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BMP7 Promotes Adipogenic but Not Osteo-/Chondrogenic Differentiation of Adult Human Bone Marrow-Derived Stem Cells in High-Density Micro-Mass Culture

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Abstract The objective of our study was to elucidate the potential of bone morphogenetic protein-7 (BMP7) to initiate distinct mesenchymal lineage development of human adult mesenchymal stem cells (MSC) in three-dimensional micro-mass culture. Expanded MSC were cultured in high-density micro-masses under serum-free conditions that favor chondrogenic differentiation and were stimulated with 50–200 ng/ml BMP7 or 10 ng/ml transforming growth factor-β3 (TGFβ3) as control. Histological staining of proteoglycan with alcian blue, mineralized matrix according to von Kossa, and lipids with Oil Red O, immunostaining of type II collagen as well as real-time gene expression analysis of typical chondrogenic, adipogenic, and osteogenic marker genes showed that BMP7 promoted adipogenic differentiation of MSC. Micro-masses stimulated with BMP7 developed adipocytic cells filled with lipid droplets and showed an enhanced expression of the adipocyte marker genes fatty acid binding protein 4 (FABP4) and the adipose most abundant transcript 1 (apM1). Development along the chondrogenic lineage or stimulation of osteogenic differentiation were not evident upon stimulation with BMP7 in different concentrations. In contrast, TGFβ3 directed MSC to form a cartilaginous matrix that is rich in proteoglycan and type II collagen. Gene expression analysis of typical chondrocyte marker genes like cartilage oligomeric matrix protein (COMP), link protein, aggrecan, and types IIα1 and IXα3 collagen confirmed chondrogenic differentiation of MSC treated with TGFβ3. These results suggest that BMP7 promotes the adipogenic and not the osteogenic or chondrogenic lineage development of human stem cells when assembled three-dimensionally in micro-masses. J. Cell. Biochem. 102: 626–637, 2007. © 2007 Wiley-Liss, Inc.

Key words: mesenchymal stem cells; bone morphogenetic protein; adipogenesis; high-density culture; gene expression analysis

Bone marrow-derived stem cells (MSC) are considered as attractive progenitors for the regeneration of mesenchymal tissues using tissue engineering approaches [Ringe et al., 2002a,b; Barry and Murphy, 2004; Jorgensen et al., 2004]. Human adult MSC can be isolated and grown in vitro, remain undifferentiated when expanded extensively and may gradually loose their proliferation and differentiation capacity with an increasing number of cell passages [Mauney et al., 2005]. MSC have been shown to develop along various mesenchymal

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lineages including differentiation into bone, cartilage, and fat cells [Pittenger et al., 1999]. Especially, much attention has been drawn to the potential of MSC to regenerate bone and cartilage. Meanwhile, a few clinical studies have been reported using in vitro propagated bone marrow-derived MSC for the restoration of articular cartilage surfaces in osteoarthritic lesions [Wakitani et al., 2002] and for the regeneration of bone in maxillary sinus floor augmentation [Ueda et al., 2005].

Bone morphogenetic proteins (BMP) are members of the transforming growth factor (TGF)-β superfamily of growth and differentiation factors (GDFs) and have been shown to be important regulators of mesenchymal lineage development [Hogan, 1996; Tsumaki and Yoshikawa, 2005]. GDF5, GDF6, and GDF7 induce neotendon and ligament formation after subcutaneous or intramuscular transplantation in rats [Wolfman et al., 1997], BMP2 accelerates bone formation of bone marrow stromal cells in a murine calvarian defect model [Cowan et al., 2005], BMP2, 4, and 6 induce chondrogenesis of bone marrow-derived mesenchymal stem cells (MSCs) in vitro [Sekiya et al., 2005], and BMP2/4 recruit bone marrow-derived mesenchymal progenitors in an in vitro chemotaxis test system [Fiedler et al., 2002]. Amongst others, BMP7 is involved in the development of skeletal elements, kidney, eyes, and the heart [Jena et al., 1997; Kim et al., 2001]. BMP7 induces the transcription factor runt-related transcription factor 2 (Runx2)/*core-binding factor α1* (CBFA1) that is important for osteogenic differentiation of mesenchymal progenitors [Tou et al., 2003] and forms trabecular bone when expressed recombinantly by adenoviruses in bone-derived collagen carriers and implanted intramuscularly [Franceschi et al., 2000]. In chondrocytes, BMP7 promotes the re-differentiation of expanded chondrocytes in vitro and after subcutaneous transplantation of three-dimensional cultures into nude mice [Kaps et al., 2002]. As shown by antisense treatment of cartilage explants, loss of BMP7 results in a reduction of cartilage aggrecan and may disturb the normal homeostasis of articular cartilage [Soder et al., 2005].

In the present study, we analyzed the potential of BMP7 to initiate distinct mesenchymal lineage development of human bone marrow-derived MSCs in serum-free high-density micro-mass culture. BMP7 stimulated adipogenic differentiation of expanded adult MSC using a differentiation system that favors chondrogenic development. BMP7 failed stimulating MSC to undergo osteogenic and/or chondrogenic lineage development. In contrast, MSC cultured in the presence of TGFβ3, a potent chondrogenic inducer, formed a chondrocytic extracellular matrix and induced typical chondrocyte marker genes, while adipogenic and osteogenic marker genes were repressed. These results suggest that BMP7 promotes adipogenic differentiation of MSC when cultured three-dimensionally and may a potential candidate for rather adipose than cartilage tissue engineering based on mesenchymal progenitors.

METHODS

Isolation, Culture, and Differentiation of MSC

Human adult MSCs were isolated from iliac crest bone marrow aspirates of healthy donors (n = 3, age 49–77) as described previously [Haynesworth et al., 1992]. The bone marrow aspirates were derived from donors who were examined to exclude hematopoietic neoplasmas. The bone marrow samples were histologically diagnosed as normal. In brief, bone marrow aspirates (3–4 ml per sample) were washed with phosphate buffered saline (PBS, Biochrom, Berlin, Germany) and resuspended in complete Dulbeccos's modified eagle (DME)-medium (Biochrom) containing 2 ng/ml basic fibroblast growth factor (bFGF, Tebu, Offenbach, Germany) and 10% fetal bovine serum (FBS, Perbio, Bonn, Germany). Cells were purified using a percoll gradient of a density of 1.073 g/ml and resuspended in complete DME-medium. Cells were plated at a density of 300,000 cells/cm^2; medium was exchanged after 72 h and then every 2–3 days thereafter. Reaching 90% confluence, cells were detached by the addition of a solution containing 0.5% trypsin-ethylenediaminetetraacetic acid (EDTA; Biochrom) and replated at a density of 5,000 cells/cm^2.

High-density cultures of MSC (passage 3) were generated as described previously [Johnstone et al., 1998]. In brief, MSC of the individual donors (n = 3) were mixed in equal amounts and 250,000 MSC were centrifuged to form a pelleted high-density culture. Differentiation was induced for up to 4 weeks with DME-medium supplemented with

Insulin-Transferrin-Selenium (ITS + 1, Sigma, Taufkirchen, Germany), 0.1 μM dexamethasone (Sigma), 1 mM sodium pyruvate (Sigma), 0.17 mM ascorbic acid-2-phosphate (Sigma), 0.35 mM proline (Sigma), and 10 ng/ml TGFβ3 (R&D Systems, Wiesbaden, Germany), or 50–200 ng/ml BMP7 (R&D Systems). DME-medium supplemented with ITS + 1, dexamethasone, sodium pyruvate, ascorbic acid, and proline served as control. The medium was exchanged every other day. The study was approved by the ethical committee of the Charité Universitätsmedizin Berlin.

Polymerase Chain Reaction (PCR)

Total RNA from 25 pellets per individual experiment per point in time was isolated as described previously [Chomczynski, 1993]. Subsequently, total RNA (3 μg) was reverse transcribed with the iScript cDNA Synthesis Kit according to the manufacturer's recommendations (BioRad, München, Germany). The relative expression level of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used to normalize marker gene expression in each sample in different concentrations [Gorzelniak et al., 2001]. Real-time PCR using the i-Cycler PCR System (BioRad) was performed with 1 μl of cDNA sample using the SYBR Green PCR Core Kit (Applied Biosystems, Karlsruhe, Germany). Relative quantitation of marker gene expression (Table I) was performed and is given as percentage of the GAPDH product. For statistical evaluation, the *t*-test was applied.

Histological Methods and Immunohistochemistry

For histological and immunohistochemical analyses, high-density cultures (n = 3 per point in time and per individual experiment) were embedded in OCT compound, frozen, and cryosections (6 μm) were prepared. Proteoglycans were visualized by staining with Alcian Blue 8GS (Roth, Karlsruhe, Germany) at pH 2.5. Adipocytes filled with lipid droplets were identified by staining with Oil Red O (Sigma) after fixation with formaldehyde for 45 min at room temperature. Mineralization of the extracellular matrix was analyzed by von Kossa staining. For histological analysis, nuclei were either counterstained with Nuclear Fast Red (SIGMA) or hematoxylin.

For immunohistochemical analysis of type II collagens, cryosections (6 μm) were incubated for 1 h with primary antibodies (rabbit anti-human type II collagen, Acris). In parallel, sections were incubated for 1 h with rabbit IgG (DAKO, Hamburg, Germany) as controls. Subsequently, sections were processed using the EnVision System Peroxidase Kit (DAKO) according to the manufacturer's instructions, followed by counterstaining with hematoxylin (Merck, Darmstadt, Germany). Sections stained with rabbit IgG showed no color reaction and

TABLE I. Chondrogenic, Adipogenic, and Osteogenic Marker Genes

Gene name	Gene symbol	Accession number	Oligonucleotides (5′ → 3′) (up/down)	Product size (bp[a])
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	NM_002046	GGC GAT GCT GGC GCT GAG TAC TGG TCC ACA CCC ATG ACG A	149
Type IIα1 collagen	COL2A1	NM_001844	CCG GGC AGA GGG CAA TAG CAG GTT CAA TGA TGG GGA GGC GTG AG	128
Type IXα3 collagen	COL9A3	NM_001853	AAT CAG GCT CTC GAA GCT CAT AAA A CCT GCC ACA CCC CCG CTC CTT CAT	100
Cartilage oligomeric matrix protein	COMP	NM_000095	GGG TGG CCG CCT GGG GGT CTT CTT GCC GCA GCT GAT GGG TCT C	116
Cartilage link protein	CRTL	NM_001884	GCG TCC GCT ACC CCA TCT CTA GCG CTC TAA GGG CAC ATT CAG TT	145
Aggrecan	AGC1	NM_013227	CCA GTG CAC AGA GGG GTT TG TCC GAG GGT GCC GTG AG	146
Peroxisome proliferative activated receptor gamma	PPARG	NM_138712	GCC TTG CAG TGG GGA TGT CTG CCT CGC CTT TGC TTT GGT CAG	194
Adipose most abundant gene transcript 1	APM1	NM_004797	GGC CTG CCC AGC TCT CGT AT CTC TCC TCT TTG GGC ATC ACC	83
Fatty acid binding protein 4, adipocyte	FABP4	NM_001442	CCT TAG ATG GGG GTG TCC TGG TA AAT GTC CCT TGG CTT ATG CTC TC	156
Core-binding factor α1	CBFA1	NM_004348	AGA ACC CAC GGC CCT CCC TGA ACT GTG GAA GAC AGC GGG GTG GTA GAG	181
Osteocalcin	BGLAP	NM_199173	GAG CCC CAG TTC CCC TAC CC GCC TCC TGA AAG CCG ATG TG	103

[a]bp = base pairs.

documented specificity of the type II collagen antibodies and the peroxidase detection system.

Image Analysis

Image analysis of Oil Red O stained sections of high-density micro-masses was performed using the Photoshop software as described previously [Kaps et al., 2002]. Briefly, a standard was defined that represents the particular color of the specific staining. The tools 'magic wand' and 'select similar' were used to select areas of that particular color. The amount of stained pixels in relation to the total amount of pixels of the micro-mass section gives the percentage of the Oil Red O stained area. Image analysis was performed with 2–3 independent micro-masses for each individual experiment. More precisely, one representative section obtained from the center of each individual micro-mass was analyzed and the *t*-test was applied.

RESULTS

Histological Analysis of Bone Morphogenetic Protein-Mediated Differentiation of MSC in High-Density Micro-Masses

Human bone marrow-derived MSC were analyzed routinely for the presence of typical MSC-related cell surface antigens by flow cytometric analysis. Cells showed the typical antigen profile of MSC and were positive for CD166, CD105, CD90, and CD73. MSC were negative for the antigens CD45, CD34, and CD14.

Propagated bone marrow-derived MSC (passage 3) were stimulated for up to 4 weeks in high-density micro-mass cultures with TGFβ3 or BMP7. Differentiation along the chondrogenic and adipogenic lineage was characterized histologically and immunohistochemically by staining of proteoglycan with alcian blue, detection of type II collagen, and staining of lipids with Oil Red O (Fig. 1). After 4 weeks, MSC treated with TGFβ3 showed the typical chondrogenic lineage development with deposition of proteoglycan (Fig. 1A) and type II collagen (Fig. 1B). Adipogenic differentiation or cells filled with lipids were not evident (Fig. 1C). MSC exposed to increasing concentrations of BMP7 exhibited compact micro-masses, albeit to a lesser extent than MSC treated with TGFβ3, and showed weak staining of proteoglycan (Fig. 1D, 1G, 1J). Cartilage-specific type II collagen was virtually absent in MSC treated with BMP7 (Fig. 1E, 1 H, 1K). Addition of BMP7 gave rise to the development of adipogenic cells that were mainly located at the border zone of the high-density cultures and were filled with lipids stained by Oil Red O. Lipid-filled cells were evident after stimulation with rising concentrations of BMP7 from 50 ng/ml (Fig. 1F), 100 ng/ml (Fig. 1I) to 200 ng/ml (Fig. 1L). Control MSC micro-masses, not exposed to TGFβ3 or BMP7, were small in size, appeared fibrous and showed only a faint staining of proteoglycan (Fig. 1M). Type II collagen (Fig. 1N) and cells filled with lipid droplets (Fig. 1O) were not detected in untreated control MSC.

High power magnification images of MSC (Fig. 2) documented the development of adipogenic cells filled with lipid droplets in the outer layers of the high-density micro-mass after prolonged culture in the presence 50 (Fig. 2A), 100 (Fig. 2B), and 200 ng/ml (Fig. 2C) BMP7. In contrast, control MSC cultured in the absence of BMP7 or TGFβ3 showed no adipogenic cells but few diffuse droplets stained by Oil Red O (Fig. 2D).

Quantification of adipogenic differentiation by computer-assisted image analysis of histological specimens stained with Oil Red O (Fig. 3) showed that adipogenesis was negligibly low in control MSC and MSC stimulated with TGFβ3. In contrast, MSC stimulated with low or high doses of BMP7 showed significantly ($P < 0.05$) higher amounts of lipids than untreated controls or MSC stimulated with TGFβ3. Since adipogenic development was achieved with 50 ng/ml BMP7 and was not significantly enhanced by higher amounts of BMP7 (100 or 200 ng/ml), further analysis was performed with MSC stimulated with 50 ng/ml BMP7.

Adipogenic Development of MSC Upon Stimulation With BMP7 in High-Density Culture

The time-dependent adipogenic developmental behavior of MSC in high-density was analyzed after stimulation of micro-mass cultures with 50 ng/ml BMP7 by Oil Red O staining (Fig. 4). After 7 days, MSC cultured in the presence of BMP7 developed a compact micro-mass with only few cells filled with lipids. Oil Red O positive cells were located exclusively in outer layers of the high-density culture (Fig. 4A, black triangles). At day 14 (Fig. 4B), the amount of cells filled with lipid droplets increased and

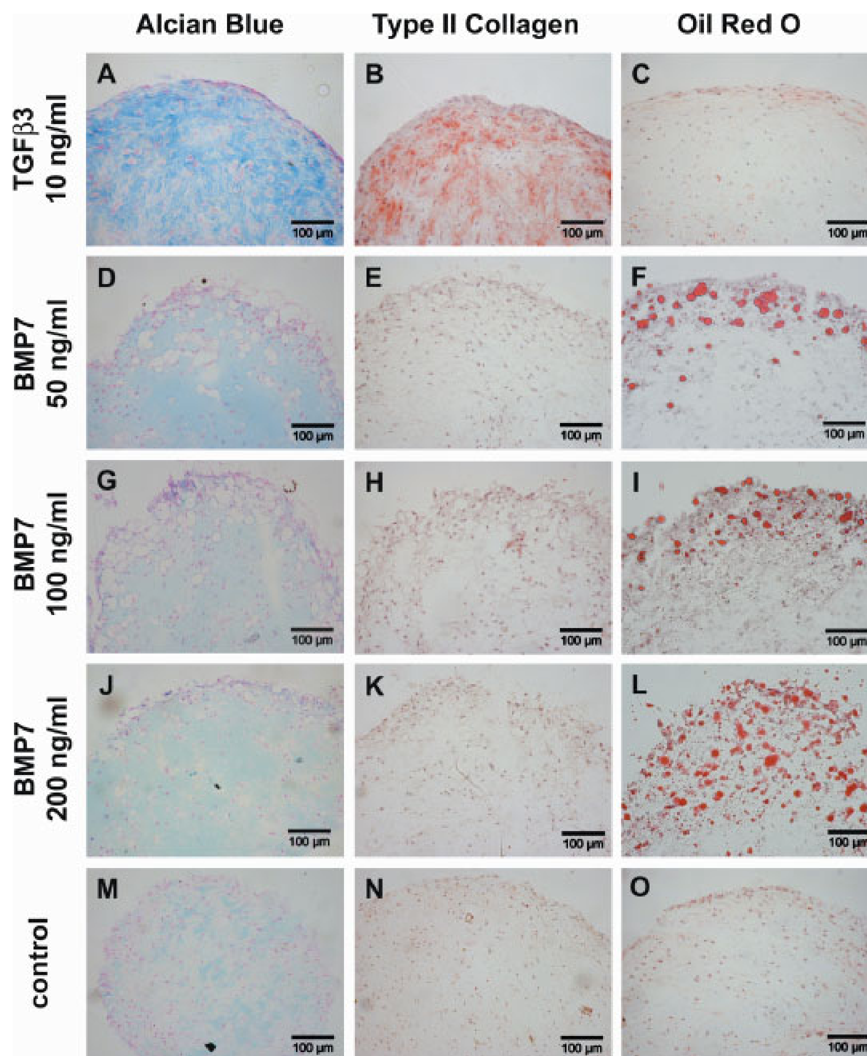

Fig. 1. Histochemical analysis of MSC undergoing mesenchymal lineage development upon stimulation with TGFβ3 or BMP7 in high-density micro-masses. At day 28, stimulation of MSC with 10 ng/ml TGFβ3 resulted in the deposition of proteoglycan stained by alcian blue (**A**) and cartilage-related type II collagen (**B**). Cells filled with lipid droplets stained by Oil Red O were not evident (**C**). MSC cultured in the presence of 50, 100, or 200 ng/ml BMP7 showed weak staining of proteoglycan (**D**, **G**, **J**), type II collagen immunostaining was negative (**E**, **H**, **K**) and Oil Red O stained adipocytes filled with lipid droplets were present in outer layers of micro-masses treated with 50 (**F**), 100 (**I**), and 200 (**L**) ng/ml BMP7. Control MSC not treated with growth factors developed small micro-masses that were characterized by faint staining of proteoglycan (**M**) and absence of type II collagen (**N**). Adipocytic cells were not evident in human control MSC cultured in micro-masses in the absence of TGFβ3 and BMP7 (**O**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

were also found scattered in deeper layers of the micro-mass (Fig. 4B, black triangles). At day 21 (Fig. 4C) and day 28 (Fig. 4D), adipogenic MSC as well as the volume of lipids stored by adipogenic cells increased and still showed a dominant staining in outer layers with only few cells located in the central part of the high-density culture.

To verify the potential of BMP7 to mediate osteogenic development, high-density cultures of MSC induced with TGFβ3 or BMP7 were analyzed for the presence of mineralized matrix molecules (Fig. 5). Von Kossa staining demonstrated that TGFβ3-mediated chondrogenesis (Fig. 5A) and BMP7-mediated adipogenesis (Fig. 5B/C) were not accompanied by the formation of a bone-related mineralized extracellular matrix. Control MSC micro-mass cultures were also negative for mineralized matrix components stained by von Kossa (Fig. 5D).

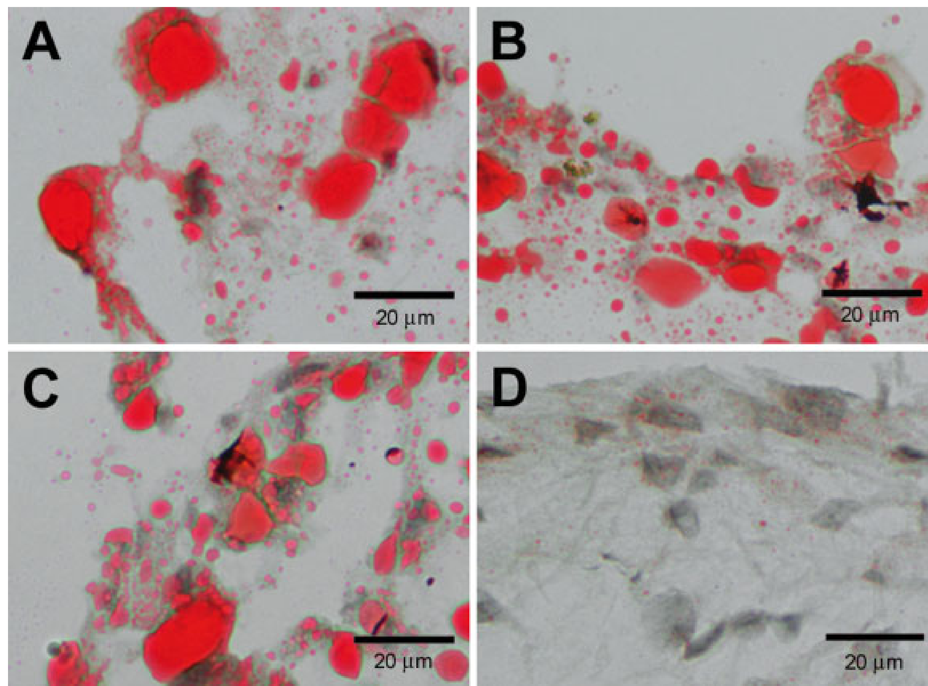

Fig. 2. Oil Red O staining of MSC treated with BMP7. At day 28, high magnification images of MSC treated with 50 (**A**), 100 (**B**), and 200 ng/ml BMP7 (**C**) showed single adipocytic cells filled with lipid droplets in the outer layers of the micro-masses. Control MSC cultured in the absence of BMP7 (**D**) were negative showing diffuse spots stained by Oil Red O. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Semi-Quantitative Real-Time Gene Expression Analysis of MSC Micro-Masses Stimulated With TGFβ3 and BMP7

The potential of bone marrow-derived MSC to undergo adipogenic differentiation upon stimulation with BMP7 as well as TGFβ3-mediated chondrogenesis was further documented by comparative real-time gene expression analyses of distinct adipogenic, osteogenic, and chondrogenic marker genes (Fig. 6). Compared to untreated controls, stimulation of MSC in high-density cultures with TGFβ3 for up to 14 days induced the expression of typical chondrogenic

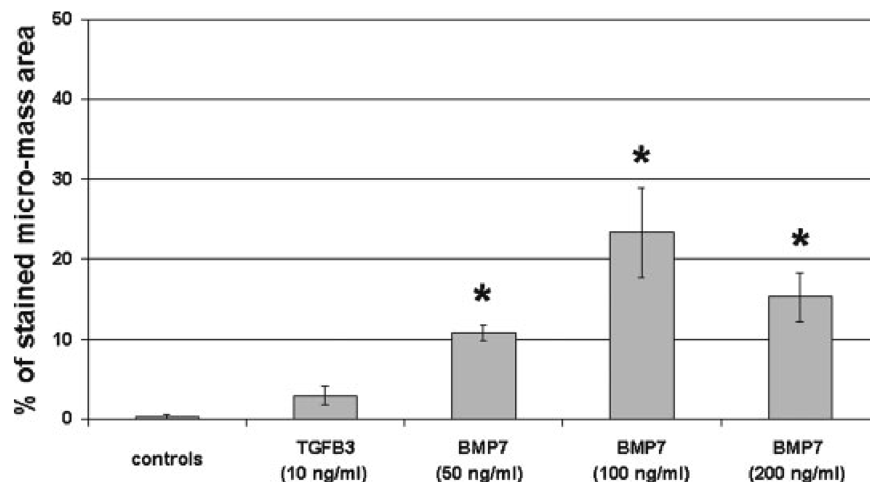

Fig. 3. Image analysis of histological specimen stained with Oil Red O. At day 28, in untreated control MSC Oil Red O positive cells were virtually absent. MSC stimulated with TGFβ3 showed a weak staining of adipocytes. Stimulation with rising amounts of BMP7 induced adipogenic differentiation and showed significantly enhanced ($P < 0.05$, asterisks) amounts of lipids compared to untreated controls and MSC stimulated with TGFβ3. The amount of lipids was not significantly increased in MSC treated with 100 or 200 ng/ml BMP7 compared to MSC stimulated with 50 ng/ml BMP7.

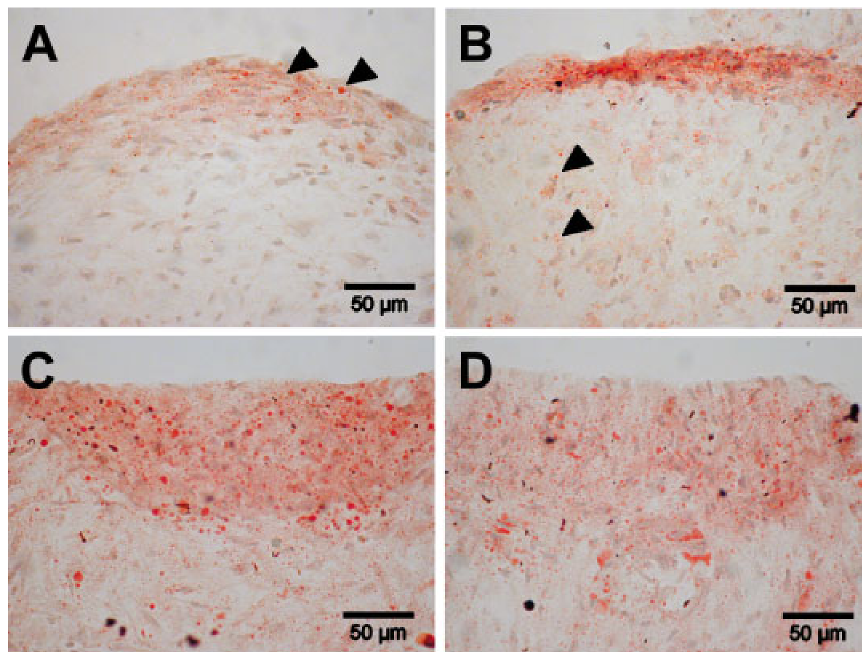

Fig. 4. Histological analysis of BMP7-mediated adipogenesis of MSC micro-masses. At day 7 (**A**), MSC cultured in the presence of 50 ng/ml BMP7 developed a compact pellet structure with few cells in the outer cell layers stained positive with Oil Red O (A, black triangles). At day 14 (**B**), the amount of adipocytes increased and lipid-filled cells were also sporadically evident in deeper layers of the micro-masses (B, black triangles). At day 21 (**C**) and day 28 (**D**), Oil Red O staining documented the prominent development of adipocytes in outer layers of the high-density culture. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

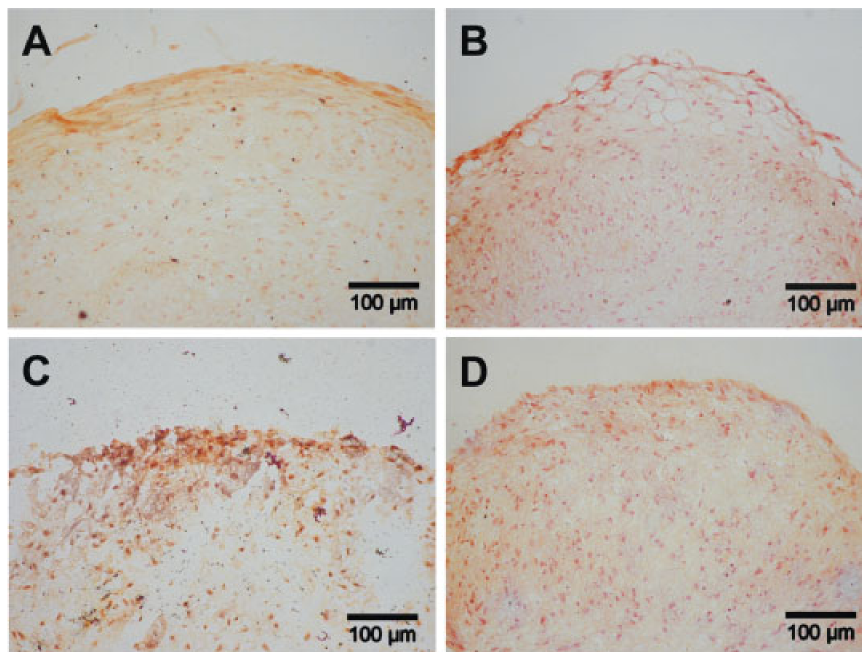

Fig. 5. Von Kossa staining of MSC high-density cultures stimulated with TGFβ3 and BMP7. By day 28, von Kossa staining of mineralized extracellular matrix was negative in MSC cultured with 10 ng/ml TGFß3 (**A**), with 50 ng/ml BMP7 (**B**), with 200 ng/ml BMP7 (**C**), and untreated control MSC (**D**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

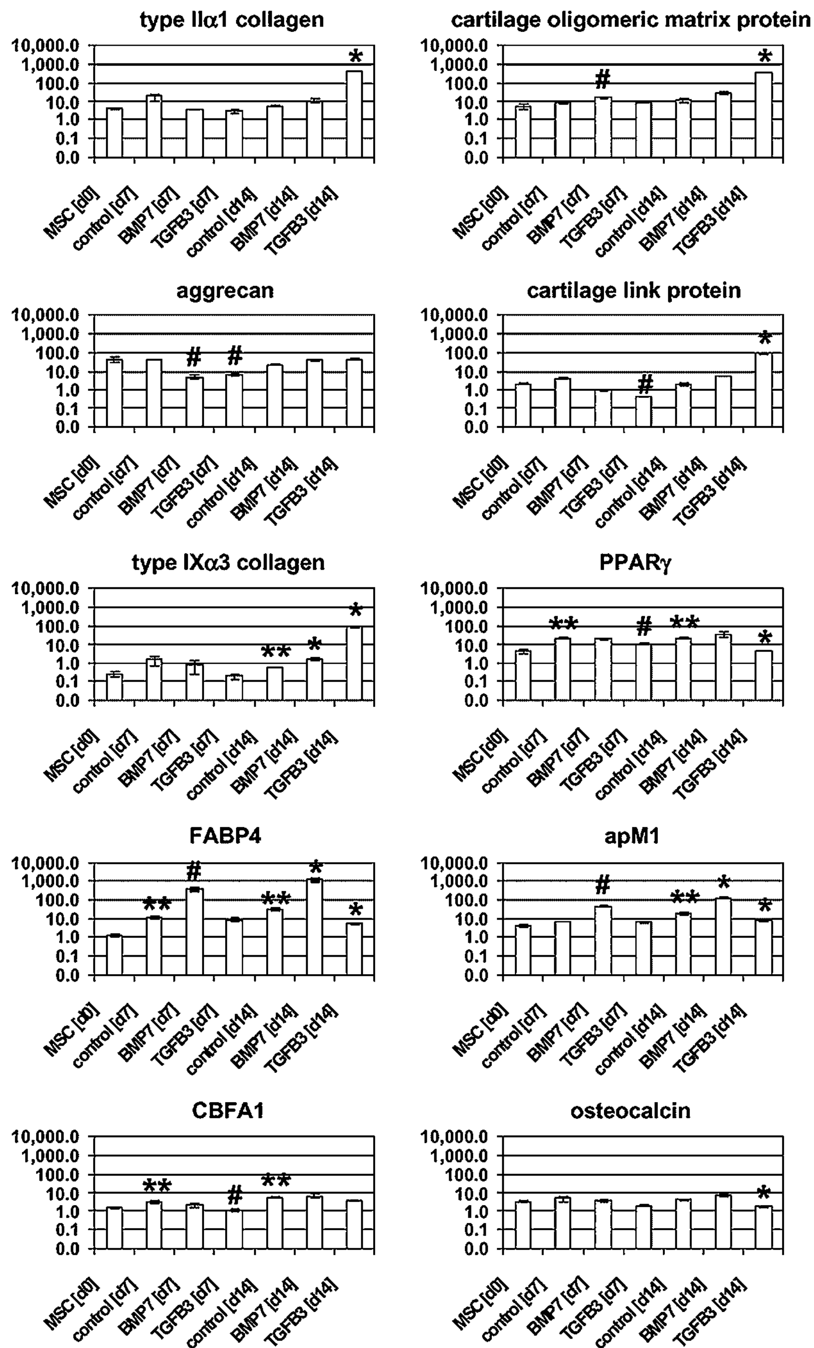

Fig. 6. Semi-quantitative real-time gene expression analysis of chondrogenic, adipogenic, and osteogenic marker genes in human MSC micro-mass cultures. Chondrogenic development of MSC was analyzed by gene expression analysis of the typical chondrocytic marker genes *type IIα1 collagen, COMP, aggrecan, cartilage link protein* and *type IXα3 collagen*. Adipogenic differentiation was assessed by gene expression analysis of *PPARγ*, *FABP4*, and the *apM1*. Osteogenic differentiation was evaluated by the expression of the *CBFA1* and *osteocalcin*. The expression of marker genes was calculated as the percentage of the expression of the housekeeping gene *GAPDH*. The mean of each triplicate well is plotted and the error bars represent SD. Significant ($P < 0.005$) differential expression of marker genes in treated MSC compared to untreated controls (>1.5-fold) is indicated by "#" for day 7 and "*" for day 14. Differential expression of genes in untreated controls (day 7 or day 14) compared to undifferentiated MSC (>1.5-fold) at day 0 is indicated by "**" with $P < 0.005$.

marker genes like *collagen types IIα1* and *IXα3*, *cartilage oligomeric matrix protein* (*COMP*), and *cartilage link protein*. The adipogenic marker genes *peroxisome proliferative activated receptor γ* (*PPARγ*), the *adipose most abundant gene transcript 1* (*apM1*), and the *adipocyte-specific fatty acid binding protein 4* (*FABP4*) were repressed upon application of TGFβ3 compared to controls and MSC stimulated with BMP7. Osteogenic marker genes like *CBFA1* showed a reduced expression at day 7 and *osteocalcin* showed negligible regulation in MSC cultured in the presence of TGFβ3.

By day 14, chondrogenic marker genes, except type IXα3 collagen, were not regulated BMP7-dependently and showed comparable expression levels in MSC treated with 50 ng/ml BMP7 and controls not stimulated with any of the growth factors. Stimulation of MSC with BMP7 resulted in an increased expression of the adipogenic markers *FABP4* and *apM1* as early as on day 7. The expression of *PPARγ* remained stable and showed an expression level as high as in untreated controls. The expression levels of the osteogenic markers *CBFA1* and *osteocalcin* in BMP7 treated MSC was equivalent to their levels in untreated controls. At day 7 and/or day 14, control MSC showed elevated levels of the adipogenic markers genes *PPARγ, FABP4*, and *apM1* as well as the osteogenic marker gene *osteocalcin* compared to control MSC at day 0.

DISCUSSION

In the present study, the cellular in vitro differentiation model of mesenchymal progenitors cultured in micro-masses was utilized and demonstrated the adipogenic potential of BMP7 on human MSC micro-masses derived from bone marrow. Adipogenesis was verified by histological staining and gene expression analysis with emphasis on adipocyte-related genes. Histological staining of mineralized bone matrix, immunostaining of type II collagen, typical for chondrocytes, and gene expression analysis of osteogenic and chondrogenic marker genes documented that BMP7 stimulated adipogenic lineage development and not chondrogenic and/or osteogenic differentiation of human MSC cultured in three-dimensional micro-masses. In contrast to BMP7, TGFβ3 induced the chondrogenic and repressed the adipogenic developmental sequence of human bone marrow-derived MSC as shown by histological staining, immunohistochemistry, and gene expression analysis of typical chondrogenic and adipogenic marker genes.

MSCs have been shown to develop into distinct mesenchymal tissues including bone, cartilage, and fat in different species [Pittenger et al., 1999; Ringe et al., 2002a,b; Sekiya et al., 2004]. Especially in the field of tissue engineering and regenerative medicine, much attention has been drawn to the chondrogenic developmental capacity of MSC. Chondrogenic lineage development of human MSC have been shown by culturing MSC three-dimensionally and by initiating chondrogenesis with all isoforms of TGF [Johnstone et al., 1998; Barry et al., 2001; Sekiya et al., 2002] and selected BMPs including BMP2 [Schmitt et al., 2003], BMP6 [Sekiya et al., 2001], and BMP9 [Majumdar et al., 2001].

As shown here, TGFβ3 induced the chondrogenic development of human MSC and repressed genes related to osteogenic and/or adipogenic differentiation. This is consistent with the inhibition of adipocyte development in pre-adipocyte BMS2 cells during BMP2-mediated osteogenesis [Gimble et al., 1995] and during TGFβ1-mediated chondrogenesis of human bone marrow-derived MSC [Zhou et al., 2004]. Usually, adipogenic differentiation of human mesenchymal stem and precursor cells is achieved in monolayer cultures by stimulating the cells with a mixture of insulin, dexamethasone, indomethacin, and methylxanthine [Pittenger et al., 1999; Janderova et al., 2003]. Interestingly, using cell propagation methods [Solchaga et al., 2005] and high-density micro-mass culture conditions, which favor chondrogenic development in a serum-free environment, BMP7 stimulated the adipogenic differentiation of human MSC. BMP7-dependent stimulation of the chondro- and/or osteogenic differentiation of MSC in micro-masses could neither be documented by histological staining nor gene expression analysis of typical osteo-/chondrogenic marker genes.

The role of BMP7 in the initiation or promotion of distinct mesenchymal lineage development in mesenchymal progenitors is still unclear. BMP7 has been shown to be a potent inducer of osteogenic development of mesenchymal progenitors, induced the osteogenic differentiation of primary murine multipotent muscle satellite cells [Asakura et al., 2001], and caused a strong osteogenic induction, albeit to a lesser extent than BMP6, in human bone

marrow-derived stem cells cultured in monolayer [Friedman et al., 2005]. In embryonic mouse C3H10T1/2 multipotent mesenchymal precursors cultured in monolayer, both osteogenic and chondrogenic differentiation was apparent after stimulation with BMP7, while application of BMP7 and co-culturing of these cells with chondrocytes induced osteogenic development only [Gerstenfeld et al., 2002]. Using the bipotent murine bone marrow stromal cell line BMS2, BMP7 has been shown to stimulate cell proliferation and induced adipocyte and osteoblast development. Remarkably, BMP7-mediated development was dose-dependent, leading to adipocytic cells when using low doses of BMP7 and inducing osteogenesis in expense of adipocyte differentiation in monolayer when high doses of the inducer were applied [Chen et al., 2001]. Interestingly, in this study, using comparable doses of BMP7 to induce mesenchymal lineage development of human MSC in high-density micro-masses enhanced adipocyte differentiation and failed to stimulate osteogenesis and chondrogenesis. Similar effects were also observed in C3H10T1/2 cells cultured in monolayers, which developed into adipocytes upon treatment with low doses of BMP7. Instead, high doses of BMP7 caused the cells to undergo adipogenic and chondrogenic development simultaneously [Asahina et al., 1996]. Here, BMP7 stimulated the adipogenic but not chondrogenic or osteogenic differentiation of adult human MSC from bone marrow cultured in high-density using different concentrations of the inducer under conditions that support chondrogenesis. BMP-directed or growth factor-dependent guidance of mesenchymal progenitors along a distinct lineage depend on the differentiation state of the particular cells, their developmental potential and commitment as well as on the dose-dependency of differentiation events. In vitro conditions like monolayer versus three-dimensional culturing, that may mimic tissues and potentially mesenchymal condensations, may also of special importance for the evaluation of growth factor-mediated developmental sequences. For instance, BMP2 induces osteo-/chondro- and adipogenic development of early stage murine mesenchymal progenitors when applied in monolayer [Ahrens et al., 1993], while three-dimensional assembly of the cells initiated chondrogenic differentiation [Denker et al., 1999]. Similar effects were also observed for TGFβ1 that induced the formation of micromasses containing type II collagen but failed to induce this particular cartilage-related collagen in monolayer cultures [Denker et al., 1995]. Since lineage development of mesenchymal progenitors varied depending on whether cells were cultured in monolayer or in high density, BMP-mediated chondrogenic or mesenchymal lineage decision may also depend on whether cell–cell or cell–matrix-interaction prevail. The importance of cell–cell contact has been shown in a chicken limb bud model, in which the BMP-dependent chondrogenesis was blocked by the inhibition of gap junction-mediated intercellular communication [Zhang et al., 2002]. Taking the data about mesenchymal differentiation of MSC in monolayer and in high density into account, we suggest that BMP7 may induce multiple lineages when monolayer cultures of MSC with distinct cell–matrix-interaction are used. The culture of MSC in high density favors cell–cell-interaction that may favor development of a distinct lineage on expense of the other. In addition, in a murine model, the differentiation capability of bone marrow mesenchymal stroma cells has been recently shown to depend on aging that increased the commitment of these cells to the adipogenic lineage. Moerman and colleagues [Moerman et al., 2004] report that the intrinsic differentiation capacity of MSC may change with aging leading to a reduced osteogenic and to an enhanced adipogenic potential of the progenitors. The age-dependent commitment of progenitors may also account for the BMP7-mediated adipogenic differentiation of adult human MSC derived from bone marrow. As shown here, prolonged cultivation of MSC derived from adult donors in high-density without stimulation with growth factors was accompanied by elevated levels of adipogenic and osteogenic transcription factors like CBFA1 and PPARγ. Since CBFA1 and PPARγ are central regulators of osteogenic and adipogenic development [Lian et al., 2004; Rosen, 2005], the induction of these transcription factors suggest that bone marrow-derived MSC from adult donors may have spontaneously differentiated without stimulation with BMP7 or TGF along the adipogenic and/or osteogenic lineage under the conditions used in this study. However, although adult MSC may have been committed to the adipogenic lineage by aging, BMP7 enhanced the expression of typical adipogenic

marker genes and mediated a significantly increased deposition of lipids compared to untreated/committed controls and MSC treated with TGF. Therefore, BMP7 promotes the adipogenic and not the osteo-/chondrogenic differentiation of adult human MSC in high-density culture.

In conclusion, using a differentiation system that favors chondrogenic differentiation, BMP7 induced adipocyte development of human bone marrow-derived MSC in three-dimensional micro-mass culture. After stimulation of MSC with BMP7, neither osteogenic nor chondrogenic cells were evident in our in vitro high-density differentiation system. With respect to adipose tissue regeneration approaches, the capacity of BMP7 to induce solely adipocyte and not osteo-/chondrogenic lineage development of mesenchymal progenitors has to be further elucidated in appropriate animal model systems.

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