

# Cartilage-Like Gene Expression in Differentiated Human Stem Cell Spheroids

## A Comparison of Bone Marrow–Derived and Adipose Tissue–Derived Stromal Cells

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**Objective.** To compare the chondrogenic potential of human bone marrow–derived mesenchymal stem cells (BMSC) and adipose tissue–derived stromal cells (ATSC), because the availability of an unlimited cell source replacing human chondrocytes could be strongly beneficial for cell therapy, tissue engineering, in vitro drug screening, and development of new therapeutic options to enhance the regenerative capacity of human cartilage.

**Methods.** Quantitative gene expression of common cartilage and cell interaction molecules was analyzed using complementary DNA array technology and reverse transcription–polymerase chain reaction during optimization of cell differentiation, in order to achieve a molecular phenotype similar to that of chondrocytes in cartilage.

**Results.** The multilineage potential of BMSC and ATSC was similar according to cell morphology and histology, but minor differences in marker gene expression occurred in diverse differentiation pathways. Although chondrogenic differentiation of BMSC and ATSC was indistinguishable in monolayer and remained partial, only BMSC responded (with improved chondrogenesis) to a shift to high-density 3-dimensional

cell culture, and reached a gene expression profile highly homologous to that of osteoarthritic (OA) cartilage.

**Conclusion.** Hypertrophy of chondrocytes and high matrix-remodeling activity in differentiated BMSC spheroids and in OA cartilage may be the basis for the strong similarities in gene expression profiles between these samples. Differentiated stem cell spheroids represent an attractive tool for use in drug development and identification of drug targets in OA cartilage–like tissue outside the human body. However, optimization of differentiation protocols to achieve the phenotype of healthy chondrocytes is desired for cell therapy and tissue engineering approaches.

In contrast to bone, skin, liver, brain, or muscle, cartilage seems to be devoid of an internal tissue-specific stem cell compartment and shows a very limited capacity for tissue homeostasis and regeneration in response to damage. Chronic degeneration of articular cartilage often occurs even in the absence of evident injury. Until now, no regenerative therapeutic option was available to prevent, delay, halt, or heal osteoarthritis (OA; also called degenerative joint disease), which is the most common musculoskeletal disorder in developed countries (1). Self-replenishing and easily accessible new sources of human chondrocytes could be very beneficial for use in cell therapy, tissue engineering, cartilage repair, and drug development in OA, and may allow the development of new therapeutic options for cartilage regeneration.

Recent evidence suggests that stem cells residing in one tissue can contribute to the repair of another because of a previously unrecognized degree of plasticity, and that such cell fate changes seem to be a natural

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property of adult stem cells (2). A population of stem cells with the capacity to differentiate toward osteogenic (3,4), chondrogenic, (5,6) or adipogenic (7) lineages in vitro and in vivo (8,9) persists in adult bone marrow. Depending on the author, and in the absence of well-established markers, these cells have been called either colony-forming units (10), bone marrow-derived mesenchymal stem cells (BMSC) (11–13), marrow mesodermal progenitor cells (14), or marrow stromal stem cells (15). They may be isolated from the bone marrow and expanded by standard culture techniques while retaining their multilineage capacity (16), and protocols have been devised to use them for the treatment of defects in articular cartilage (17).

In addition to bone marrow, periosteum (18), muscle (19), and adipose tissue (20,21) appear to be sources of mesenchymal stem cells. Subcutaneous adipose tissue is a particularly attractive reservoir for progenitor cells, because it is easily accessible, rather abundant, and self-replenishing. A multipotent progenitor cell population, which we refer to herein as adipose tissue-derived stromal cells (ATSC), is derived after collagenase digestion from adipose tissue (22). These cells can be induced to differentiate toward the adipogenic, chondrogenic, osteogenic, and myogenic lineages (23,24).

The differentiation and multilineage potential of distinct stem cell populations has previously been judged according to cell morphology, histochemistry, and detection of a few tissue-specific differentiation molecules (6,25). Although the expression of main cartilage matrix components such as COL2A1, aggrecan, decorin, biglycan, and cartilage oligomeric matrix protein (COMP) has been revealed after induction of chondrogenesis in BMSC (5), no such information is available for chondroinduction in ATSC. To our knowledge, no in vitro studies are available comparing the chondrogenic capacity of multipotent progenitor cells obtained from different tissue sources under standardized conditions. The completeness of chondroinduction on the molecular level in mesenchymal stem cell populations is unknown, as is which genes are induced or suppressed during chondrogenesis, and whether gene expression profiles similar to those of cultured chondrocytes or native cartilage can be achieved.

The aim of this study was to compare the multilineage potential and chondrogenic capacity of BMSC and ATSC at the molecular level, and to optimize cell differentiation toward a molecular phenotype similar to that of native chondrocytes. By utilizing gene expression profiling with a custom-made cartilage complementary

DNA (cDNA) array and a commercial cell interaction cDNA array, we intended to characterize chondrogenesis in stromal cell populations in order to obtain improved vital components for use in cell therapy, tissue engineering, and drug development in artificial human cartilage outside the human body.

## MATERIALS AND METHODS

**Bone marrow samples.** Bone marrow samples for isolation of mesenchymal stem cells were obtained from patients (mean age 44.6 years) undergoing total hip replacement or iliac bone graft harvest. Adipose tissue stromal cells were isolated from lipoaspirates generated during elective liposuction procedures or from adipose tissue samples obtained from patients (mean age 55.3 years) undergoing total hip arthroplasty. Normal articular knee cartilage from 5 donors (mean age 40.6 years) was obtained either at autopsy (within 48 hours of death) or from patients undergoing amputation for tumor resection. Articular OA cartilage was obtained from the tibial plateaus of 9 patients (mean age 70.9 years) undergoing total knee replacement surgery. The studies were approved by the local ethics committee. Informed consent was obtained from all individuals included in the study.

**Cell isolation and cultivation.** Mesenchymal stem cells were isolated from fresh bone marrow samples as previously described (13). Briefly, cells were fractionated on a Ficoll-Paque Plus density gradient (Amersham Pharmacia, Uppsala, Sweden), and the low-density MSC-enriched fraction was washed and seeded in culture flasks in MSC culture medium (14) containing 2% or 10% fetal calf serum (FCS), with or without recombinant human epidermal growth factor (Strathmann Biotech, Hamburg, Germany) and recombinant human platelet-derived growth factor BB (Sigma-Aldrich, Deisenhofen, Germany). After 24–48 hours, cultures were washed with phosphate buffered saline (PBS) to remove nonadherent material. During expansion, medium was replaced twice a week.

ATSC were isolated according to the method described by Hauner et al (20). Briefly, minced tissue or lipoaspirates were digested with Krebs-Ringer solution buffered with 25 mM HEPES, 20 mg/ml bovine serum albumin (BSA), and 1.5 mg/ml collagenase (CLS type I; Worthington, Freehold, NJ) and filtered with a 250- $\mu$ m nylon mesh. Erythrocytes were removed using erythrocyte lysis buffer (0.154M NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1 mM EDTA). The remaining cells were seeded in culture flasks and maintained under conditions identical to those for BMSC. Human chondrocytes were obtained from cartilage regions with no macroscopically evident degeneration, using digestion with collagenase B (1.5 mg/ml) (Roche Diagnostics, Mannheim, Germany) and hyaluronidase (0.1 mg/ml) (Serva, Heidelberg, Germany), as described previously (26). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 100 units/ml of penicillin, and 100  $\mu$ g/ml of streptomycin, and were maintained at 37°C in a humidified atmosphere and 6% CO<sub>2</sub>.

**Induction of differentiation.** ATSC or BMSC expanded in monolayer were plated at a cell density of 1–3  $\times$  10<sup>4</sup> cells/cm<sup>2</sup> for monolayer cultures. For induction of chondro-

genesis at high cell density in 3-dimensional (3-D) culture, cells were seeded at  $3\text{--}5 \times 10^5$  cells/well on a 48-well plate, where the cells formed a multilayer within 1 hour. Individual cell spheroids formed spontaneously within 1–2 days by cell aggregation and were then moved to 96-well U-bottomed plates to reduce the costs for medium and growth factors due to lower volume at feeding (twice weekly). They were maintained in induction medium for 2 weeks, unless stated otherwise. Chondrogenic medium consisted of DMEM high glucose (DMEM-HG; DMEM containing 4.5 gm/liter glucose) supplemented with 5  $\mu\text{g/ml}$  insulin, 5  $\mu\text{g/ml}$  transferrin, 5  $\mu\text{g/ml}$  selenous acid, 0.1  $\mu\text{M}$  dexamethasone, 0.17 mM ascorbic acid–2-phosphate, 1 mM sodium pyruvate, 0.35 mM proline, 1.25 mg/ml BSA, and 10  $\mu\text{g/ml}$  transforming growth factor  $\beta_3$  (TGF $\beta_3$ ; Sigma-Aldrich).

In a pilot study involving 2 donors, comparison of spontaneously forming spheroids and classic pellet culture revealed that consistent changes in gene expression were confined to up-regulation of type I collagen (1.26-fold), biglycan (1.17-fold), and osteonectin (1.13-fold) in spheroids at 2 weeks after induction. All other genes showed no changes or were discordant between donors for spheroid versus pellet culture. Thus, their different expression levels should not be a result of the culture system used. We therefore consider our culture form as being equivalent to the classic technique, although ours is associated with decreased cost, time, and effort. For osteogenic induction, MSC growth medium (Poietics; BioWhittaker, Taufkirchen, Germany) supplemented with 0.1  $\mu\text{M}$  dexamethasone, 0.05 mM ascorbic acid–2-phosphate, and 10 mM  $\beta$ -glycerophosphate was used. Adipogenic medium was composed of DMEM-HG containing 10% FCS, 0.01 mg/ml insulin, 1  $\mu\text{M}$  dexamethasone, 0.2 mM indomethacin, 0.5 mM 3-isobutyl-1-methyl xanthine, 100 units/ml penicillin, and 100  $\mu\text{g/ml}$  streptomycin.

**Cell staining.** Cells grown in monolayers were fixed in 4% paraformaldehyde before histologic examination. Staining procedures were performed using standard protocols. Briefly, adipogenic cell layers were stained with 0.3% oil red O/60% isopropanol for 15 minutes. Von Kossa's staining was performed by using an aqueous 5% AgNO<sub>3</sub> solution, followed by fixation for 2 minutes in 5% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. Aqueous 0.1% Safranin O or 0.03% toluidine blue staining solution was incubated for 6–8 minutes on chondrogenic cells, and hematoxylin or nuclear fast red/Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> was used for counterstaining. For immunohistochemical staining, chondrogenic BMSC and ATSC spheroids were fixed in PBS containing 4% paraformaldehyde for 2 hours at 20°C, dehydrated in alcohol, washed in acetone, and infiltrated with paraffin.

Paraffin sections (3–4  $\mu\text{m}$ ) were dried, deparaffinized using XEM-200 (Vogel, Giessen, Germany), rehydrated in alcohol, then pretreated with 2 mg/ml of hyaluronidase (Merck, Darmstadt, Germany) for 15 minutes at 37°C and subsequently with 1 mg/ml of pronase (Roche Diagnostics) for 30 minutes at 37°C. Nonspecific background was blocked using PBS containing 5% BSA for 30 minutes. Sections were incubated overnight at 4°C with a monoclonal mouse anti-human type II collagen (ICN Biomedicals, Aurora, OH) in PBS containing 1% BSA. Following washing with Tris buffered saline, reactivity was detected using biotinylated donkey anti-mouse secondary antibody (1:200) (Dianova, Hamburg, Germany), streptavidin–alkaline phosphatase (Dako, Glostrup,

**Table 1.** Composition of the cartilage cDNA array

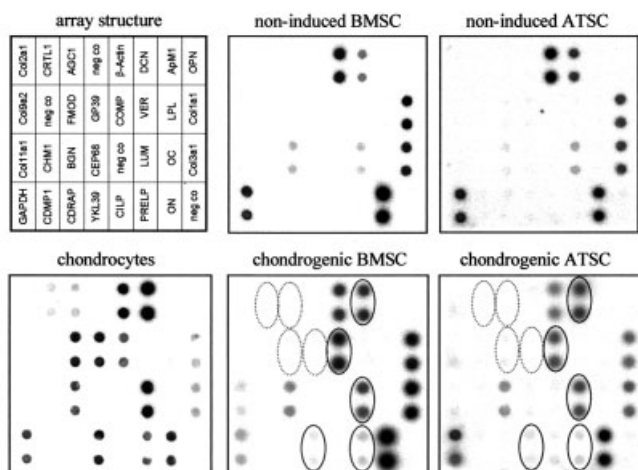
Gene	UniGene cluster
$\beta$ -actin	Hs.288061
Adiponectin/adipose most abundant gene transcript 1	Hs.80485
Aggrecan 1	Hs.2159
Biglycan	Hs.821
Cartilage acidic protein 1/chondrocyte-expressed protein 68	Hs.326444
Cartilage-derived morphogenetic protein 1	Hs.1573
Cartilage-derived retinoic acid-sensitive protein	Hs.279651
Cartilage glycoprotein 39/chitinase 3-like 1/YKL40	Hs.75184
Cartilage intermediate-layer protein	Hs.151407
Cartilage-linking protein 1	Hs.2799
Cartilage oligomeric matrix protein	Hs.1584
Chitinase 3-like 2	Hs.154138
Chondromodulin 1	Hs.97932
COL1A1	Hs.172928
COL2A1	Hs.81343
COL3A1	Hs.119571
COL9A2	Hs.37165
COL11A1	Hs.82772
Decorin	Hs.76152
Fibromodulin	Hs.230
GAPDH	Hs.169476
Lipoprotein lipase	Hs.180878
Lumican	Hs.79914
Osteocalcin	Hs.2558
Osteonectin	Hs.111779
Osteopontin	Hs.313
Proline/arginine-rich end leucine-rich repeat protein	Hs.76494
Versican/chondroitin sulfate proteoglycan 2	Hs.81800

Denmark) for 30 minutes at 20°C, and fast red (F4648; Sigma-Aldrich, Bornem, Belgium) for 20 minutes at 20°C. Sections were permanently mounted with Aquatex (Merck) and examined by light microscopy.

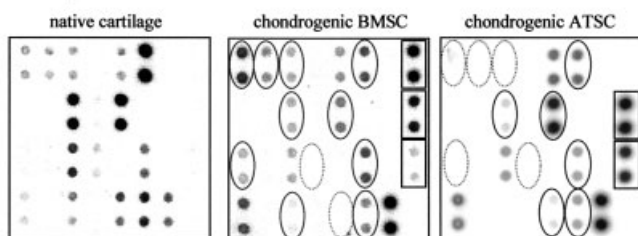
**RNA isolation.** Total RNA was isolated from cultured cells using a standard guanidinium thiocyanate/phenol extraction technique (peqGOLD TriFast; peqLab, Erlangen, Germany). Stem cell spheroids and cartilage samples had been minced previously. Polyadenylated messenger RNA (mRNA) was isolated using oligo(dT) coupled to magnetic beads (Dynabeads; Dynal Biotech, Oslo, Norway), according to the manufacturer's instructions.

**Complementary DNA array production.** Analyzed genes included cartilage-, bone-, and adipose tissue-specific genes, as well as housekeeping genes (GAPDH and  $\beta$ -actin) and negative controls (*Arabidopsis thaliana*) (Table 1). Selected cDNA (size range 400–850 bp) were cloned into pBlue-script SK<sup>+</sup> vector (Stratagene, Amsterdam, The Netherlands). Complementary DNA was PCR-amplified using vector-specific primers and 50 ng of plasmid as template. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and concentrated, and standardized amounts were arrayed onto positively charged Hybond-N<sup>+</sup> nylon filters (Amersham Pharmacia Biotech, Freiburg, Germany). Gene fragments (10 ng/dot) were spotted twice on each filter, as depicted in Figure 1.

### A Monolayer Culture



### B 3D-spheroids



**Figure 1.** Gene expression profiles by cDNA array analysis. **A**, Array location of genes (neg co = negative control genes from *Arabidopsis thaliana*), and representative gene expression patterns of noninduced bone marrow-derived mesenchymal stem cells (BMSC) and adipose tissue-derived stromal cells (ATSC) ( $n = 8$ ), and of chondrocytes ( $n = 5$ ), chondrogenic BMSC ( $n = 3$ ), and chondrogenic ATSC ( $n = 3$ ) 2 weeks after induction of differentiation. **B**, Representative gene expression profiles of native healthy cartilage ( $n = 5$ ), and of spheroid cultures of BMSC ( $n = 3$ ) and ATSC ( $n = 3$ ) 2 weeks after chondrogenic induction. Genes that were up-regulated toward the expression levels in cultured chondrocytes or native cartilage are in solid-line ovals, and genes for which induction failed are in broken-line ovals. Genes that were not expressed in cartilage but were positive in induced spheroids are in rectangles. Although the expression profile of BMSC was improved toward the molecular phenotype of native chondrocytes when induced in 3-dimensional (3-D) spheroid culture, ATSC induction profiles were identical in monolayer and 3-D cultures after 2 weeks. CDMP1 = cartilage-derived morphogenetic protein 1; CHM1 = chondromodulin 1; CRTL1 = cartilage-linking protein 1; CDRAP = cartilage-derived retinoic acid-sensitive protein; BGN = biglycan; FMOD = fibromodulin; AGC1 = aggrecan 1; YKL39 = chitinase 3-like 2; CEP68 = cartilage acidic protein 1/chondrocyte-expressed protein 68; GP39 = glycoprotein 39; CILP = cartilage intermediate-layer protein; PRELP = proline/arginine-rich end leucine-rich repeat protein; LUM = lumican; VER = versican/chondroitin sulfate proteoglycan 2; DCN = decorin; ON = osteonectin; OC = osteocalcin; LPL = lipoprotein lipase; APM1 = adiponectin/adipose most abundant gene transcript 1; OPN = osteopontin.

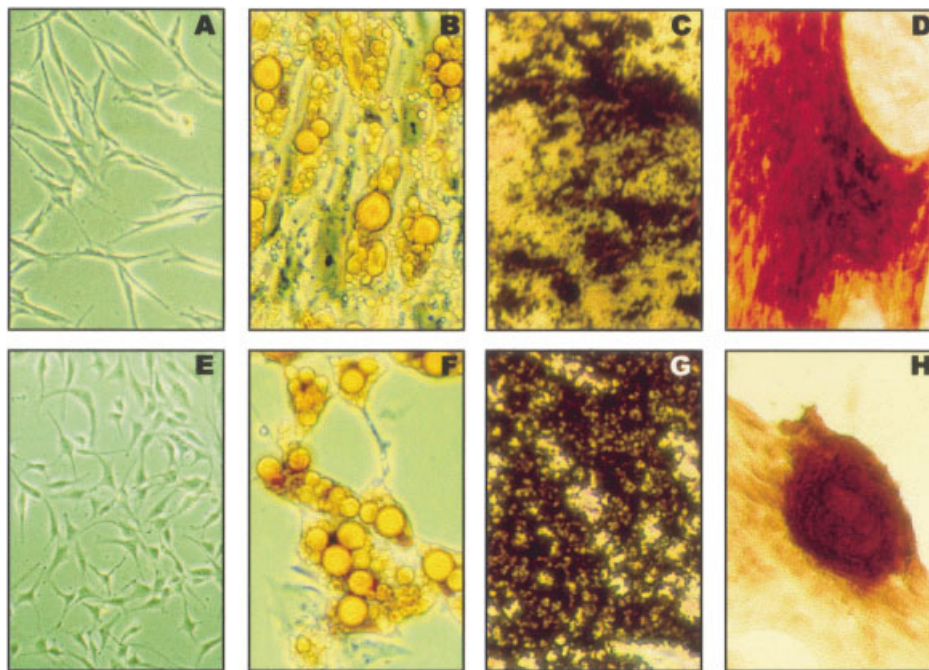
**Complementary DNA array hybridization.** Complementary DNA ( $^{32}\text{P}$ -dATP-labeled) probes were prepared from isolated mRNA according to the manufacturer's protocol (SuperScript II; Life Technologies, Karlsruhe, Germany). The labeled cDNA was denatured and hybridized to cDNA arrays overnight at  $68^\circ\text{C}$ . Arrays were washed 3 times (for 30 minutes each time) in  $0.04\text{M}$  phosphate buffer (pH 7.2)/1% sodium dodecyl sulfate at  $68^\circ\text{C}$  before being exposed to an imaging plate (Fuji Photo Film, Dusseldorf, Germany) for 18 hours. Images were captured on the BAS-1800 II Bio-imaging Analyzer, using BAS Reader 2.26 beta software (Fuji/Raytest, Straubenhardt, Germany) and were analyzed using AIDA software (Fuji/Raytest). Expression levels in mRNA samples from different sources were normalized to the signal strength of the housekeeping genes.

Standardization experiments suggested setting the lower cutoff for reliable quantification to 5% of the mean of the housekeeping gene expression levels. Inter- and intra-assay variation of several independent experiments indicated that only alterations in gene expression levels of 3-fold or higher should be considered. Alternatively, cDNA probes prepared using the Atlas SMART Probe Amplification Kit (Clontech, Palo Alto, CA) were hybridized to Clontech Atlas human cell interaction arrays, and data were evaluated as described above (Genbank accession numbers are available at [www.clontech.com](http://www.clontech.com)).

**LightCycler real-time reverse transcription-polymerase chain reaction (RT-PCR).** Single-stranded cDNA was generated from mRNA using reverse transcriptase (SuperScript II; Life Technologies) and oligo(dT) primers and was purified using QIAquick (Qiagen). To quantify the mRNA levels with the LightCycler (Roche Diagnostics), aliquots of the single-stranded cDNA were amplified, and real-time fluorimetric intensity of SYBR Green I was monitored. The  $\text{MgCl}_2$  and the cycling parameters were optimized according to the LightCycler protocol (LightCycler operator's manual, version 3.5; Roche Diagnostics). The forward (F) and reverse (R) primers used for amplification were as follows: for GAPDH-F, 5'-GGG-AAG-CTT-GTC-ATC-AAT-GG-3'; for GAPDH-R, 5'-ATC-ATC-TCT-GCC-CCC-TCT-G-3'; for COL2A1-F, 5'-TGG-CCT-GAG-ACA-GCA-TGA-C-3'; for COL2A1-R, 5'-AGT-GTT-GGG-AGC-CAG-ATT-GT-3'; for CEP-68-F, 5'-CTC-TGG-ACG-CTA-CTC-TAT-CT-3'; and for CEP-68-R, 5'-GAG-GGC-ATG-GAG-AAC-TTG-G-3'. The concentration of GAPDH was used to control for input RNA (determined once for each cDNA sample) and to normalize all other genes tested from the same cDNA sample. The copy ratio of each analyzed cDNA was determined as the mean of 3 experiments. Following PCR,  $5\ \mu\text{l}$  of the samples was loaded onto a 1.5% agarose gel containing ethidium bromide, electrophoresed, and visualized under ultraviolet light.

## RESULTS

**Multilineage potential of BMSC and ATSC.** Human BMSC and ATSC (Figures 2A and E) exhibited a fibroblast-like phenotype in monolayer cultures, without any obvious variation in cell morphology between samples derived from proximal femur, iliac bone, or adipose tissue. In the presence of specific induction media,



**Figure 2.** Representative phase-contrast photomicrographs of bone marrow–derived mesenchymal stem cells (A–D) and adipose tissue–derived stromal cells (E–H), showing the multilineage potential of expanded cells. Undifferentiated cells (A and E) were grown in induction media for 2 weeks prior to fixation with 4% paraformaldehyde. Oil red O staining of adipogenic cultures shows cell bodies filled with numerous lipid vesicles (B and F). Osteogenic cultures deposited mineralized extracellular matrix, as indicated by von Kossa’s staining (C and G). Matrix synthesized under chondrogenic differentiation is strongly stained by Safranin O (D and H).

BMSC and ATSC adopted a differentiated phenotype. By day 14 of adipogenic induction, cells had accumulated lipid droplets typical for the adipocyte phenotype (Figures 2B and F). In osteogenic medium, mineralized matrix components were deposited on top of the network-like monolayers, as evident by von Kossa’s staining (Figures 2C and G). Cells induced with chondrogenic medium developed a round phenotype and aggregated spontaneously to spheroid-like or rod-like cell agglomerates, especially at high cell density, and cultures became positive for Safranin O (Figures 2D and H) and toluidine blue (results not shown). According to cell morphology and staining, BMSC and ATSC had a similar phenotype and multilineage potential.

**Gene expression analysis of tissue-specific marker genes.** In order to dissect the multilineage potential of BMSC and ATSC at the molecular level, selected fat, bone, and cartilage marker genes were assessed by real-time RT-PCR and cDNA array analysis on a small-scale cartilage cDNA array (26). Osteonectin, osteopontin (OPN), and osteocalcin (OC) were selected to analyze osteogenic differentiation; adiponectin and

lipoprotein lipase were used as adipose tissue–specific genes; and COL2A1, COMP, biglycan, and lumican were used as cartilage-specific genes. The mean expression levels in noninduced versus chondrogenic, adipogenic, and osteogenic cultures are summarized in Table 2.

In adipogenic cultures, strong induction of adiponectin and lipoprotein lipase was evident, with higher levels for lipoprotein lipase obtained in ATSC compared with BMSC. In osteogenic BMSC cultures, weak induction of adipocyte-specific genes occurred, together with the appearance of a few lipid-accumulating cells in the culture. A certain degree of overlap between adipocyte and osteoblast phenotypes and a reversible differentiation into osteoblast and adipocyte lineages have previously been suggested for cells in bone marrow (23,27). In ATSC cultures, however, osteogenic conditions completely suppressed adipogenesis, and no lipid-accumulating cells were observed.

In spite of mineralized matrix deposition within 2 weeks of osteogenic induction, only weak up-regulation of osteonectin and no evident change in OPN and OC expression occurred, compared with the BMSC and

**Table 2.** Relative mRNA expression levels in monolayer cultures of BMSC and ATSC before and 2 weeks after induction of differentiation, by cDNA array analysis\*

Gene	Noninduced		Adipogenic		Osteogenic		Chondrogenic	
	BMSC (n = 8)	ATSC (n = 4)	BMSC (n = 3)	ATSC (n = 3)	BMSC (n = 3)	ATSC (n = 3)	BMSC (n = 3)	ATSC (n = 3)
APM1	–	–	149	300	6	–	–	–
LPL	–	–	113	595	27	–	–	–
ON	141	145	312	438	296	267	666	493
OPN	–†	–	–	–	6	–	–†	–
OC	–†	–	–	–	–†	–	–	–
COL2A1	–	–	–	–	–	–	–	–
COMP	–	–	34	143	25	13	386	202
BGN	7	–	16	13	19	9	42	64
LUM	5	11	12	21	26	87	213	106
PRELP	–	–	10	12	11	–	21	14

\* Values are the mean level of expression (% relative to housekeeping genes). BMSC = bone marrow–derived mesenchymal stem cells; ATSC = adipose tissue–derived stromal cells; APM1 = adiponectin/adipose most abundant gene transcript 1; LPL = lipoprotein lipase; ON = osteonectin; OPN = osteopontin; OC = osteocalcin; COMP = cartilage oligomeric matrix protein; BGN = biglycan; LUM = lumican; PRELP = proline/arginine-rich end leucine-rich repeat protein.

† Positive in real-time reverse transcription–polymerase chain reaction.

ATSC starting cultures. Real-time RT-PCR analysis, which has higher sensitivity than cDNA array analysis, indicated that some OPN and OC was already expressed in noninduced BMSC cultures (data not shown). Both OPN and OC disappeared under adipogenic conditions, and OC was down-regulated in chondrogenic differentiation medium (which contains TGF $\beta_3$ ). Thus, noninduced BMSC cultures may contain some OPN-positive and OC-positive cells that are sensitive to TGF $\beta_3$  and adipogenic conditions. The inhibitory effects of TGF $\beta_3$  on osteogenic differentiation of stromal cells and osteoblast maturation are well known from the literature (28–31) and may account for the reversion of osteoblast-like gene expression in these cultures.

Overall, osteonectin, OPN, and OC were unsatisfactory markers of early osteogenic induction and did not reflect matrix mineralization in BMSC and ATSC cultures at the molecular level. In contrast, COMP, lumican, and biglycan were good markers of chondroinduction. They were up-regulated to similar levels in ATSC and BMSC. COL2A1 expression, however, remained below the detection level in all chondrogenic monolayer cultures and was similar to expression in cultured chondrocytes (Figure 1A), which undergo a rapid switch to dedifferentiation and lose type II collagen expression after extended time in monolayers (26). In summary, marker gene analysis revealed some minor differences in noninduced and osteogenic cultures of BMSC versus ATSC, while similar expression profiles occurred after adipogenic and chondrogenic differentiation in monolayer.

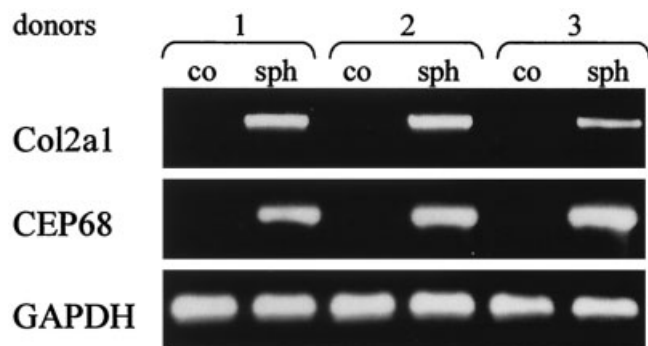
**Gene expression in stem cells after chondrogenic induction compared with chondrocytes and native cartilage.** Gene expression profiles of cultured chondrocytes and native cartilage were assessed on a small-scale custom-made cDNA array composed of >20 common cartilage-expressed molecules (26). Monolayer-expanded chondrocytes (Figure 1A) showed significant down-regulation of differentiation markers (COL2A1, COMP, proline/arginine-rich end leucine-rich repeat protein [PRELP], fibromodulin, and cartilage intermediate-layer protein) and induction of expansion-related genes (COL1A1 and COL3A1, chitinase 3–like 2 [YKL39], cartilage glycoprotein 39 [gp39], and lumican) compared with chondrocytes from native cartilage (Figure 1B).

Noninduced BMSC and ATSC expressed only COL1A1, COL3A1, decorin, osteonectin, lumican, and biglycan (plus the housekeeping genes). Standard chondrogenic induction in monolayer resulted in up-regulation of cartilage differentiation markers such as COMP, PRELP, and decorin, and of expansion-related genes YKL39 and lumican (Figure 1A). Compared with cultured chondrocytes, however, induction was incomplete, because cartilage-linking protein 1 (CRTL1), aggrecan, fibromodulin, and gp39 remained negative (Figure 1A). In addition, genes expressed in cartilage, such as COL2A1 and chondrocyte-expressed protein 68 (CEP-68) (32), were undetectable in monolayer chondrocytes and in the induced stromal cells, according to cDNA array analysis. Similar to the noninduced starting

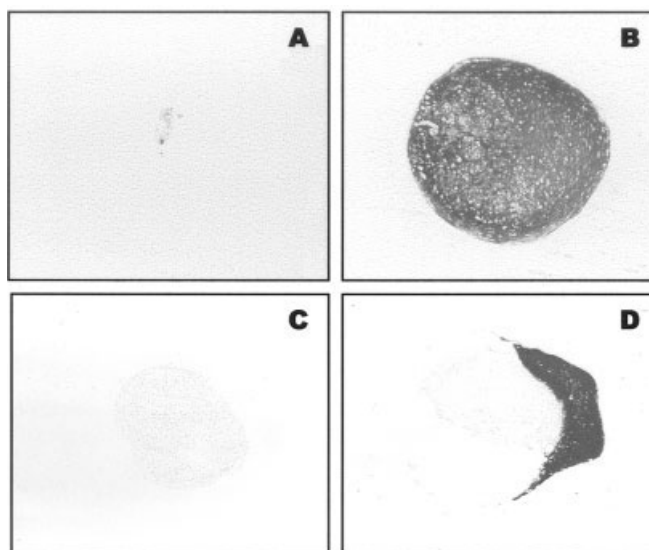
cultures, chondrogenic lineages of BMSC and ATSC had comparable expression profiles in monolayer.

Variation of cell density, replacement of growth factors (growth differentiation factor 5, TGF $\beta_1$ , TGF $\beta_3$ ), and shift to 3-D culture were applied to improve chondrogenesis of stromal cells toward a molecular phenotype typical for cultured chondrocytes or native human cartilage. Best results were achieved by a shift of cells to high density ( $0.8\text{--}1 \times 10^6$  cells/ml) 3-D spheroid culture at the time of chondrogenic induction with TGF $\beta_3$ . Additional cartilage-relevant genes, including COL2A1, aggrecan, CRTL1, and fibromodulin, were induced in BMSC (Figure 1) after 2 weeks, and expression of only 2 genes, CEP-68 and cartilage intermediate layer protein, was lacking compared with healthy native human cartilage (Figure 1B). Up-regulation of several genes, including COL2A1, was confirmed by real-time RT-PCR analysis. Induction of CEP-68, a recently described marker gene for cultured chondrocytes, became evident in BMSC spheroids, using this highly sensitive technique (Figure 3).

Compared with monolayer conditions, a shift to 3-D culture at high cell density, surprisingly, did not improve chondrogenesis of ATSC, because by day 14 they remained negative for COL2A1, aggrecan, CRTL1, fibromodulin, and cartilage-derived retinoic acid-sensitive protein (Figure 1B). Immunohistochemistry



**Figure 3.** Real-time reverse transcription-polymerase chain reaction (PCR) analysis of gene expression in noninduced bone marrow-derived mesenchymal stem cells (BMSC) (control; co) versus BMSC spheroids differentiated for 2 weeks (sph). Cells obtained from 3 donors were analyzed, and PCR products were visualized by agarose gel electrophoresis. COL2A1 and chondrocyte-expressed protein 68 (CEP-68) gene expression levels in BMSC spheroids were 0.94-fold and 0.02-fold, respectively, that of GAPDH, whereas no expression was detected in adipose tissue-derived stromal cell (ATSC) spheroids and noninduced BMSC and ATSC. Up-regulation of CEP-68 expression parallel to that of COL2A1 in differentiated BMSC spheroids confirms that this gene is a good marker for chondrogenic induction (see ref. 32) and emphasizes the higher sensitivity of PCR compared with cDNA array analysis.



**Figure 4.** Immunohistochemical analysis of type II collagen expression, showing bone marrow-derived mesenchymal stem cell (BMSC) (A and B) and adipose tissue-derived stromal cell (ATSC) (C and D) spheroids after chondrogenic differentiation for 2 weeks (A and C) or 4 weeks (B and D). Immunostaining of paraffin sections indicates that induction of type II collagen expression occurs earlier in BMSC spheroids than in ATSC spheroids. Color figure can be viewed in the online issue, which is available at <http://www.arthritisrheum.org>.

revealed earlier expression of the COL2A protein in BMSC versus ATSC spheroids after chondroinduction (Figure 4). COL2A staining was partial in all positive ATSC spheroids, while full or partial staining was obtained for spheroids generated from BMSC cultures. In summary, molecular differences in chondrogenic induction between ATSC and BMSC became apparent in high-density 3-D cell culture. Improved differentiation to a molecular phenotype similar to that of cartilage was achieved only for BMSC.

**Expression of cell interaction molecules during chondrogenesis.** Cell condensation by reduction of intercellular spaces precedes chondrogenesis in vivo, and this event, known as precartilage condensation, may be a necessary step in cartilage differentiation (33). Condensation of cells to spheroids occurred spontaneously in our high-density cell cultures that were exposed to chondrogenic medium, with no evident variation between BMSC and ATSC. Because spheroid culture had strong effects on the chondrogenesis of BMSC, we assessed the gene expression profile of cell interaction molecules in BMSC before and after chondrogenic induction in 3-D spheroids. Expression levels of 265 cell interaction molecules were studied using a commercial cell interaction cDNA array on BMSC obtained from 3

**Table 3.** Alteration in gene expression of cell interaction molecules in BMSC before and 2 weeks after chondrogenic induction\*

Gene	Changes vs. day 0, fold	Gene	Changes vs. day 0, fold
<b>Up-regulated genes</b>		<b>Unaltered genes</b>	
Aggrecan 1	17.9	Laminin $\beta$ 2 subunit precursor	-1.5
$\beta$ 1-catenin	4.7	Liver GAPDH	1.5
Biglycan	5.7	Low-density lipoprotein receptor-related protein 1	-1.3
Bullous pemphigoid antigen 1	16.4	Major histocompatibility complex class I C	-1.1
CD4 antigen	4.0	Matrix metalloproteinase 2	1.5
COL11A1	11.4	Matrix metalloproteinase 14	-1.2
COL11A2	17.2	nm23-H4; nucleoside-diphosphate kinase	2.0
COL16A1	3.3	Nucleoside diphosphate kinase A	1.0
Contactin	23.2	Nucleoside diphosphate kinase B	1.2
Decorin	12.2	Phospholipase A2	1.0
Envoplakin	7.5	Ras homolog gene family member A	1.0
Ephrin type B receptor 1	10.4	Ras homolog gene family member B	1.4
Insulin-like growth factor binding protein 5	8.9	Ras homolog gene family member C	1.0
$\alpha$ L integrin; CD11a antigen	71.2	Rho GDP-dissociation inhibitor 1	-1.4
$\beta$ 1 integrin	8.4	60S ribosomal protein L13A	1.0
Jagged homolog 1	13.2	40S ribosomal protein SA	1.3
Manic fringe homolog	18.2	40S ribosomal protein S9	-1.1
Matrix metalloproteinase 13	78.6	Tenascin	1.4
Matrix metalloproteinase 17	8.3	Tissue inhibitor of metalloproteinase 1	-1.9
COL2A1	15.4	Tissue inhibitor of metalloproteinase 2	-1.6
Protein tyrosine phosphatase receptor type F	7.9	Tissue inhibitor of metalloproteinase 3	-1.3
Rho-associated coiled-coil containing protein kinase	7.2	Tissue-type plasminogen activator	2.2
Secreted protein, acidic, cysteine-rich; osteonectin	4.6	Tubulin $\alpha$ 1	-1.4
Wingless-type MMTV integration site 8B protein	5.4	Ubiquitin C	1.0
Wingless-type MMTV integration site 13 protein	29.2	Versican core protein; chondroitin sulfate proteoglycan core protein 2	1.4
<b>Unaltered genes</b>		<b>Down-regulated genes</b>	
$\alpha$ 1-catenin	-2.4	Caveolin 1	-15.7
Basigin; CD147 antigen	-2.9	COL6A1	-4.0
CD44 antigen; hyaluronate receptor	1.2	COL6A3	-3.2
CD59 glycoprotein	-1.1	COL18A1	-3.4
CDC42 homolog	1.9	Fibronectin receptor alpha subunit; CD49E antigen	-9.2
COL1A2	2.7	Insulin-like growth factor binding protein 2	-12.4
COL3A1	1.7	Insulin-like growth factor binding protein 6	-7.2
Cytoplasmic $\beta$ -actin	-1.4	Laminin $\gamma$ 1 subunit precursor	-4.4
Endothelial plasminogen activator inhibitor 1	-2.0	Metalloproteinase/disintegrin/cysteine-rich protein	-4.7
Fibronectin 1	-1.2	Ras-like protein TC10	-4.0
Hypoxanthine-guanine phosphoribosyltransferase 1	-1.2	Tissue inhibitor of metalloproteinase 4	-4.2
Insulin-like growth factor binding protein 4	-1.7	Tumor necrosis factor receptor I	-14.9
Laminin $\alpha$ 4 subunit precursor	-1.7	Tumor necrosis factor superfamily member 7	-42.8
		Zyxin 2	-8.3

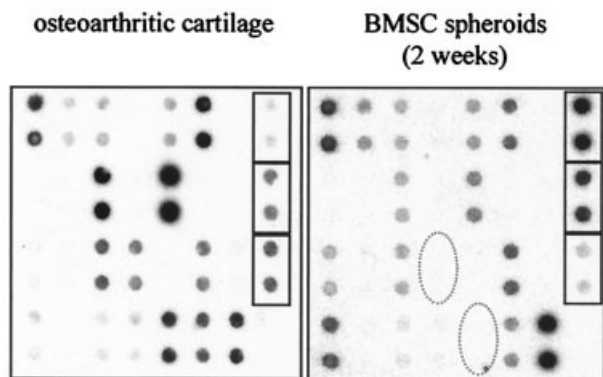
\* Alteration is defined as only genes with expression levels  $>5\%$  of the mean levels of housekeeping genes. BMSC = bone marrow-derived mesenchymal stem cells.

healthy donors (ages 8–48 years). Mean RNA expression levels were calculated, and genes with changes  $>3$ -fold were classified as being up-regulated or down-regulated (Table 3).

Before induction, 48 genes were expressed at levels  $>5\%$  of the mean level of expression of all housekeeping genes, and after 2 weeks of chondrogenic differentiation, 57 such genes were detected. A total of 25 genes were up-regulated ( $>3$ -fold) after cell condensation and chondrogenesis, whereas 14 genes were down-regulated ( $>3$ -fold). Besides the up-regulation of cartilage-relevant genes such as type II collagen, type XI

collagen, and aggrecan,  $\alpha$ L integrin,  $\beta$ 1 integrin, contactin, Wnt-13, the bullous pemphigoid antigen 1, as well as matrix metalloproteinase 13 (MMP-13) were induced. Down-regulated genes included tumor necrosis factor (TNF) superfamily member 7, TNF receptor I, caveolin 1, zyxin 2, and insulin-like growth factor-binding proteins 2 and 6.

**Stem cell spheroids with a gene expression profile similar to that of OA cartilage.** The limited self-repair capability of articular cartilage prevents substantial reconstitution of degenerated joint areas and is a major cause for development of OA. In an attempt to



**Figure 5.** Representative gene expression patterns of cartilage obtained from osteoarthritic (OA) joints ( $n = 9$ ) and of bone marrow-derived mesenchymal stem cell (BMSC) spheroids ( $n = 3$ ), as evaluated by cDNA array analysis. Expression profiles are similar for all genes except chondrocyte-expressed protein 68 and cartilage intermediate-layer protein (broken-line ovals). In contrast to healthy cartilage, OA cartilage and chondrogenic BMSC spheroids were positive for osteopontin, COL1A1, and COL3A1 (rectangles). Expression of cartilage oligomeric matrix protein varied among different OA samples.

support a healing process in the tissue, articular cartilage chondrocytes shift their gene expression pattern during the course of OA development (34). As a response to degeneration, expression of COL1A1, COL2A1, and COL3A1 is significantly up-regulated (35,36), and genes such as tenascin, OPN, and COL10 are induced in at least some individuals (37–39). Interestingly, cartilage cDNA array data of cartilage specimens obtained from patients with late-stage knee OA revealed a high degree of similarity to those of differentiated BMSC spheroids in 3-D culture (Figure 5). In contrast to healthy cartilage, OA cartilage and chondrogenic BMSC spheroids were positive for OPN, COL1A1, and COL3A1 (Figure 5) and showed strong expression of COL2A1. RT-PCR analysis demonstrated that COL10 was induced in all BMSC and ATSC cultures after chondrogenic induction (data not shown). In conclusion, BMSC-derived chondrogenic spheroids adopted a molecular phenotype resembling that of human chondrocytes in OA cartilage and thus of cells that attempt to support cartilage matrix repair in vivo.

## DISCUSSION

This study directly compares the chondrogenic potential of BMSC and ATSC at the level of gene expression, and judges their multilineage potential along the adipogenic, chondrogenic, and osteogenic pathways,

using several marker genes. Our data suggest that, although the multilineage potential of BMSC and ATSC was similar according to cell morphology and histology, some minor differences in marker gene expression occurred before and after induction of diverse differentiation pathways. Only BMSC cultures were susceptible to adipogenic differentiation under osteogenic conditions and contained some OPN- and OC-expressing cells, which were still sensitive to transdifferentiation, because these genes were suppressed under adipogenic conditions. Although chondrogenic differentiation of BMSC and ATSC was indistinguishable in monolayer and remained partial, BMSC responded with improved chondrogenesis to a shift to high-density 3-D cell culture, and reached a gene expression profile highly homologous to that of OA cartilage. Thus, our data corroborate the hypothesis of Zuk et al (25), that adipose tissue contains an omnipotent cell population in which expression of type II collagen protein can be induced. However, ATSC were less sensitive to chondroinductive culture manipulations, and over 2 weeks, their differentiation was less complete than that of BMSC.

Complementary DNA array analysis is a powerful tool for quantitative mRNA profiling of a large number of genes at the same time. Complementary DNA arrays are particularly useful for gene expression profiling of cultured cell populations and tissues containing only one cell type, such as cartilage. The isolation and expansion protocols applied in this study yielded ~99% pure cell populations, according to staining with common surface markers (13,20,40). Nevertheless, heterogeneity may be a minor issue in primary stem cell cultures. All stem cells may not share exactly the same state of commitment, and for ATSC cultures obtained after liposuction, contamination with some pericytes, endothelial cells, or smooth muscle cells was suspected (25,41). Additionally, donor-dependent heterogeneity such as age (42,43), site of harvest (44,45), hormone status (45), and disease (46) may influence the yield and proliferative capacity of stem cells or their osteogenic (47,48), adipogenic (49), and chondrogenic potential (46). We were able to obtain highly homogeneous BMSC and ATSC populations that were negative for CD34 and von Willebrand factor (data not shown).

We intended to focus on robust findings in gene expression analysis which were not prone to bias because of contamination with other cells or were confined to a small, well-defined population of donors. For this reason, we selected BMSC and ATSC samples from individuals with a broad range of ages (8–85 years) and obtained cells from either femoral bone ( $n = 25$ ), iliac

crest ( $n = 11$ ), or subcutaneous adipose tissue obtained at surgery ( $n = 6$ ) or after liposuction ( $n = 10$ ). We eliminated minor effects of our evaluation in cDNA array analysis by selecting a relatively high cutoff for gene expression (5% of the mean level of expression of all housekeeping genes) and considered only changes  $>3$ -fold as valid. Possible donor-specific and site-specific variations were averaged by including 3–8 samples from different donors in each analysis. By using these approaches, we somewhat underestimate variations in gene expression in different groups. In addition, expression of selected genes was confirmed using a second independent method. Thus, incomplete induction of ATSC in chondrogenesis and lack of COL2A expression after 2 weeks was consistent in all tested samples by cDNA array and RT-PCR analyses, and was confirmed by immunohistochemistry.

It is important to note that our study was confined to a single time point (2 weeks after induction of chondrogenesis). Preliminary data suggest that chondrogenesis not only is delayed in ATSC compared with BMSC but also is qualitatively different in spheroid culture. Although the hypertrophic marker COL10 was induced in both BMSC and ATSC, OPN expression was induced only in BMSC, suggesting possible further differentiation toward hypertrophic chondrocytes or osteoblast-like cells. A shift of dedifferentiated chondrocytes toward an osteogenic phenotype has previously been reported in chickens (50), and it will be necessary to study later differentiation stages and the stability of the induced gene expression profiles *in vitro* and *in vivo* before conclusions can be drawn about the inferiority of ATSC as a source of chondrocytes.

Condensation of cells by reduction of intercellular spaces is favorable for chondrogenesis, because the cells that aggregate first will be the first to differentiate, and inhibition of cell aggregation delays chondrogenic differentiation (33). Spontaneous condensation of all cells to one spheroid usually occurred within 1 day in chondrogenic medium, with no obvious differences between ATSC and BMSC cultures. Cell interaction molecules strongly up-regulated in spheroids under chondrogenic conditions included the CD11a antigen  $\alpha$ L integrin, as well as  $\beta$ 1 integrin, which is a ubiquitously expressed integrin that has been previously detected in chondrocytes (51). The  $\alpha$ L integrin, also called lymphocyte function-associated antigen 1 (LFA-1), is a membrane glycoprotein strongly expressed on leukocytes. LFA-1 mediates cell adhesion to activated epithelium expressing intercellular adhesion molecule 1 (52). With respect to cartilage, LFA-1 has been detected in os-

teodestructive joint lesions in rat collagen-induced arthritis (CIA), almost exclusively at sites associated with cartilage erosion and in the vicinity of bone infiltration (53). Compared with control animals, in CIA animals, the LFA-1 phenotype was greatly increased in bone marrow cells (53). This argues in favor of an up-regulation of LFA-1 on marrow mesenchymal stem cells when there is a need for cell condensation as an early step in cartilage regeneration. According to the 71-fold up-regulation in our BMSC spheroid cultures, expression of LFA-1 in pre-cartilage condensation deserves further investigation.

Contactin and bullous pemphigoid antigen 1 (BPAG1) are other contact-mediating molecules up-regulated in the differentiated spheroids. BPAG1 is expressed in epithelial tissues localized to hemidesmosomes and has an actin-binding, an intermediate filament-binding, and a plakin-binding domain. Both molecules have not been detected in chondrocytes or cartilage thus far and may be characteristic for early chondrocytic differentiation stages. Among the wingless- and int-related proteins (Wnts), which have an important role during embryonic development and limb patterning (54,55), Wnt-13 and Wnt-8b were up-regulated in differentiated stem cell spheroids, while other members (Wnt-2, Wnt-5a, and Wnt-10b) were below the 5% expression level. Wnts differentially regulate colony growth and differentiation in chondrogenic rat calvaria cells, and negative effects on colony size and matrix synthesis have been described for Wnt-1, Wnt-3a, Wnt-4, Wnt-7a, and Wnt-7b, while positive effects were observed for Wnt-7a and Wnt-5a (56).

The strongest up-regulation observed in chondrogenic BMSC spheroids (78.6-fold) was for MMP-13 (collagenase 3). MMP-13 is involved in triple helical collagen degradation during matrix remodeling, and its activity was reported to be enhanced in OA articular cartilage (57). Recent data suggest that proteolysis involving MMP-13 is required for chondrocyte differentiation into a hypertrophic state (58) and occurs together with up-regulation of COL10 (which we detected by RT-PCR in BMSC spheroids) but before OC expression (which was suppressed by chondrogenic conditions) (Table 2). OPN expression is a further sign for induction of hypertrophy in differentiated BMSC spheroids (59). Unlike most cartilage, articular cartilage differentiation is arrested before terminal hypertrophic differentiation. Hypertrophy of chondrocytes and high matrix-remodeling activity, however, have been reported to occur in OA (35,37,60), and both effects may be the basis for the strong similarities in gene expression profiles observed

between OA cartilage and differentiating BMSC spheroids in this study. Interestingly, inhibition of MMP-13 activity prevented hypertrophy of chondrocytes, suppressed COL10 expression, and inhibited calcium incorporation into the extracellular matrix (61). MMP-13 inhibition may, therefore, be a means to manipulate hypertrophy in differentiating BMSC spheroids, such as in OA (62).

In conclusion, we generated from mesenchymal stem cells differentiated spheroids exhibiting an OA-like gene expression profile *in vitro*. This *ex vivo* system, providing unlimited sources of human OA-like cartilage tissue, will allow investigators to define in more detail the developmental program from poorly differentiated stromal cells to cartilage, will permit its manipulation by diverse culture conditions, and may facilitate drug development and identification of drug targets in OA outside the human body. According to our data, further optimization of chondrogenic induction will be required to achieve a molecular phenotype similar to that of healthy chondrocytes, as is desired for cartilage cell therapy and tissue engineering.

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