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The Role of BMP-7 in Chondrogenic and Osteogenic Differentiation of Human Bone Marrow Multipotent Mesenchymal Stromal Cells In Vitro

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ABSTRACT

This study addresses the role of bone morphogenetic protein-7 (BMP-7) in chondrogenic and osteogenic differentiation of human bone marrow multipotent mesenchymal stromal cells (BM MSCs) in vitro. BM MSCs were expanded and differentiated in the presence or absence of BMP-7 in monolayer and three-dimensional cultures. After 3 days of stimulation, BMP-7 significantly inhibited MSC growth in expansion cultures. When supplemented in commonly used induction media for 7–21 days, BMP-7 facilitated both chondrogenic and osteogenic differentiation of MSCs. This was evident by specific gene and protein expression analyses using real-time PCR, Western blot, histological, and immunohistochemical staining. BMP-7 supplementation appeared to enhance upregulation of lineage-specific markers, such as type II and type IX collagens (*COL2A1*, *COL9A1*) in chondrogenic and secreted phosphoprotein 1 (*SPP1*), osteocalcin (*BGLAP*), and osterix (*SP7*) in osteogenic differentiation. BMP-7 in the presence of TGF-β3 induced superior chondrocytic proteoglycan accumulation, type II collagen, and SOX9 protein expression in alginate and pellet cultures compared to either factor alone. BMP-7 increased alkaline phosphatase activity and dose-dependently accelerated calcium mineralization of osteogenic differentiated MSCs. The potential of BMP-7 to promote adipogenesis of MSCs was restricted under osteogenic conditions, despite upregulation of adipocyte gene expression. These data suggest that BMP-7 is not a singular lineage determinant, rather it promotes both chondrogenic and osteogenic differentiation of MSCs by co-ordinating with initial lineage-specific signals to accelerate cell fate determination. BMP-7 may be a useful enhancer of in vitro differentiation of BM MSCs for cell-based tissue repair. J. Cell. Biochem. 109: 406–416, 2010.

KEY WORDS: MESENCHYMAL STROMAL CELLS; BONE MORPHOGENETIC PROTEIN-7; CHONDROGENIC DIFFERENTIATION; OSTEOGENIC DIFFERENTIATION

Bone marrow multipotent mesenchymal stromal cells (BM MSCs) have considerable potential for use in the therapeutic regeneration of tissues due to their ability to differentiate into various cell lineages, including osteoblasts and chondrocytes [Brooke et al., 2007; Basem and Abdallah, 2009]. The targeted differentiation of BM MSCs into lineage-determined cells for transplantation into injury sites is of interest in many different fields of regenerative medicine. Its therapeutic potential for intervertebral disc regeneration holds out new hope to reverse a painful and debilitating condition suffered by millions of people globally [Brisby et al., 2004; Tao et al., 2008]. The approaches for differentiation of stem cells into a specific cell type for tissue repair are the subject of much research. Although BM MSCs could differentiate according to signaling received in vivo, lineage-directed differentiation of the MSCs in vitro prior to cell transplantation may achieve improved phenotypic stability in vivo [Mehlhorn et al., 2006]. To understand the essential differentiation factors required for specific lineage differentiation and to improve in vitro techniques are of particular importance for successful clinical application of BM MSCs.

Bone morphogenetic proteins (BMPs), as members of the transforming growth factor-β (TGF-β) superfamily, are multi-functional cellular regulators with a pivotal role in numerous

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biological processes in both the developing embryo and adult [Chimal-Monroy et al., 2003; Chen et al., 2004], but are best known for their ability to induce bone formation [Urist, 1965; Reddi, 1998; Wozney, 2002]. Indeed recombinant human BMP-2 and BMP-7 have been approved for clinical use in the regeneration of bone in fracture healing and vertebral arthrodesis [Biase and Capanna, 2005]. In addition to their activity in stimulating bone formation, we have recently demonstrated that BMP-2 enhances TGFβ3-mediated chondrogenic differentiation of BM MSCs in vitro [Shen et al., 2008]. Similarly BMP-7, also known as osteogenic protein-1 (OP-1), has shown strong anabolic activity in both bone and cartilage [Geesink et al., 1999; Chubinskaya and Kuettner, 2003]. In spite of its main clinical use in spinal fusion and fracture non-unions [Giannoudis and Tzioupis, 2005], BMP-7 demonstrated synergism with microfractures to stimulate cartilage repair in vivo [Kuo et al., 2006]. BMP-7 can stimulate a chondrogenic phenotype in adipose tissue derived stem cells [Knippenberg et al., 2006] and enhances the chondrogenesis of synovial mesenchymal stem cells when combined with TGF-β1 [Miyamoto et al., 2007]. Interestingly, BMP-7 initiated adipogenic instead of osteo-/chondrogenic differentiation of BM MSCs in high-density micromass cultures, which generally favor chondrogenic differentiation [Neumann et al., 2007]. Thus, BMP-7 appears to have the ability to promote an array of lineage commitments, but the limited number of studies and somewhat contradictory results warrant further investigation.

The objective of the present study was to determine the effects of BMP-7 on chondrogenic and osteogenic differentiation of BM MSCs in vitro by supplementation of BMP-7 into individual standard differentiation media. Each lineage phenotype development was examined by analyzing the specific gene and protein expression, proteoglycan deposition, or matrix mineralization. All results were compared to undifferentiated and differentiated MSCs without BMP-7 stimulation.

MATERIALS AND METHODS

CELL ISOLATION AND CULTIVATION

Human bone marrow was collected from the surgically discarded tissues of patients undergoing total hip replacement following written informed consent under ethical approval from Human Research Ethics Committees of St. George Hospital and St. Vincent's Hospital, Sydney, Australia. BM MSCs were isolated from three donors by immunodepletion, Ficoll-Paque density gradient centrifugation, and plastic adhesion as previously described [Shen et al., 2008]. The cells were grown in growth medium consisting of 60% Dulbecco's modified Eagle's medium—low glucose (DMEM-LG; Invitrogen, Carlsbad, CA), 40% MCDB-201 medium, 1% insulin transferrin selenium (ITS), 1% linoleic acid/bovine serum albumin (BSA), 1 nM dexamethasone, 32 μg/ml ascorbate-2-phosphate (Sigma–Aldrich, St. Louis, MO), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS; Invitrogen). Cultures were incubated at 37°C in 5% CO_2 in air with medium changed twice weekly.

CELL GROWTH ASSAY

BM MSCs at passage 3 were seeded in culture flasks in duplicate at the density of 2×10^3 cells/cm^2. After 24 h attachment, the cells were treated with 100 ng/ml of recombinant human BMP-7 (Stryker Biotech, Hopkinton, MA) in growth medium for 1, 3, 5, and 7 days. Following treatment, cells were collected at each time point and counted by trypan blue exclusion assay under a light microscope. The relative cell number, calculated as fold of cell number at day 1, was used for comparison between BMP-7 treated and non-treated MSCs.

CHONDROGENIC AND OSTEOGENIC DIFFERENTIATION

BM MSCs at Passages 3–4 were harvested by the standard trypsinization method. Chondrogenic differentiation was performed in both alginate bead and pellet cultures. Alginate bead culture was set up as previously described [Shen et al., 2008]. Briefly, MSCs were suspended in a solution of 1.2% (w/v) sodium alginate in 150 mM NaCl, at the density of 5×10^6/ml for differentiation and 1×10^6/ml for undifferentiated controls. The beads were formed by pressing the cell suspension dropwise into 102 mM $CaCl_2$ solution in 12-well culture plates followed by washing with 150 mM NaCl solution. For pellet culture, 1×10^6 cells/tube were pelleted at 1,500 rpm for 5 min (Beckman GS-6 centrifuge) in 15 ml polypropylene conical tubes. Embedded or pelleted MSCs were cultured in chondrogenic differentiation medium consisting of DMEM—high glucose, 40 μg/ml L-proline, 1% ITS solution, 5.35 μg/ml linoleic acid, 50 μg/ml ascorbate-2-phosphate, 100 nM dexamethasone, 1.25 mg/ml BSA, and either 10 ng/ml of recombinant human TGF-β3 (R&D Systems, Minneapolis, MN) or 100 ng/ml of BMP-7 (Stryker Biotech) individually and in combination (TGF-β3 + BMP-7).

Osteogenic differentiation of MSCs was induced in confluent monolayer cultures in 24-well culture plates using a standard induction medium [Caterson et al., 2002] containing DMEM-LG, 10% FBS, 10 mM β-glycerophosphate, 50 μg/ml ascorbate-2-phosphate, 100 nM dexamethasone in the presence and absence of BMP-7 (0–200 ng/ml) for 7–14 days. For both differentiation procedures, undifferentiated MSCs were cultured in parallel in growth medium as negative controls. Cell cultures were incubated at 37°C, 5% CO_2 for 7–21 days and the media were changed twice weekly.

RNA EXTRACTION, cDNA SYNTHESIS, AND REAL-TIME PCR

Total RNA was isolated from MSCs using TRIzol reagent (Invitrogen) and RNeasy kit (Qiagen, Dusseldorf, Germany) following manufacturers' instructions. The cDNA was prepared using SuperScript III first-strand synthesis system (Invitrogen) from 1 μg of total RNA. The 1:40 diluted cDNA was used in 20 μl reactions for real-time PCR analysis using a Rotor-Gene RG3000 system (Corbett Life Science, Sydney, Australia). All primers were designed according to published mRNA sequences. To exclude possible genomic DNA contamination, the RNA was treated with DNase and primers were designed to be intron spanning. The thermal profile was as follows: 1 cycle of 95°C for 5 min, followed by 40 amplification cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Data were analyzed by Rotor-Gene 6.0 software. The GenBank access numbers, real-time PCR primers, and product sizes are listed in Table I. Relative

TABLE I. Primer Pairs Used for Real-Time PCR

Gene symbol	GenBank accession number	Sequence (5′–3′)	Product size (bp)
COL1A1	NM_000088	F-GAGAGCATGACCGATGGATT R-ATGTAGGCCACGCTGTTCTT	149
COL2A1	NM_033150	F-GTGACAAAGGAGAGGCTGGA R-ACCTCTAGGGCCAGAAGGAC	146
COL9A1	NM_001851	F-CAGGATATCCAGGCCTACCA R-TCCCTGGTCACCTTCTTCAC	104
COL10A1	NM_000493	F-CACCTTCTGCACTGCTCATC R-GGCAGCATATTCTCAGATGGA	104
ACAN	NM_013227	F-TCAACAACAATGCCCAAGAC R-AAAGTTGTCAGGCTGGTTGG	128
SOX9	NM_000346	F-GTACCCGCACTTGCACAAC R-GTAATCCGGGTGGTCCTTCT	139
COMP	NM_000095	F-GGAGATCGTGCAGACAATGA R-GAGCTGTCCTGGTAGCCAAA	147
BGN	NM_001711	F-CAGCCCGCCAACTAGTCA R-GGCCAGCAGAGACACGAG	93
RUNX2	NM_004348	F-GCCTAGGCGCATTTCAGA R-CTGAGAGTGGAAGGCCAGAG	66
WWTR1	NM_015472	F-CAGCAATGTGGATGAGATGG R-TGGGGATTGATGTTCATGG	66
ALPL	NM_000478	F-GACAAGAAGCCCTTCACTGC R-AGACTGCGCCTGGTAGTTGT	120
SPP1	NM_000582	F-GCCGAGGTGATAGTGTGGTT R-TGAGGTGATGTCCTCGTCTG	101
BGLAP	NM_199173	F-GGCGCTACCTGTATCAATGG R-TCAGCCAACTCGTCACAGTC	106
SP7	NM_152860	F-ATGTCTTGCCCCAAGATGTC R-TATCCACCACTACCCCCAGT	115
PPARG	NM_015869	F-CAGGAAAGACAACAGACAAATCA R-GGGGTGATGTGTTTGAACTTG	94
ADIPOQ	NM_004797	F-CCTGGTGAGAAGGGTGAGAA R-CTCCTTTCCTGCCTTGGATT	124
LPL	NM_000237	F-GTGGCCGAGAGTGAGAACAT R-GAAGGAGTAGGTCTTATTTGTGGAA	66

expression levels were calculated as a ratio to the average value of house-keeping genes, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) [Shen et al., 2008].

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

The MSC pellets or alginate beads were rinsed with PBS, fixed in 4% paraformaldehyde for 30 min, and embedded in paraffin. Sections of 4 μm thickness were cut and mounted on slides. Before staining, sections were dewaxed in xylene and hydrated with graded ethanol.

Proteoglycans were stained with 1% Alcian blue and nuclei were counterstained with 0.1% nuclear fast red solution. Type II collagen and SOX9 were analyzed by immunohistochemical staining using primary goat anti-human type II collagen (1:200 dilution) and rabbit anti-human SOX9 (1:80 dilution) polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. The slides were treated with Multilink solution (Dako, Sydney, Australia) followed by streptavidin-conjugated peroxidase incubation. The sections were visualized with 3,3′-diaminobenzidine hydrochloride solution and counterstained with hematoxylin. The primary antibody was omitted for negative controls.

The activity of cell surface alkaline phosphatase (ALP) was determined at day 14 of osteogenic differentiation using Fast blue dye staining. Calcium mineralization of the extracellular matrix was detected at days 7 and 14 by Alizarin red S staining for osteogenic differentiated MSCs. Adipocytes were detected using Oil red O staining, specific for intracellular lipids [Hung et al., 2002].

WESTERN BLOT ANALYSIS

Cells were rinsed with cold PBS and lysed in CelLytic-M solution containing protease inhibitors (Sigma–Aldrich). Equal quantities of protein samples (20 μg) were electrophoresed in 8–12% gradient SDS–polyacrylamide gels. Proteins were transferred by electroblotting to PVDF membranes and the membranes were blocked with 5% skim milk in Tris–HCl buffered saline (TBS; 20 mM Tris, pH 7.6, 0.15 M NaCl) overnight at 4°C. Membranes were incubated with goat anti-human type II collagen (1:200 dilution) and rabbit anti-human SOX9 (1:200 dilution) polyclonal antibodies (Santa Cruz Biotechnology) in TBS buffer containing 0.1% Tween-20 (TTBS) for 2 h at room temperature. Alpha-tubulin was detected as the reference protein using its specific antibody (Santa Cruz Biotechnology). After washing and incubation with fluorescent dye-conjugated secondary antibodies, immunolabeling was detected using the Odyssey infrared imaging system (LI COR Biosciences, Lincoln, NE).

STATISTICS

Comparisons between multiple experimental groups were performed with analysis of variance (ANOVA) and Student's *t*-test using Sigma Stat software (SPSS, Inc., San Rafael, CA) for analysis of gene expression and cell growth data. All values are presented as mean ± SEM. Differences between undifferentiated and differentiated BM MSCs (±BMP-7) are considered statistically significant at $P < 0.05$ (*). Statistical differences among differentiated BM MSC groups were not labeled in figures, but stated in the Results Section.

RESULTS

EFFECTS OF BMP-7 ON MSC GROWTH

BM MSCs grown in monolayer expansion culture presented a typical fibroblast-like morphology similar to that previously reported [Shen et al., 2008]. BM MSCs initially seeded at equal density were cultured in growth medium containing 10% FBS with (100 ng/ml) and without (control) BMP-7 stimulation for 1–7 days. The cell counting at each time point indicated that there was no difference between the control and BMP-7-treated MSCs at days 1 and 3 (20–50% confluence), but BMP-7-treated cell numbers were significantly reduced at days 5 and 7 (60–90% confluence), with the respective cell counts $30.0 \pm 1.3\%$ and $28.7 \pm 0.43\%$ less than those of untreated control cells (Fig. 1). A similar BMP-7-mediated growth retardation was detected in cultures containing 2% FBS (data not shown).

EFFECTS OF BMP-7 ON INDUCTION OF CHONDROCYTIC PHENOTYPE OF BM MSCs

The chondrogenic differentiation of BM MSCs was induced using specific serum-free media supplemented with TGF-β3 only, BMP-7 only, or TGF-β3 + BMP-7 in alginate bead culture for 14–21 days and compared to undifferentiated MSCs in growth medium. When cells were liberated from alginate beads at the termination of the experiment, the centrifuged cell pellets had notably different sizes, with TGF-β3 + BMP-7 > TGF-β3 > BMP-7 (Fig. 2A). The largest TGF-β3 + BMP-7 pellet seemed to have the lowest cell density, observed to be loosely pelleted and easily floating compared to other pellets. Real-time PCR analysis demonstrated that undifferentiated MSCs expressed low or undetectable levels of chondrocytic genes (Fig. 2B). Following chondrogenic differentiation for 21 days, the expression levels of chondrocytic cell specific genes were upregulated, such as aggrecan (*ACAN*), type II collagen (*COL2A1*), type IX collagen (*COL9A1*), type X collagen (*COL10A1*), and *SOX9*. Chondrocytic collagen gene expression, *COL2A1* and *COL9A1*, was further elevated ($P < 0.05$) by TGF-β3 + BMP-7 by 3,370-fold (*COL2A1*) and 2,500-fold (*COL9A1*), respectively, compared to 300- and 140-fold by TGF-β3 alone (Fig. 2B). However, there are no significant differences between TGF-β3 + BMP-7 and TGF-β3 alone for the expression of *ACAN*, *COL10A1*, and *SOX9*. Treatment with 100 ng/ml of BMP-7 alone did not upregulate chondrogenic gene expression, even under conditions that favored chondrogenic differentiation. The addition of BMP-7 suppressed ($P < 0.05$) TGF-β3-mediated upregulation of gene expression of type I collagen (*COL1A1*), cartilage oligomeric matrix protein (*COMP*), and biglycan (*BGN*), which likely represents neutralization of the effects of TGF-β3 by BMP-7 (TGF-β3 increases and BMP-7 decreases the expression).

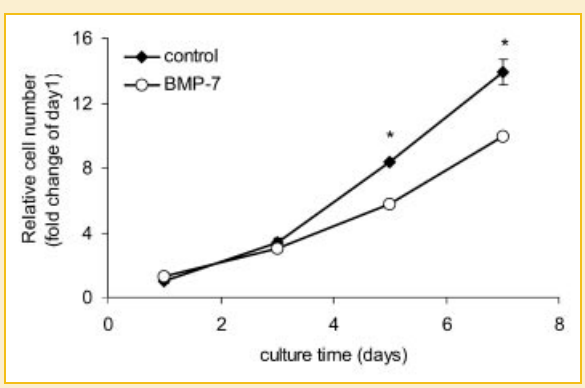

Fig. 1. Growth rate of BM MSCs in expansion culture. The effect of BMP-7 on MSC growth rate was examined at days 1, 3, 5, and 7 by counting cells using trypan blue exclusion assay under a hemocytometer. Comparison of relative cell number as a fold change of day 1 counts showed that BMP-7 did not affect cell growth up to 3 days, but retarded cell growth by 30% compared to control after 5 days in culture.

Histological and immunohistochemical analysis was applied to assess the extracellular matrix production and accumulation in chondrogenic differentiated MSCs in both alginate bead (Fig. 3A) and pellet (Fig. 3B) cultures. Alcian blue staining demonstrated greater proteoglycan deposition in the extracellular matrix of TGF-β3 + BMP-7 treated MSCs than those treated with TGF-β3 or BMP-7 alone. Similarly, the protein expression of type II collagen and SOX9 was greater in TGF-β3 + BMP-7 treated MSCs using immunohistochemistry (Fig. 3A,B) and Western blotting (Fig. 4). Taken together, TGF-β3 + BMP-7 showed enhanced chondrogenic capacity, while BMP-7 treatment without TGF-β3 had little chondroinductive activity.

EFFECTS OF BMP-7 ON INDUCTION OF OSTEOBLASTIC PHENOTYPE OF BM MSCs

The specific gene expression of MSCs cultured in osteogenic differentiation media with BMP-7 for 14 days showed elevated expression levels of the osteogenic genes *SPP1* (also known as osteopontin), osteocalcin (*BGLAP*), and osterix (*SP7*), compared to osteogenic medium alone (Fig. 5). BMP-7 supplementation showed limited effects on runt-related transcription factor 2 (*RUNX2*), WW domain-containing transcription regulator 1 (*WWTR1*, also known as transcriptional co-activator with PDZ-binding motif, *TAZ*), and alkaline phosphatase (*ALPL*) genes. The expression of *COL1A1*, which is considered as an osteogenic gene marker, was downregulated in differentiated MSCs with or without BMP-7 stimulation. The expression of *COL2A1* was slightly upregulated in the presence of BMP-7.

The histological analysis showed that BMP-7 supplementation increased cell surface ALP activity and accelerated calcium mineralization of extracellular matrix of osteogenic differentiated BM MSCs (Fig. 6A). Mineralization in differentiated MSCs could be detected as early as day 7 in the presence of BMP-7, much earlier than that of MSCs in osteogenic differentiation medium without BMP-7. The levels of mineralization were promoted by BMP-7 in a dose-dependent manner (Fig. 6B).

EFFECTS OF BMP-7 ON ADIPOGENIC GENE EXPRESSION AND RESTRICTED PHENOTYPE DEVELOPMENT UNDER OSTEOGENIC CONDITIONS

There are reports in the literature that MSCs are prone to adipogenic differentiation by BMP-7 stimulation even under unfavored differentiation conditions [Neumann et al., 2007] and that adipogenic/osteogenic differentiation can occur simultaneously with BMP-7 treatment [Chen et al., 2001]. We wondered whether

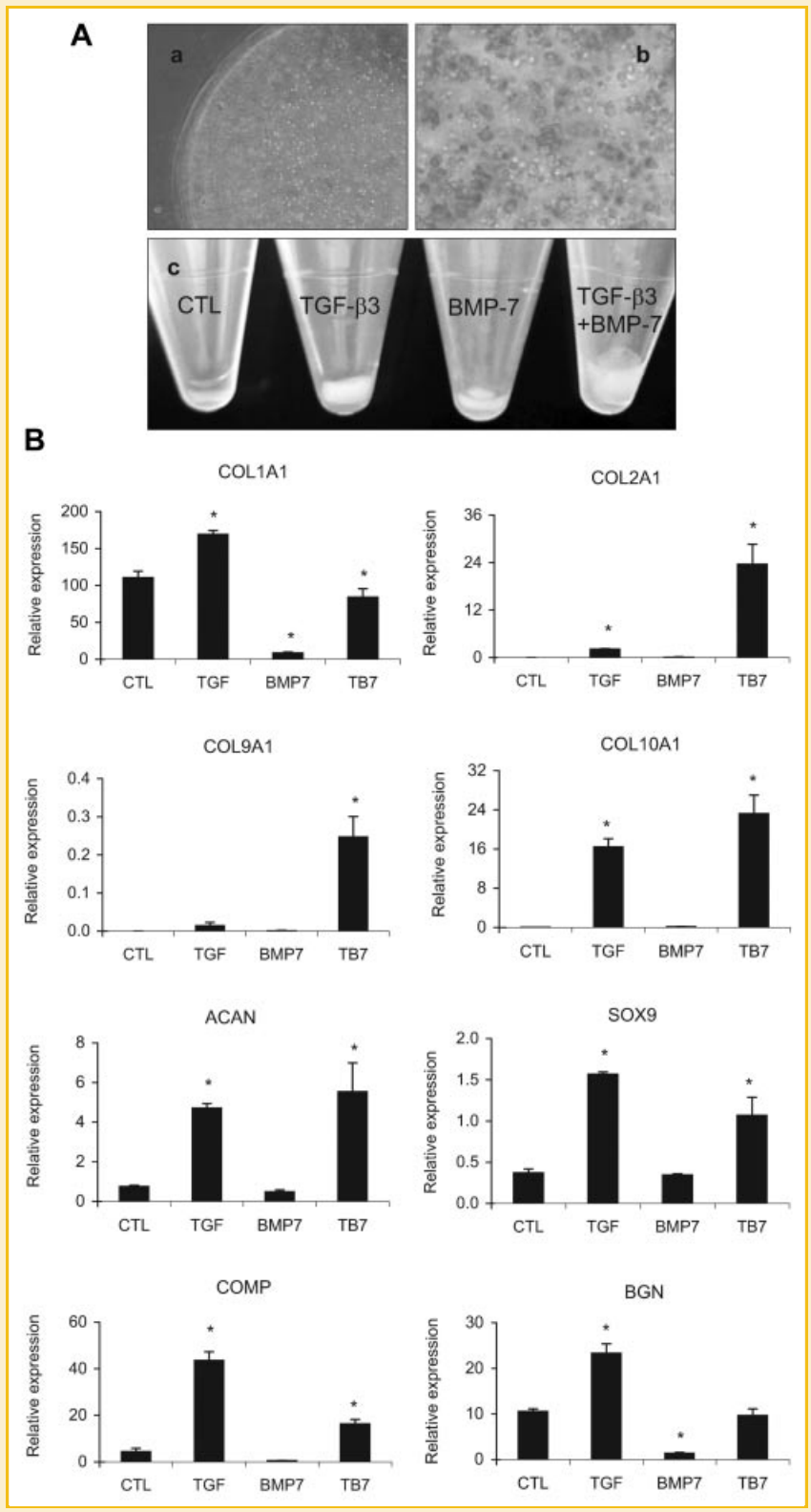

Fig. 2. The appearance of BM MSCs in alginate bead culture and their gene expression analysis. A: BM MSCs were cultured in alginate beads in either growth medium (CTL) or chondrogenic differentiation medium supplemented with TGF-β3 (TGF) alone, BMP-7 alone (BMP7), or TGF-β3 + BMP-7 (TB7) for 21 days (a,b). The cells were collected by centrifugation after liberation from beads at the termination of chondrogenic differentiation and pellet sizes were compared: TGF-β3 + BMP-7 > TGF-β3 > BMP-7 (c). B: The expression of chondrocytic genes was determined using real-time PCR analysis. Levels of gene expression shown were normalized to house-keeping genes *GAPDH* and *HPRT*. The data were derived from triplicate cultures.

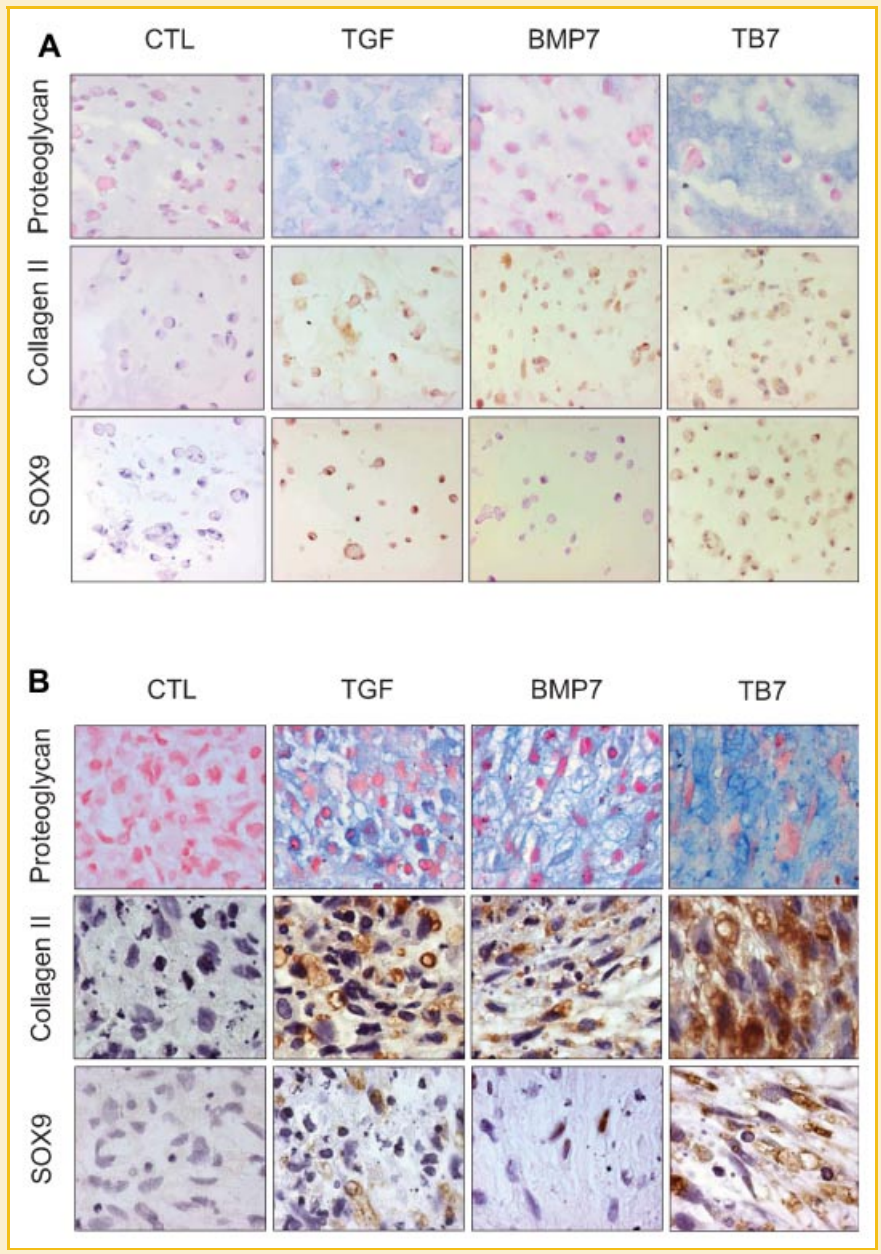

Fig. 3. Histological and immunohistochemical analysis of chondrogenic differentiated BM MSCs in alginate bead culture (A) and pellet culture (B). Alcian blue staining revealed that the proteoglycan-positive area (blue coloration) was increased in the extracellular matrix of MSC cultures containing TGF-β3 + BMP-7 (TB7) compared to that of other cultures. Increased levels of SOX9 and type II collagen expression in cultures containing TGF-β3 + BMP-7 (TB7) were detected by immunohistochemistry (original magnification 100×). Representative photos of two independent experiments.

BMP-7 divergently induced adipogenic differentiation in non-adipogenic inductive medium. The analysis of adipocyte specific gene expression and Oil red O staining were performed on osteogenic differentiated MSCs with or without BMP-7 stimulation at day 14.

The results showed that significant upregulation ($P < 0.05$) of peroxisome proliferator-activated receptor gamma (*PPARG*), adiponectin (*ADIPOQ*), and lipoprotein lipase (*LPL*) gene expression could be observed at higher levels in BMP-7-stimulated MSCs under osteogenic differentiation conditions (Fig. 7A). However, despite elevated mRNA levels detected by real-time PCR analysis, Oil red O staining indicated only very few positive cells displaying the lipid-rich adipogenic phenotype in the osteogenic differentiation culture at day 14 (Fig. 7B).

DISCUSSION

In the present study, we report that BMP-7 can promote both chondrogenic and osteogenic differentiation of BM MSCs in vitro when supplemented in commonly used induction media.

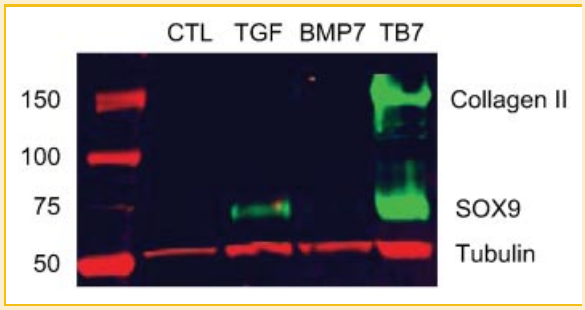

Fig. 4. Western blot analysis of chondrogenic differentiated BM MSCs. The differentiated MSCs were liberated from alginate beads and lysed using standard methods. Enhanced protein expression of type II collagen and SOX9 relative to reference tubulin is visible in the TGFβ3 + BMP-7-treated MSCs. A representative image of two independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Chondrogenic differentiation of BM MSCs can generally be induced by stimulation with all isoforms of TGF-β in serum-free medium at high cell density [Mackay et al., 1998; Pittenger et al., 1999]. However, previous reports have suggested that TGF-β alone may not be sufficient for chondrogenic induction, and that a combination of growth factors is necessary [Sekiya et al., 2001; Xu et al., 2006; Shen et al., 2008]. We present here that BMP-7, when supplemented in chondrogenic differentiation medium containing TGF-β3, enhanced the mRNA and/or protein expression of chondrogenic markers *COL2A1*, *COL9A1*, *COL10A1*, and *SOX9*, and increased extracellular matrix proteoglycan deposition. BMP-7 enhanced TGF-β3-mediated chondrogenesis but could not induce chondrogenic differentiation on its own. Interestingly, increased expression of chondrocytic-associated genes *COMP* and *BGN* mediated by TGF-β3 was downregulated by the addition of BMP-7, showing an apparent neutralization effect. The importance of this observation is unclear. The distinct levels of *COMP* expression in disc chondrocytic cells (NP cells) and in cartilage chondrocytes [Steck et al., 2005] might associate with differential BMP-7 regulation. In the disc, biglycan levels increase during aging, while collagen and proteoglycan contents decrease [Singh et al., 2009]. Further, biglycan stimulation can inhibit *ACAN* and *COL2A1* gene expression in human chondrocytes (Cs-Szabo et al., Presentation at 53rd Orthopaedic Research Society Annual Meeting 2007, Paper No. 176), suggesting that downregulation of *BGN* expression by BMP-7 could benefit the chondrogenic differentiation.

BMP-7 did not increase cell growth in high-density culture, yet the observed enlarged pellet size of liberated cells from alginate beads exhibited an obviously thicker extracellular matrix around TGF-β3 + BMP-7-treated MSCs, with less tightly packed cells in the pellet than other treatments. This observation was confirmed by histological staining. Our findings are consistent with Miyamoto's report that BMP-7 in combination with TGF-β1 enhances chondrogenesis of synovial mesenchymal stem cells [Miyamoto et al., 2007]. Additionally, a published in vivo study demonstrated the regenerative capacity of BMP-7 in injured disc cells [Masuda et al., 2006], potentially due to the anti-apoptotic [Wei et al., 2008] and anabolic activity [Stove et al., 2006] of BMP-7. We wonder whether BMP-7 could induce local stem/progenitor cell differentiation by co-ordinating with trophic factors in situ. If this is the case, the combination of TGF-β3 and BMP-7 for the treatment of degenerative disc disease might be better than BMP-7 alone based on the results in this study. The mechanism of BMP-7 enhancement for TGF-β3-mediated chondrogenic differentiation is not clear. It has been reported that the TGF-β type III receptor functions as a BMP cell surface receptor which binds to multiple members of the BMP subfamily including BMP-7 [Kirkbride et al., 2008], and TGF-β3 mediates the upregulation of the type I BMP receptor, ALK6 [Xu et al., 2006] through which BMP-7 and other BMPs are known to signal [Yamashita et al., 1996]. Taken together, the recent advance suggests that mutual regulation or receptor activation may amplify and enhance the effects of TGF-β and BMPs.

The general approach for in vitro osteogenic induction of BM MSCs relies on chemically formulated medium containing 10% FBS [Caterson et al., 2002]. In our study, supplementation of BMP-7 into standard osteogenic differentiation medium enhanced the gene expression of key osteogenic markers *SPP1*, *BGLAP*, and *SP7* and accelerated the process of matrix mineralization of BM MSCs. Previous reports have suggested that BMP-7 stimulates mouse BM MSCs [Gruber et al., 2000] or progenitor cell lines [Erlacher et al., 1998; Yeh et al., 2002, 2004; Shea et al., 2003] to differentiate along the osteogenic pathway, predominantly based on increase of ALP activity in basal culture media. As one step further, our data were derived under osteogenic inductive conditions. The osteogenic marker genes, *SPP1*, *BGLAP*, and *SP7*, were more specifically upregulated by BMP-7 than the transcription factor *RUNX2* and its activator *WWTR1*, both upregulated in osteogenic conditions but not further promoted with the addition of BMP-7 at day 14. Since the *RUNX2* expression is transient and an early event in osteogenesis, its enhancement by BMP-7 has been observed at day 7 (data not shown) instead of day 14 (Fig. 5). Interestingly, the most sensitively upregulated gene expression in response to BMP-7 stimulation was the transcription factor *SP7*, which was recently reported to promote osteogenic differentiation in vitro and in vivo using the gene overexpression approach in mouse models [Fu et al., 2007]. In contrast to its upstream gene *RUNX2*, *SP7* expression was largely and stably enhanced by BMP-7 supplementation up to 14 days, which suggests a potential mechanism of BMP-7 function in the osteogenic differentiation of BM MSCs. It remains elusive that *COL1A1* expression was downregulated under osteogenic conditions and *ALPL* expression was not enhanced by BMP-7 in this study, both genes that are commonly considered to be osteogenic marker genes. We have observed that BMP-7 stimulation could largely upregulate *ALPL* expression in growth medium, but such upregulation neither was proportional to other osteogenic gene expression nor led to mineralization of extracellular matrix (data not shown). Expression of *COL1A1* and *ALPL* may not be specific to osteogenesis, especially at an advanced differentiation stage when compared to markers such as *SPP1*, *BGLAP*, and *SP7*. Marker gene upregulation, only together with an osteoblastic phenotype, can delineate osteogenic differentiation.

The addition of BMP-7, apart from increasing cell surface ALP staining, dramatically accelerated the calcium mineralization of

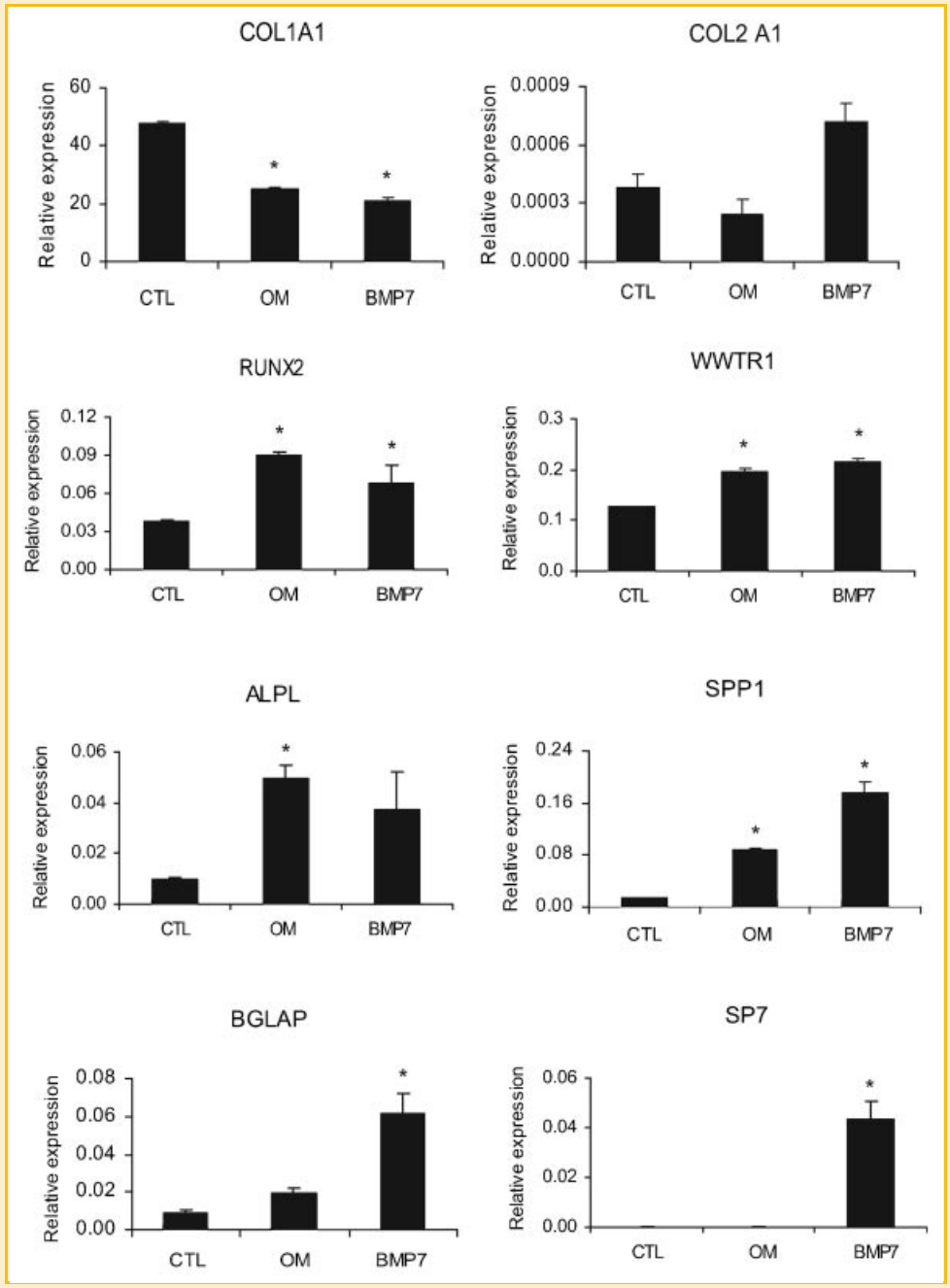

Fig. 5. Specific gene expression of osteogenic markers detected by real-time PCR. BM MSCs were cultured in 6-well plates in growth medium (control, CTL) or osteogenic differentiation medium with (BMP7) and without (OM) BMP-7 (100 ng/ml) for 14 days of differentiation. Levels of gene expression shown were normalized to house-keeping genes *GAPDH* and *HPRT*. Data shown were derived from duplicate cultures of differentiation.

differentiated MSCs in a dose-dependent manner, which is the relevant clinical response to BMP-7 administration. Our data suggest that 100 ng/ml of BMP-7 supplementation will accelerate in vitro osteogenic differentiation of BM MSCs by 50% of the standard time course, consistent with a recent report using mouse adipose-derived MSCs in osteogenic or growth medium stimulated with BMP-7 [Al-Salleeh et al., 2008]. BMP-7 has also been reported to enhance both osteogenic and chondrogenic differentiation of periosteum-derived mesenchymal cells [Gruber et al., 2001].

Our data imply that the combination of BMP-7 with other lineage-specific factors could significantly increase differentiation efficiency and shorten the process of in vitro manipulation prior to clinical application. However, the function of BMP-7 in MSC differentiation seems to be enhancement instead of lineage determination and therefore requires other lineage-specific factors to be present. This highlights the importance of culture conditions in studies. We also found that BMP-7 inhibited MSC proliferation at days 5 and 7 of stimulation in the growth medium, which is different

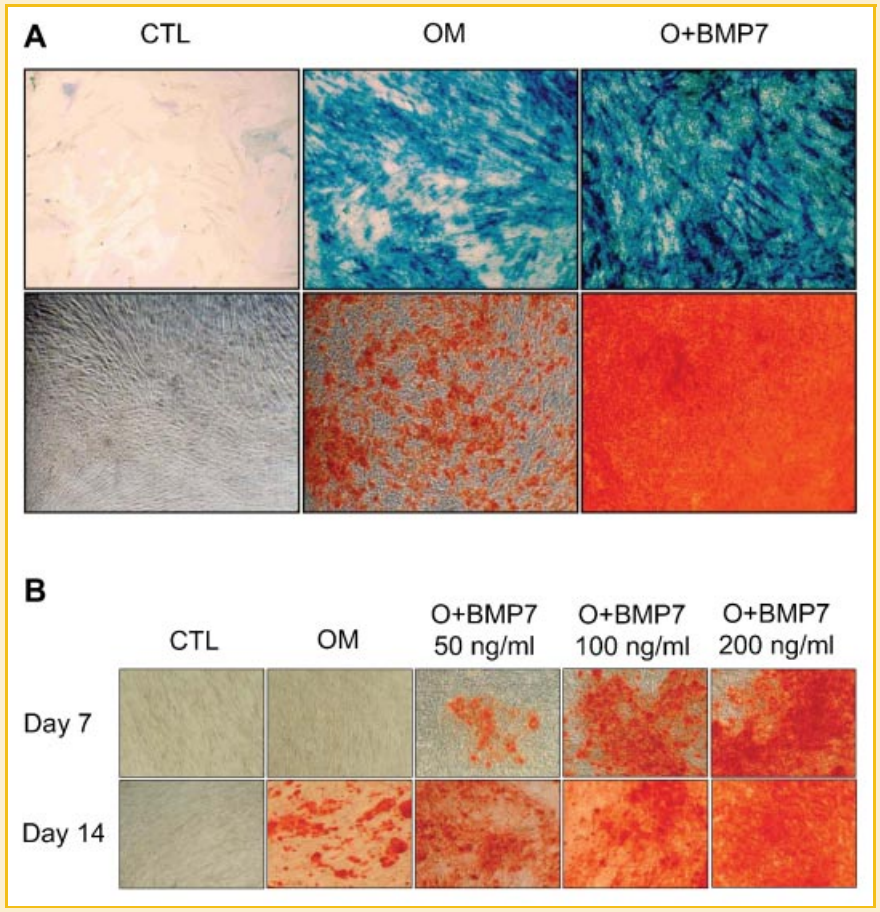

Fig. 6. Histological analysis of osteogenic differentiated BM MSCs. A: BM MSCs were cultured in 24-well plates in growth medium (control, CTL), osteogenic differentiation media (OM), or osteogenic media + BMP-7 (O + BMP7) at 100 ng/ml for 14 days. Osteogenic marker ALP was detected using Fast blue staining (top panel) and calcium mineralization was detected using Alizarin red S (bottom panel). B: BMP-7 supplementation at 50, 100, and 200 ng/ml in osteo-inductive medium for 7 (top panel) and 14 (bottom panel) days of differentiation showed dose-dependent acceleration of calcium mineralization. Representative photos of three independent experiments.

from previous reports where a mouse mesenchymal stem cell line [Shea et al., 2003] or calf periosteum cells [Gruber et al., 2001] were stimulated to proliferate, yet similar to a study with mouse bone marrow stromal cells [Gruber et al., 2000] which did not proliferate with BMP-7 stimulation in culture conditions that were different from ours. Clearly different cell types and culture conditions need to be taken into account when drawing conclusions as to the function of any growth factor in vitro.

Considering the cross talk of signaling pathways between adipogenic and osteogenic differentiation [Muruganandan et al., 2009] and the susceptibility of adipogenesis through BMP-7 stimulation [Chen et al., 2001; Neumann et al., 2007], we included examination of adipogenic phenotype development of MSCs under osteogenic differentiation conditions. In our study, we found that the expression of adipocyte specific genes *PPARG*, *ADIPOQ*, and *LPL* was elevated by BMP-7 stimulation in non-adipogenic culture conditions, but the amount of adipocytes detected with positively stained lipid droplets was negligible. BMP-7 reportedly induced mouse bone marrow stromal cell line BMS2 to differentiate into osteoblasts and adipocytes simultaneously under adipogenic conditions [Chen et al., 2001], and promoted the formation of adipocytes from BM MSCs in culture conditions favorable for chondrogenic differentiation, but lacking chondrogenic factor TGF-β [Neumann et al., 2007]. We report that osteogenic differentiation of BM MSCs was favored under the conditions used here, and that the level of adipogenic gene upregulation was insufficient to alter MSC phenotypes. Our data also suggest that without TGF-β inclusion in the chondrogenic conditions of Neumann et al. BMP-7 could not induce a chondrogenic phenotype. Instead, BMP-7 may have co-ordinated with insulin and dexamethasone in the medium (also active ingredients in adipo-indictive medium) to shift the balance toward adipogenesis. Our observations demonstrate that the cellular microenvironment is critical for the executive function of BMP-7 and imply that signaling pathways promoting one cell fate may exclude the full activation of other lineage determining signaling pathways. The regulatory control for lineage differentiation is more complex than initially anticipated.

In summary, our study provided important information for the potential use of BMP-7 to enhance in vitro induction of MSC differentiation into chondrocyte-like or osteoblast-like cells prior to

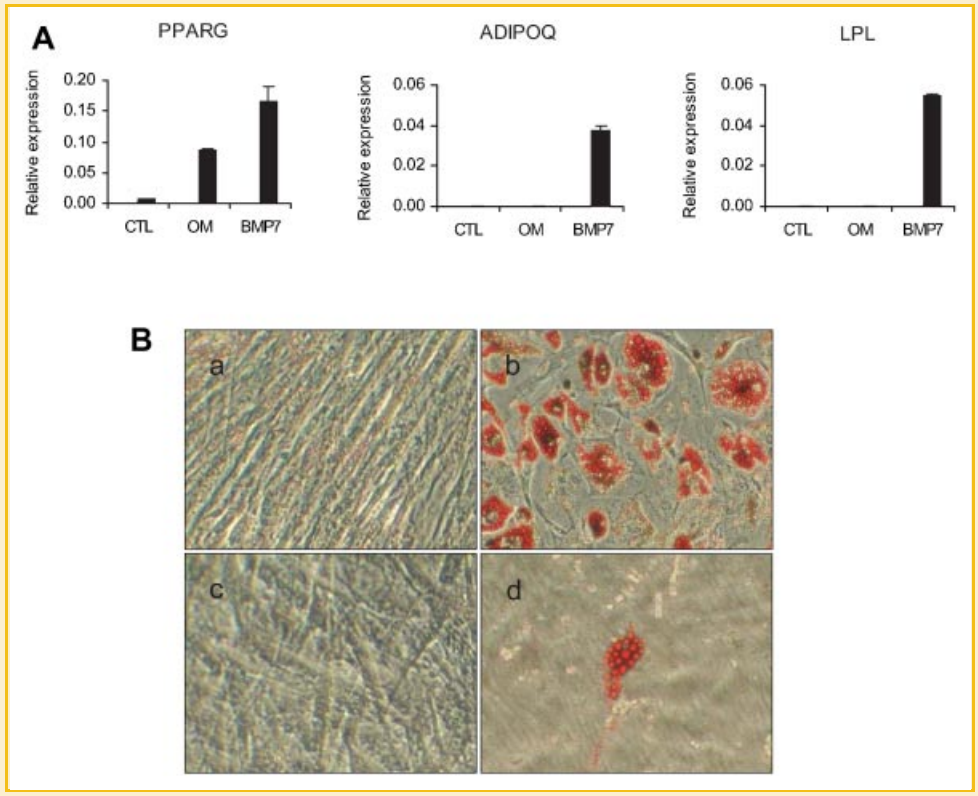

Fig. 7. Adipocyte specific gene expression analysis and adipocyte detection in osteogenic differentiated BM MSCs. A: After osteogenic differentiation for 14 days, the gene expression of adipogenic markers *PPARG*, *ADIPOQ*, and *LPL* was analyzed using real-time PCR. B: BM MSCs cultured in growth medium (negative control, a), adipogenic differentiation medium (positive control, b), osteogenic differentiation medium (c), or osteogenic medium supplemented with 100 ng/ml of BMP-7 (d) for 14 days were stained specifically for adipocytes containing lipid droplets using Oil red O. Representative results of two independent experiments.

MSC-based tissue repair. However, BMP-7 alone is insufficient to direct the lineage commitment and other lineage determinants are required to co-ordinate with it.

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