor complexes consisting of multiple different type I and type II receptors. Increasing the diversity of BMP/activin receptor complexes at the cell surface may create a more dynamic and robust signaling environment that alters the quality and/or quantity of BMP heterodimer signaling of relative to BMP homodimers. This altered signaling of BMP heterodimers may be reflected at the molecular level by increased SMAD 1/5 phosphorylation, or by increased activation of other signaling pathways, such as MAPK. BMP heterodimers may also have reduced affinity for soluble BMP antagonists, resulting in increased BMP activity relative to homodimers. Our re-

sults suggest that co-administration of BMPs does not mimic the effects of cell-autogenous production of heterodimers.

At the transcriptional level, the regulatory control mechanisms governing BMP-6-induced hMSC osteoblast differentiation appear similar to those involved in early skeletal development. BMP-6-regulated expression of the osteoblast lineage "master" regulator transcription factors, Osterix, and Dlx-5, controlled terminal differentiation through activation of the bone-associated proteins osteocalcin, osteopontin, and bone sialoprotein II and through the selective downregulation of transcription factors such as krox-20, shown to negatively regulate ECM biosynthesis. However, other markers (FosB/ΔFosB, Gli-1, and Gli-2) are not detected or are relatively unchanged (Cbfa1/Runx-2 and Col I), implying that changes in their expression levels may not be required for hMSC differentiation to osteoblasts. In particular, formation of mature Collagen I and deposition into the ECM may be regulated by BMP at the post-translational level.

While the presence of serum in rodent based culture systems makes direct comparison to our hMSC system difficult, it is nonetheless important to note that a number of differences exist between human and rodent MSC-osteoblast differentiation. In hMSC, we observed moderate to high-level expression of a number of osteoblast genes and proteins, including type I collagen, osteocalcin, and Cbfa1/Runx-2, which do not appear BMP-inducible. Our results are consistent with previously published data from serum-based hMSC culture systems [Shui et al., 2003], but are in stark contrast to rodent systems where expression of these genes and gene products is inducible and basal expression is either low or undetectable [Rickard et al., 1994; Lee et al., 1999, 2003b; Siggelkow et al., 1999]. Expression of these genes in uninduced hMSC may indicate these cells, or subpopulations of hMSC, are more committed to an osteogenic lineage. However, it is equally possible that expression of these genes is characteristic of multipotent adult hMSC. In the case of Cbfa1/Runx-2, it is plausible that BMP regulation in murine cells occurs primarily at the level of transcription, while in human cells, regulation occurs post-translationally through modifications or changes in subcellular localization [Zaidi et al., 2004]. The regulation of osteoblast differentiation is species specific, and great care is needed in comparing these systems.

While hMSC are a heterogeneous cell population, clonal multipotency has been previously established [Pittenger et al., 1999]. Our results further suggest that the multipotent hMSC are poised for BMP-induced osteoblastic differentiation because of high type I BMP-receptor expression and the very low percentage of alkaline phosphatase positive cells prior to BMP treatment. The pattern of gene expression in these cells, particularly the high level of collagen I α1, and moderate levels of osteopontin and osteocalcin, indicates that hMSC are involved in ECM biosynthesis and maintenance. This basal production of ECM is likely partially regulated by Cbfa1/Runx-2 [Ducy et al., 1997; Karsenty et al., 1999; Karsenty, 2000; Kern et al., 2001]. These undifferentiated cells have little deposition and accumulation of bone-like ECM. By contrast, the defining characteristics of differentiated hMSC at the gene expression and protein levels are cells with increased alkaline phosphatase activity and increased ECM secretion and deposition, especially BSP. The increased expression of ECM proteins in differentiated cells may be regulated by increased expression of the transcription factors Osterix and DLX-5, and decreased expression of Krox-20. Thus, while Cbfa1/Runx-2 may regulate basal ECM production in hMSC, additional BMP-induced transcriptional cascades are required for terminal differentiation. At the terminal stages of differentiation, collagen I α1, osteopontin, osteocalcin, and BSP facilitate mineralization and are incorporated into a mineralized ECM. Further studies of the transcriptional regulation of BMP signaling and MSC differentiation will improve our understanding of osteoblast development in health and disease.

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