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Premature Induction of Hypertrophy During In Vitro Chondrogenesis of Human Mesenchymal Stem Cells Correlates With Calcification and Vascular Invasion After Ectopic Transplantation in SCID Mice

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Objective. **Functional suitability and phenotypic stability of ectopic transplants are crucial factors in the clinical application of mesenchymal stem cells (MSCs) for articular cartilage repair, and might require a stringent control of chondrogenic differentiation. This study evaluated whether human bone marrow–derived MSCs adopt natural differentiation stages during induction of chondrogenesis in vitro, and whether they can form ectopic stable cartilage that is resistant to vascular invasion and calcification in vivo.**

Methods. **During in vitro chondrogenesis of MSCs, the expression of 44 cartilage-, stem cell–, and bone-related genes and the deposition of aggrecan and types II and X collagen were determined. Similarly treated, expanded articular chondrocytes served as controls. MSC pellets were allowed to differentiate in chondrogenic medium for 3–7 weeks, after which the chondrocytes were implanted subcutaneously into SCID mice; after 4 weeks in vivo, samples were evaluated by histology.**

Results. **The 3-stage chondrogenic differentiation cascade initiated in MSCs was primarily characterized by sequential up-regulation of common cartilage genes. Premature induction of hypertrophy-related molecules (type X collagen and matrix metalloproteinase 13) occurred before production of type II collagen and was followed by up-regulation of alkaline phosphatase activity. In contrast, hypertrophy-associated genes were not induced in chondrocyte controls. Whereas control chondrocyte pellets resisted calcification and vascular invasion in vivo, most MSC pellets mineralized, in spite of persisting proteoglycan and type II collagen content.**

Conclusion. **An unnatural pathway of differentiation to chondrocyte-like cells was induced in MSCs by common in vitro protocols. MSC pellets transplanted to ectopic sites in SCID mice underwent alterations related to endochondral ossification rather than adopting a stable chondrogenic phenotype. Further studies are needed to evaluate whether a more stringent control of MSC differentiation to chondrocytes can be achieved during cartilage repair in a natural joint environment.**

The need to improve therapeutic options for the treatment of focal articular cartilage defects has led clinical researchers to focus on tissue regeneration through autologous cell transplantation. However, to achieve an effective and sustained cartilage repair response, the desired phenotype must be adopted by the transplanted cells, and long-term stability of the cells must be ensured.

Transplantation of chondrocytes has demonstrated clinical success in the treatment of injured knee joints (1–4). However, removal of articular chondrocytes from their natural microenvironment confers a risk of morbidity at the donor site. In addition, ex vivo monolayer expansion before transplantation leads to rapid cell dedifferentiation, including loss of type II collagen ex-

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pression and conversion to a flattened, fibroblast-like phenotype (5–7). Adult mesenchymal stem cells (MSCs) were proposed as an alternate cell source for transplantation because of their promising potential for proliferation and differentiation and their availability from different tissue sources (8,9).

Common in vitro models of chondrogenesis have utilized high cell density and transforming growth factor β (TGFβ) as differentiation-stimulating factors. Our previous studies and those of other investigators provide evidence that bone marrow–derived MSC pellets can be stimulated to up-regulate many genes commonly found in hyaline cartilage, including those for type II collagen (COL2A1) and aggrecan (AGC1), and to produce a proteoglycan-rich extracellular matrix (10–12). However, molecular studies assessing the cell differentiation stages adopted by MSCs in the course of chondrogenesis have yielded variable results, and the patterns of gene expression seemed to depend on the specific induction conditions (10–12). Thus, a better knowledge of the molecular events occurring in response to stimulation with chondrogenic factors is imperative for the future use of MSCs in cartilage repair. It will not be possible to improve experimental protocols and ensure successful tissue repair until the differentiation cascades underlying MSC chondrogenesis are understood in detail and the protocols can guarantee a controlled induction and guidance of cell differentiation toward the desired phenotype.

To finally achieve these goals and to define a set of quality criteria for the differentiation stages, an obvious approach is to compare the molecular phenotype of MSCs upon chondrogenic stimulation with that occurring during natural differentiation of MSCs in vivo. During endochondral ossification, which includes condensation of MSCs, chondrogenic differentiation, hypertrophy, and, eventually, tissue mineralization, the cells transit through successive differentiation stages, each of which is characterized by the expression of particular genes (13,14). Whereas condensation and proliferation of chondroprogenitor cells are accompanied by expression of the type I collagen gene (COL1A1), differentiation into mature chondrocytes includes down-regulation of COL1A1 and induction of COL2A1 as well as the genes for type IX collagen (COL9A1), type XI collagen (COL11A1), link protein, and aggrecan (AGC1). Initiation of the synthesis of the type X collagen gene (COL10A1) and a decrease in the expression levels of type II collagen are characteristic of the morphogenetic transition to the hypertrophic stage. Because of its restriction to hypertrophic cartilage and its calcium-binding properties (15,16), type X collagen is thought to play a role in the transformation of cartilage into bone (17,18). Chondrocytes in the late stage of hypertrophy in calcifying cartilage show some similarities to the molecular phenotype of osteoblasts with regard to the expression of marker genes such as the matrix metalloproteinase 13 (MMP13), alkaline phosphatase, bone sialoprotein type II (IBSP), secreted phosphoprotein type 1 (SPP1, or osteopontin), and runt-related transcription factor 2 genes (19–21).

In contrast to the transient cartilage tissue associated with endochondral ossification, permanent hyaline articular cartilage is spared from hypertrophy. Articular chondrocytes typically reside in a type II collagen–rich matrix devoid of type X collagen. Remarkably, the pathophysiologic changes that occur in articular cartilage may include the induction of hypertrophy-associated genes, such as COL10A1, MMP13, or osteopontin, and the formation of mineralization foci, as, for instance, in osteoarthritis (22–25). Therefore, articular chondrocytes still have the capacity to undergo phenotypic changes and to rearrange their gene expression profile toward induction of hypertrophic marker genes. Nevertheless, in an experimental model involving ectopic transplantation of human chondrocytes into the muscle or skin of nude mice, the chondrocytes formed stable cartilage that was resistant to vascular invasion and calcification. This occurred in the absence of conditions typically found in the joint (26). Since dedifferentiated late-passage chondrocytes lose their ability to form ectopic cartilage and generate only fibrous-like tissue after transplantation, in vivo cartilage formation assays have been recommended for quality control of chondrocyte differentiation (26).

In the context of cartilage cell therapy, MSC-derived chondrocyte-like cells may possess a sufficient degree of plasticity to allow undesired alterations in phenotype. We and other investigators have previously detected COL10A1 messenger RNA (mRNA) by sensitive techniques (11,12) in in vitro–differentiated MSC pellets, indicating that some features of chondrocyte hypertrophy are present in these cells (10); however, no further attention has been given to this observation. When considering the application of MSCs in cartilage repair, it therefore seems to be of prime importance to evaluate the risk of graft instability, which might develop when a true hypertrophic differentiation occurs during stem cell–based cartilage regeneration.

The aim of this study was to dissect the intrinsic differentiation cascade initiated in MSCs in a common in vitro model of stem cell chondrogenesis (8,10–12,27),

Table 1. Samples included in the study

	Osteoarthritis		Normal		
	Hip	Knee	Bone deformation	Scoliosis	Tibia and femur hypoplasia
Type of sample	Bone marrow aspirate	Cartilage	Iliac bone marrow aspirate	Iliac bone marrow aspirate	Cartilage
No. of subjects	7	9	1	1	1
Age range, years	44–78	58–74	11	12	12
No. male/no. female	2/5	3/6	1/0	1/0	1/0
Treatment	Joint replacement	Joint replacement	Osteotomy	Osteotomy	Amputation

with particular focus on the traits of hypertrophic differentiation. We hypothesized that upon chondrogenic stimulation, MSCs would recapitulate natural embryonic differentiation pathways in which articular, but also hypertrophic, phenotypes may be reached. Moreover, we investigated in detail the differentiation stages of MSCs as compared with expanded human articular chondrocytes (HACs) at various time points during chondrogenic differentiation in pellet cultures, to evaluate their capacity to form stable ectopic cartilage-like tissue after transplantation in vivo.

PATIENTS AND METHODS

Collection of human bone marrow. Bone marrow samples were obtained from 9 subjects undergoing total hip replacement or iliac bone graft harvest (Table 1). Irrespective of their origin, all bone marrow samples were regarded as one group. Articular cartilage was obtained from the tibial plateaus of 9 patients with knee osteoarthritis, from regions having no macroscopically evident degeneration, and from the knee of 1 normal subject (Table 1). All participants provided their informed consent, and all studies were approved by the local ethics committee.

Cell isolation and cultivation. MSCs were isolated as described previously (10). Briefly, cells were fractionated by density-gradient centrifugation and seeded in culture flasks in MSC expansion medium (high-glucose Dulbecco's modified Eagle's medium [DMEM], 40% MCDB201, 2% fetal calf serum [FCS], $2 \times 10^{-8}M$ dexamethasone, $10^{-7}M$ ascorbic acid-2-phosphate, 5 μg/ml insulin, 5 μg/ml tranferrin, 5 μg/ml selenous acid, 100 units/ml penicillin–streptomycin, 10 ng/ml recombinant human platelet-derived growth factor BB [all from Sigma-Aldrich, Deisenhofen, Germany], and 10 ng/ml recombinant human epidermal growth factor [Strathmann Biotech, Hamburg, Germany]). Nonadherent cells were removed after 24–48 hours. Colonies of adherent MSCs were expanded and replated 2–3 times at a density of 5×10^3 cells/cm^2. The expansion reached ~16–18 population doublings (PDs) in culture.

HACs were obtained from cartilage regions with no macroscopically evident degeneration, and digested with collagenase B (1.5 mg/ml; Roche Diagnostics, Mannheim, Germany) and hyaluronidase (0.1 mg/ml; Serva, Heidelberg, Germany) as described previously (6). The HACs were seeded at 1.5×10^4/cm^2 either in low-glucose DMEM with 10% FCS and 100 units/ml penicillin–streptomycin or in MSC expansion medium, and maintained in a humidified atmosphere of 6% CO_2 at 37°C. Cells were split at confluency for 2–5 passages (at ~2–6 PDs). For in vivo implantation experiments, 7.5×10^5 HACs were seeded in a T175 flask and harvested at confluency after 3–6 weeks (~3×10^6 cells, at 1.8–2.3 PDs).

Induction of chondrogenic (re)differentiation. For in vitro investigations, pellets were formed by centrifugation from $4–5 \times 10^5$ MSCs or expanded HACs. The pellets were kept in chondrogenic medium (high-glucose DMEM supplemented with 0.1 μM dexamethasone, 0.17 mM ascorbic acid-2-phosphate, 5 μg/ml insulin, 5 μg/ml tranferrin, 5 μg/ml selenous acid, 1 mM sodium pyruvate, 0.35 mM proline, 1.25 mg/ml bovine serum albumin, and 10 ng/ml TGFβ3 [Sigma-Aldrich] [10]) for up to 7 weeks.

Subcutaneous transplantation in SCID mice. Pellets of freshly isolated chondrocytes (n = 5) or of chondrocytes expanded for 2 PDs (n = 5) (1×10^6 cells) or of MSCs (5×10^5 cells) that had been subjected to chondrogenic induction for 3–7 weeks were fixed with fibrin glue to a nonresorbable surgical suture to facilitate handling and recovery at explantation. Four subcutaneous pouches prepared on the backs of male SCID mice (ages 8–10 weeks; Charles River, Sulzfeld, Germany) received identical chondrocyte or MSC implants. Samples were harvested 4–6 weeks later. Animal procedures were approved by the Karlsruhe Local Animal Experimentation Committee (AZ 35-9185.81/G-98/04).

Histologic evaluation. Pellets were fixed in 4% paraformaldehyde–phosphate buffered saline for 2 hours at 20°C, and then sectioned (4 μm) and stained according to standard procedures using toluidine blue (0.03% aqueous solution), Alcian blue (1%; Chroma, Köngen, Germany), or alizarin red (0.5%; Chroma, Münster, Germany) and counterstaining with fast green FCF (0.04% in 0.1% acetic acid; Certistan, Darmstadt, Germany). Immunohistochemical staining was performed as described previously (10) with a mouse anti-human monoclonal antibody to types I and II collagen (clones I-8H5 and II-4C11, respectively; ICN Biomedicals, Aurora, OH) or an antiaggrecan monoclonal antibody (MA75A95; Biozol, Eching, Germany), followed by counterstaining with hemalum.

The detection of type X collagen was performed as described in detail previously (28). Briefly, deparaffinized sections were pretreated with 0.02 mg/ml protease XXIV (Sigma-Aldrich) for 60 minutes at 20°C to optimize staining intensity before detection with mouse anti-human type X collagen monoclonal antibodies (X-34 and X-53, at 1:100) (22) and the Super Sensitive Detection Kit from Biogenex (DCS, Hamburg, Germany); as color substrate, 3-hydroxy-2-napthylacid 2,4-dimethylanilid was used. Nuclei were counterstained with hematoxylin and permanently mounted with Aquatex (Merck, Rahway, NJ). Untreated sections or type II collagen–immunostained sections were further processed for in situ hybridization for human Alu genomic repeats, as described previously (29).

Alkaline phosphatase activity assay. Culture supernatants (100 μl) collected during chondrogenesis of pellets from 5 independent MSC donors were stored at −80°C. For determination of alkaline phosphatase activity, samples were incubated with 100 μl substrate solution (10 mg/ml *p*-nitrophenyl phosphate [Sigma-Aldrich] in 0.1*M* glycine, 1 m*M* $MgCl_2$, 1 m*M* $ZnCl_2$, pH 9.6) and measured spectrophotometrically in a microplate reader at 405 nm after a suitable reaction time, with results referred to the standard curve made from *p*-nitrophenol (Sigma-Aldrich).

RNA isolation. After tissue homogenization of up to 8 parallel pellets in a polytron (Kinematica, Littau-Luzern, Switzerland), total RNA was isolated by guanidinium thiocyanate–phenol extraction (peqGOLD Trifast; Peqlab, Erlangen, Germany). Polyadenylated mRNA was isolated from total RNA using oligo(dT)-coupled magnetic beads (Dynabeads; Dynal, Oslo, Norway) according to the manufacturer's instructions.

Complementary DNA (cDNA) array hybridization. The cDNA arrays were prepared and hybridized as described previously (10). Gene fragments (10 ng/dot) of 48 selected genes (30), including 4 housekeeping genes and negative controls (*Arabidopsis thaliana*), were spotted twice on each filter. The ^{32}P-labeled cDNA probes were prepared from sample mRNA using gene fragment–specific reverse primers (SuperScript II; Life Technologies, Karlsruhe, Germany) and hybridized to cDNA arrays overnight at 68°C. Arrays were exposed to Fuji imaging plates for 18 hours, and images were captured on a Bio-Imaging Analyzer BAS-1800 II using BAS Reader 2.26 beta software (Fuji/Raytest, Straubenhardt, Germany), with results analyzed using AIDA software (Fuji/Raytest). Levels of mRNA expression from different sample sources were normalized to the mean signal strength of the 4 housekeeping genes. Standardization experiments indicated that for reliable quantification, a lower cutoff value for detection should be set at 5% of the mean expression level of the housekeeping genes. The inter- and intraassay variation among several independent experiments indicated that only ≥3-fold alterations in gene expression levels should be considered.

Real-time reverse transcription–polymerase chain reaction (RT-PCR). First-strand cDNA of mRNA was generated by using reverse transcriptase (SuperScript II; Life Technologies) and oligo(dT) primers and purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). To quantify the mRNA levels with the LightCycler (Roche Diagnostics), aliquots of the first-strand cDNA of samples from 5 patients were amplified, and the real-time fluorimetric intensity of SYBR green I was monitored. The $MgCl_2$ and the cycling parameters were optimized according to the LightCycler protocol (Roche Diagnostics). The following forward and reverse primers were used for amplification: for GAPDH, forward 5′-GGGAAGCTTGTCATCAATGG-3′ and reverse 5′-CAGAGGGGGCAGAGATGAT-3′; for caveolin 1 (CAV1), forward 5′-CCGTCTGTGACCCACTCTTT-3′ and reverse 5′-CAGCCAATAAAGCGATGGTT-3′; for COL2A1, forward 5′-TGGCCTGAGACAGCATGAC-3′ and reverse 5′-AGTGTTGGGAGCCAGATTGT-3′; for COL10A1, forward 5′-CCCTTTTTGCTGCTAGTATCC-3′ and reverse 5′-CTGTTGTCCAGGTTTTCCTGGCAC-3′; for MMP13, forward 5′-CTGGAGATATGATGATACTAAC-3′ and reverse 5′-CACGCATAGTCATATAGATACT-3′; for SOX9, forward 5′-CATCCCGCAGACCCACAG-3′ and reverse 5′-TCCCCTCAAAATGGTAATGAATC-3′; and for zyxin, forward 5′-ACTGTGTCCCCGACTACCAC-3′ and reverse 5′-CCTGCATCCCTAGACCATGT-3′. The GAPDH signal was determined once for each cDNA sample and used to normalize the findings for all other genes. For quality control, 5–10 μl of the samples was loaded onto a 1.5% agarose gel containing ethidium bromide, and then electrophoresed and visualized under ultraviolet light.

Statistical analysis. Results are expressed as the mean ± SD or median (interquantile range) for all outcome values. The Mann-Whitney U test and Wilcoxon's test were applied in order to determine whether the differences in outcome parameters were significant. All tests were 2-sided, and a P value less than or equal to 0.05 was considered significant. The value of cDNA array technology is that it enables the simultaneous examination of numerous genes, whereas quantitative RT-PCR is much more sensitive but is restricted to the analysis of one single gene in one reaction. Compared with RT-PCR experiments, however, the cDNA array technique has a higher probability of yielding significant results (Type I errors) during statistical analysis. In accordance with the explorative design of this study, we performed statistical analyses without power adjustments, and used RT-PCR to control relevant data. Data analysis was performed with SPSS for Windows (version 10.0.7; SPSS, Chicago, IL).

RESULTS

Premature up-regulation of hypertrophy-associated genes in MSCs during a 3-phase chondrogenic differentiation cascade in vitro. The intrinsic differentiation program executed in human MSCs was analyzed in pellets derived from different donors at days 0, 1, 2, 4, 7, 14, 28, and 42 after induction. A customized small-scale cDNA array, as described by Steck et al (30), containing a large set of cartilage-related genes was used for gene expression profiling (Figure 1A). Selected genes were analyzed by real-time RT-PCR. Overall, the expression levels of 19 of 44 genes were altered significantly upon chondrogenic induction compared with the starting cell population before induction. The earliest changes occurred directly after chondrogenic stimula-

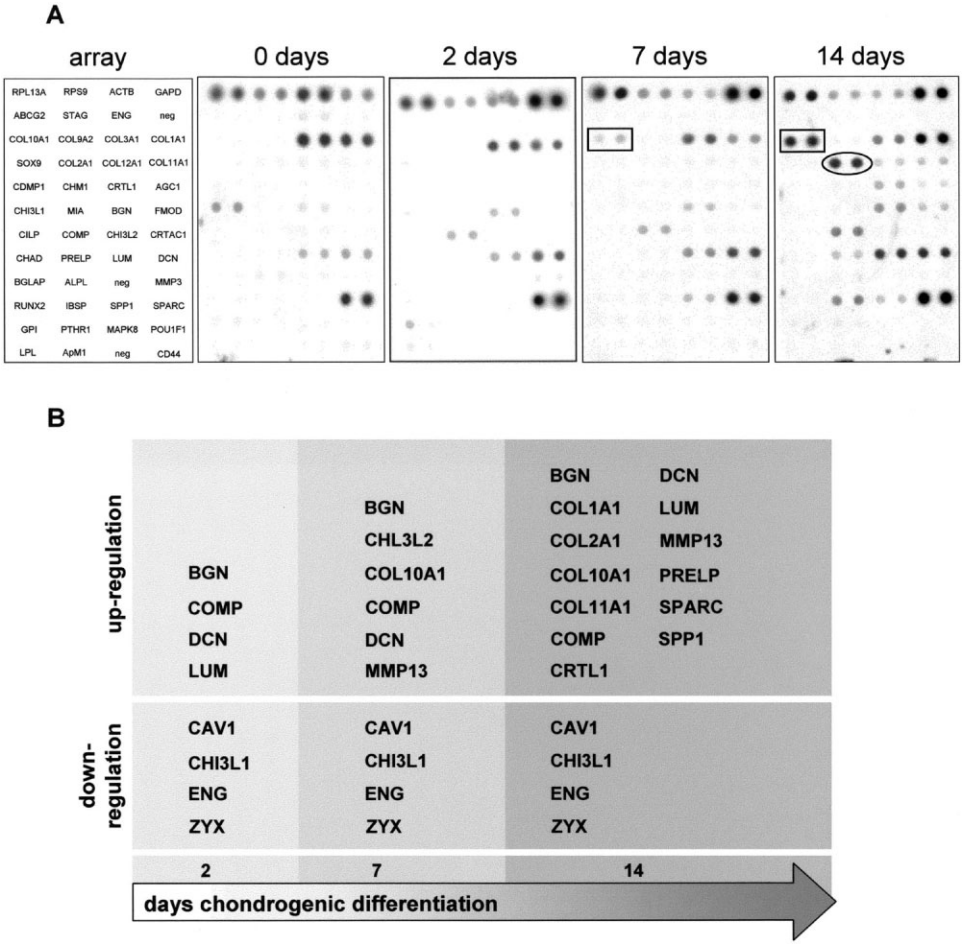

Figure 1. Gene expression profiling of human mesenchymal stem cell (MSC) pellets during chondrogenic differentiation in vitro. Messenger RNA expression profiles were analyzed before and 1, 2, 4, 7, 14, 28, and 42 days after chondrogenic induction, using a customized small-scale cDNA array (see ref. 30) or real-time reverse transcription–polymerase chain reaction (RT-PCR) (n = 5 donors). **A,** Representative gene expression profiles for noninduced MSCs (0 days) and for pellets at 2, 7, and 14 days after chondrogenic induction. COL10A1 mRNA was detectable at days 7 and 14 (rectangles), while COL2A1 transcript was expressed at day 14 only (oval). **B,** Three distinct differentiation stages of MSCs, each characterized by significant alterations in gene expression levels ($P < 0.05$ versus noninduced cells). Data were obtained by cDNA array analysis, except for the MMP13, CAV1, and ZYX genes, which were quantified using real-time RT-PCR. BGN = biglycan; DCN = decorin; ENG = endoglin; LUM = lumican; ZYX = zyxin; neg = negative.

tion, while a broad shift in expression profiles was observed at day 14 (Figure 1A).

The highly reproducible chronologic order of distinct events allowed us to define 3 differentiation phases (summarized in Figure 1B), as follows. In the first stage, 4 genes were down-regulated within 2 days after induction: the chitinase-like molecule CHI3L1 (or YKL40), the MSC marker endoglin, caveolin 1 (a membrane protein of caveolae), and the adhesion plaque protein zyxin. These genes remained in a suppressed state during the entire differentiation process. In contrast, the transcript levels of the extracellular matrix molecules biglycan, cartilage oligomeric matrix protein (COMP), decorin, and lumican increased immediately after chondrogenic induction and continued to rise in the later differentiation stages (Table 2).

The second phase of differentiation, ranging from day 2 to day 7, was characterized by induction of CHI3L2 (or YKL39) and, most remarkably, the hypertrophy-associated genes COL10A1 and MMP13 (Table 2 and Figure 1A). During natural chondrogenic differentiation cascades in vivo, COL2A1 induction always precedes the onset of COL10A1 expression that is initiated during transition to the hypertrophic stage

Table 2. Genes up-regulated in mesenchymal stem cell pellets from 5 donors during chondrogenic differentiation in vitro, according to cDNA array analysis*

	Before induction	Days after chondrogenic induction		
		2	7	14
Biglycan	5.7 ± 1.5	14.7 ± 5.6†	38.0 ± 34.0†	60.0 ± 24.0†
CHI3L2	–	–	6.8 ± 7.3†	5.0 ± 6.3
COL1A1	89.1 ± 32.7	99.0 ± 53.4	52.0 ± 48.1	284.4 ± 161.0†
COL2A1	–	–	–	159.1 ± 155.5†
COL2A1‡	0.1 ± 0.2	0.1 ± 0.1	3.9 ± 7.0	216.8 ± 134.0†
COL10A1	–	–	12.8 ± 5.9†	130.9 ± 55.7†
COL10A1‡	0.0 ± 0.1	0.3 ± 0.2†	11.4 ± 5.4†	83.0 ± 43.7†
COL11A1	–	–	5.3 ± 6.9	18.9 ± 7.2†
COMP	–	18.4 ± 13.7†	70.6 ± 69.1†	92.5 ± 49.1†
CRTL1	–	–	–	22.2 ± 26.5†
Decorin	17.9 ± 8.2	137.7 ± 97.2†	134.7 ± 49.4†	147.5 ± 74.7†
Lumican	10.1 ± 6.5	42.4 ± 31.5†	45.4 ± 30.4	109.8 ± 93.3†
MMP13‡	0.5 ± 1.0	ND	73.0 ± 66.3†	38.0 ± 46.3†
PRELP	–	–	–	20.5 ± 16.1†
SOX9‡	1.9 ± 1.3	ND	5.6 ± 2.7†	5.9 ± 2.3
SPARC	127.6 ± 34.9	198.7 ± 56.8	152.2 ± 77.0	431.3 ± 132.2†
SPP1	–	–	11.2 ± 9.5	26.0 ± 28.7†

* Values are the mean ± SD percent expression levels in relation to the mean expression level in the housekeeping genes (cutoff for detection limit 5%). ND = not determined.
† $P < 0.05$ versus noninduced cells.
‡ Levels were determined by real-time reverse transcription–polymerase chain reaction, with results expressed as a percent of the GAPDH signal.

(31,32). In the present study, COL2A1 mRNA was not yet detectable at the time of appearance of COL10A1 and MMP13. The level of COL10A1 mRNA continued to rise during the entire cultivation period (Table 2).

In the third differentiation stage, occurring during the second week after induction, additional genes commonly found in cartilage were induced or up-regulated, including COL2A1 and COL11A1 and the genes for cartilage linking protein 1 (CRTL1) and proline/arginine-rich end leucine-rich repeat protein (PRELP) (Table 2). In addition to the COL10A1 and MMP13 transcripts, the hypertrophy-associated gene osteopontin (or SPP1) as well as mRNA for osteonectin (or SPARC) and COL1A1 were significantly up-regulated at day 14. The expression pattern at days 28 and 42 was highly similar to that at day 14. Based on gene expression profiling by cDNA array, the third stage was considered to be the final stage of MSC differentiation in vitro.

The premature induction of COL10A1 was confirmed using real-time RT-PCR (Table 2), in both osteoarthritic and nonosteoarthritic donor samples. Using this highly sensitive method, transcripts for COL2A1 and COL10A1 generally appeared earlier than those detected in cDNA array analysis. In fact, COL10A1 expression was already significantly elevated by days 2 and 7, and therefore occurred before the increase in COL2A1, whose up-regulation did not reach a statistically significant level ($P < 0.05$) before day 14. Normalization of the respective signal intensities to standard curves for COL10A1 and COL2A1 demonstrated that the earlier detection of COL10A1 was not an artifact of the hybridization efficiency of the primer sets used (results not shown). In summary, a donor-independent, 3-stage differentiation cascade characterized by early induction of hypertrophic marker genes was initiated in MSCs by this standard chondrogenic stimulation protocol in vitro (Figure 1B).

Absence of induced hypertrophy in dedifferentiated HACs under chondrogenic culture conditions. The culture conditions applied in this in vitro system might be one reason for the premature induction of hypertrophy-associated genes during MSC chondrogenesis. To resolve this question, a comparative analysis with HACs was performed. After monolayer expansion for 46–62 days in MSC expansion medium or in DMEM containing 10% FCS, HAC pellets were formed, exposed to the same chondrogenic conditions as those applied to MSCs, and examined at similar time points (days 0–42). Rapid redifferentiation of HACs toward a mature articular chondrocyte phenotype was expected.

Surprisingly, and in contrast to the strictly se-

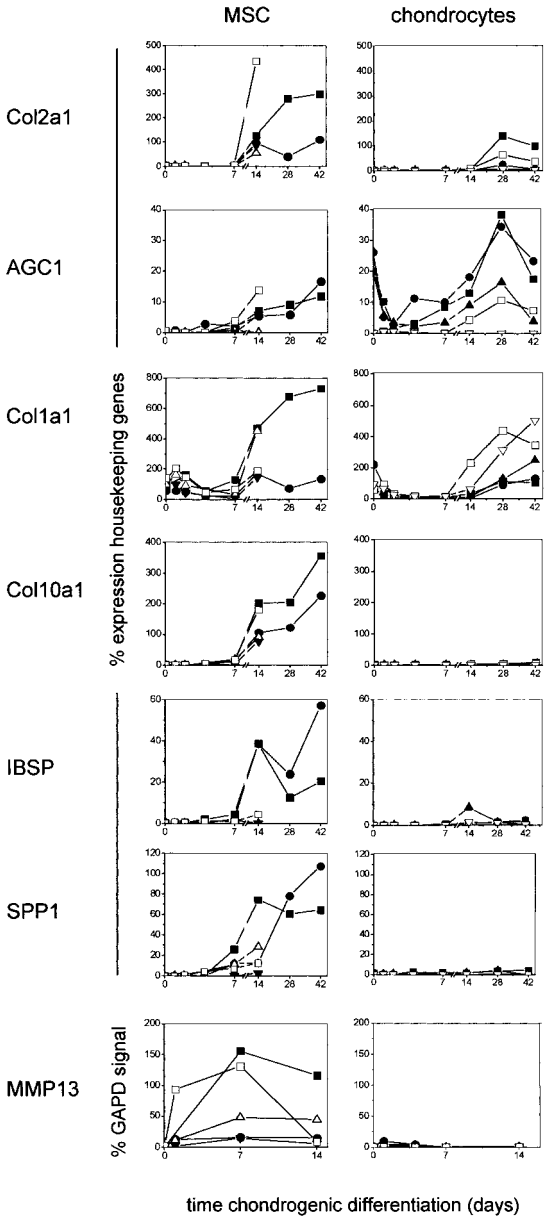

Figure 2. Hypertrophic marker genes induced in human mesenchymal stem cells (MSCs), but not in human articular chondrocytes (HACs) during redifferentiation under culture conditions identical to those for MSC chondrogenesis. Messenger RNA expression levels of genes characteristic of hyaline, fibrous, and hypertrophic cartilage were evaluated using real-time reverse transcription–polymerase chain reaction (for MMP13) or cDNA array analysis (for all other genes). MSCs were differentiated for 0, 1, 2, 4, 7, and 14 days (n = 5) and for 28 and 42 days (n = 2) in pellet culture. Results in the MSCs (left) are represented by solid symbols for osteoarthritic donor samples, and open symbols for iliac bone graft–derived samples. HACs were (re)differentiated for 0, 1, 2, 4, 7, 14, 28, and 42 days in pellet culture (n = 3 donors). Results in the HACs (right) are represented by solid symbols for expansion in Dulbecco's modified Eagle's medium/10% fetal calf serum, and open symbols for expansion in MSC expansion medium (n = 2).

quential up-regulation of gene subsets in MSCs, HACs first underwent a phase of reorganization of gene expression patterns in the course of redifferentiation, and this was characterized by a high variability among donor samples. During the first week, a temporary down-regulation of AGC1 and COL1A1 was observed (Figure 2), in addition to down-regulation of COL3A1, COL11A1, COL12A1, CRTL1, lumican, and osteonectin (results not shown). Subsequent up-regulation of hyaline cartilage markers like COL2A1 or AGC1 was delayed in pellets derived from HACs (occurring at day 28) as compared with those derived from MSCs (occurring at day 14) (Figure 2). After the transient down-regulation of COL1A1 expression, its mRNA levels increased considerably during the redifferentiation of HACs, similar to that in MSC pellets. However, unlike in MSCs, hypertrophic marker molecules like COL10A1, IBSP, osteopontin/SPP1, and MMP13 remained below the detection limit in HAC pellets throughout the observation period.

The real-time RT-PCR findings substantiated a significant difference in COL10A1 induction between MSC and HAC pellets at day 14 (83% and <1.0%, respectively [n = 5]; $P < 0.01$), which was independent of the expansion medium chosen for chondrocytes. Moreover, MMP13 expression was significantly higher in MSCs than in HACs at day 14 ($P < 0.01$), although the expression levels were highly variable among different donors (Figure 2). The fact that a majority of the cartilage-related genes (AGC1, biglycan, cartilage-derived retinoic acid–sensitive protein, cartilage acidic protein 1, COL2A1, COL11A1, COMP, CRTL1, decorin, fibromodulin, PRELP, and SOX9) were up-regulated (results not shown) indicates that the HACs were capable of extensive redifferentiation.

Deposition of type X collagen prior to type II collagen and up-regulation of alkaline phosphatase activity in differentiated MSC pellets in vitro. Immunostaining for type II collagen revealed a considerable heterogeneity both in cell composition and between samples with pellets derived from HACs compared with those derived from MSCs (Figures 3A–F). HAC pellets remained negative for type X collagen staining throughout the observation period (Figures 3J–L), whereas in most of the MSC pellets, deposition of type X collagen started at day 7 in distinct, unclustered cells (Figure 3G) and became evenly distributed after 2 weeks (Figures 3H and I). Consistent with the mRNA expression profile, type X collagen immunostaining (days 7–14) (Figures 3G and H) preceded type II collagen detection (days 14–28) (Figures 3B and C) by ~7 days in MSCs. Late-stage pellets were rich in sulfated proteoglycans

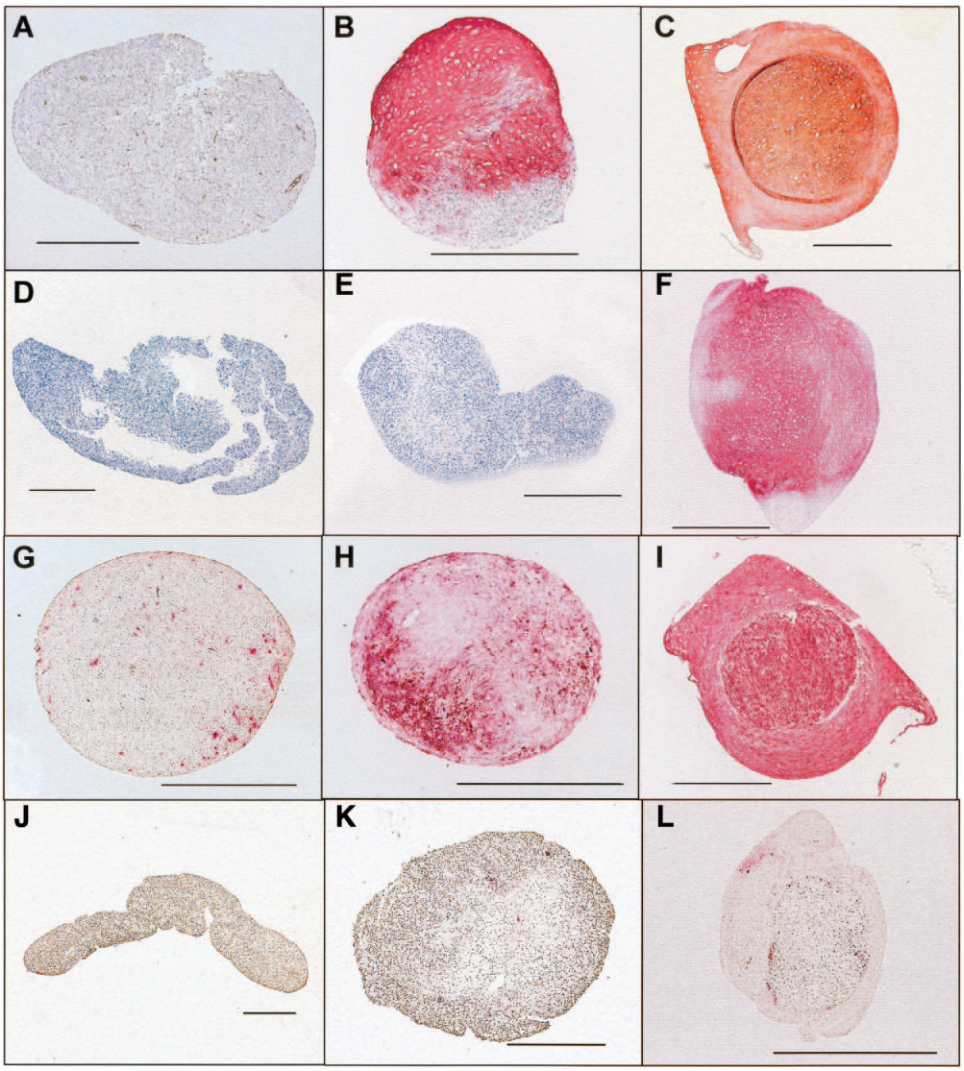

Figure 3. Premature onset of type X collagen expression in mesenchymal stem cell (MSC) pellets and inhibition of type X collagen induction in redifferentiated human articular chondrocyte (HAC) pellets. Production of type II collagen **(A–F)** and type X collagen **(G–L)** was determined by immunohistochemical analysis of paraffin sections of MSC **(A–C** and **G–I)** and HAC **(D–F** and **J–L)** pellets (n = 3 each) at days 7 **(A, D, G,** and **J)**, 14 **(B, E, H,** and **K)**, or 42 **(C, F, I,** and **L)** after chondrogenic induction. HACs were expanded in MSC expansion medium prior to chondrogenic differentiation. Representative micrographs from 3 independent experiments are shown. Note that type X collagen staining preceded type II collagen staining in MSC pellets at day 7 (compare **G** with **A)**. In HACs, the onset of type II collagen staining varied between 2 weeks and 6 weeks and often remained partial; some type II collagen–negative and –positive pellets were derived from the same HAC population. Bar = 500 μm.

and expressed the large proteoglycan aggrecan (results not shown). The third differentiation stage was thus characterized by concomitant deposition of types I, II, and X collagen, indicating the presence of markers characteristic of fibrous cartilage (types I and II collagen), hyaline cartilage (type II collagen), and hypertrophic cartilage (types II and X collagen).

In addition to the induction of hypertrophy-associated genes (COL10A1, MMP13, IBSP, and osteopontin), the transition of chondrocytes to hypertrophy and terminal differentiation involves up-regulation of alkaline phosphatase activity, considerable increase in cell volume, matrix calcification, and, eventually, cell death (33–35). Although the expression of alkaline phosphatase mRNA was below the level of detection in cDNA array analysis (n = 5 donors), alkaline phosphatase enzyme activity increased considerably in the supernatants of MSC pellet cultures at day 21 (Figure 4A),

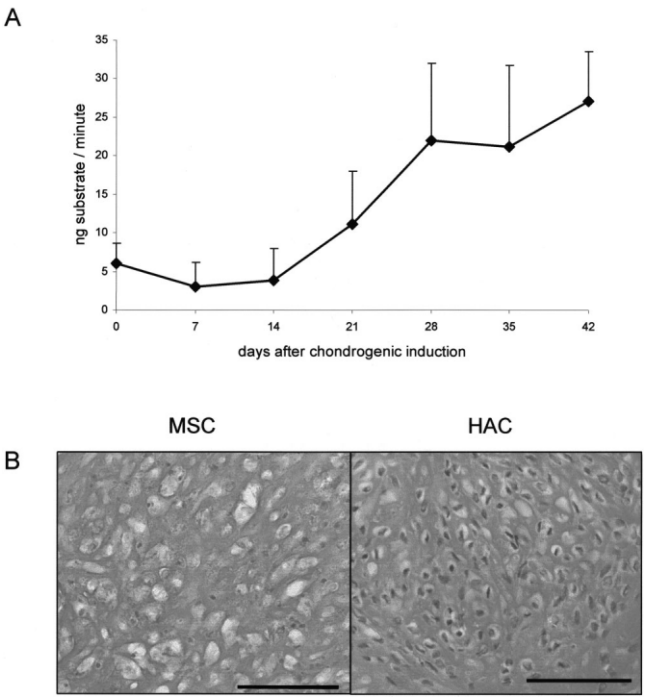

Figure 4. Alkaline phosphatase activity and cell volume in mesenchymal stem cell (MSC) pellets. **A,** Alkaline phosphatase activity in the medium of MSC pellets derived from 5 patients at various time points during chondrogenic differentiation. Enzymatic conversion of *p*-nitrophenyl phosphate was measured at 405 nm in a microplate assay, revealing a considerable increase in activity at day 21. Bars show the mean and SD. **B,** Representative micrographs of MSC and human articular chondrocyte (HAC) pellets (n = 3 each), stained for type II collagen and counterstained with hemalum at 42 days after chondrogenic induction. Bar = 100 μm. Color figure can be viewed in the online issue, which is available at http:www.arthritisrheum.org.

which was ~1 week after the major shift in gene expression defining the third stage of differentiation. Alkaline phosphatase activity remained high (~5.4-fold above baseline) for up to 6 weeks after chondrogenic induction.

During chondrogenesis, the total cell number, measured as the DNA content of the pellets, decreased 3.6- and 6-fold at 3 and 6 weeks, respectively, indicating considerable cell death during differentiation (results not shown). Thus, the enhanced alkaline phosphatase activity of induced, late-stage MSC pellets did not originate from higher cell numbers. Cells with a substantial enlargement of the cell body were, however, clearly outnumbered, similar to those in HAC pellets (Figure 4B), and no mineralization of the matrix was detectable by alizarin red staining in vitro (results not shown).

Histologic findings after subcutaneous transplantation of cartilage-like pellets in SCID mice. We speculated that differences in hypertrophy between HAC and MSC pellets during in vitro treatment may influence the fate of cartilage-like tissue after transplantation in vivo. Pellets derived from freshly isolated articular chondrocytes (n = 5 donors) or chondrocytes expanded for 2 PDs in monolayer culture (n = 5, same donors) were transplanted subcutaneously into the backs of SCID mice. A third group of animals received pellets of MSCs that had been subjected to TGFβ-mediated chondrogenic induction for 3–7 weeks (n = 3).

After 4 weeks in vivo, ectopic stable cartilage had formed from all chondrocyte-derived pellets, which stained positive for Alcian blue (Figure 5A) and type II collagen (Figure 5C) but were negative for alizarin red/fast green (Figure 5B), type X collagen (Figure 5D), and type I collagen (Figure 5E). No obvious differences in the outcome in vivo occurred between freshly isolated chondrocytes and cells that had been expanded for 2 PDs.

Depending on the time of chondrogenic induction, MSC-derived pellets either remained fibrous or disappeared (3 weeks after induction [n = 3]) (results not shown) or were maintained as fibrocartilage-like tissue that stained positive for Alcian blue (Figure 5F), type II collagen (Figure 5H), and type I collagen (Figure 5J) (4–7 weeks after induction [n = 3]). Transplants of MSC pellets were also positive for type X collagen (Figure 5I), and in most of the MSC pellets, extensive calcification of the extracellular matrix was apparent (Figure 5G). Minute ossicles had formed (Figures 5H and I) and vascular invasion was evident after 4–5 weeks in vivo (Figure 5J).

All of the pellets consisted of human-derived cells, as determined by in situ hybridization for human Alu genomic repeats. When parallel MSC pellets were kept under chondrogenic culture conditions in vitro during the time of implantation, they showed intensified deposition of proteoglycan and type II collagen, but no matrix calcification developed (results not shown).

DISCUSSION

Autologous chondrocyte transplantation has demonstrated clinical success in the treatment of focal articular cartilage defects (1,4), but donor-site morbidity, dedifferentiation of chondrocytes during expansion, and lack of quality control instruments for the cellular product remain as limitations for use in clinical practice. Stem cells were proposed as an attractive alternate cell source because of their promising growth and differen-

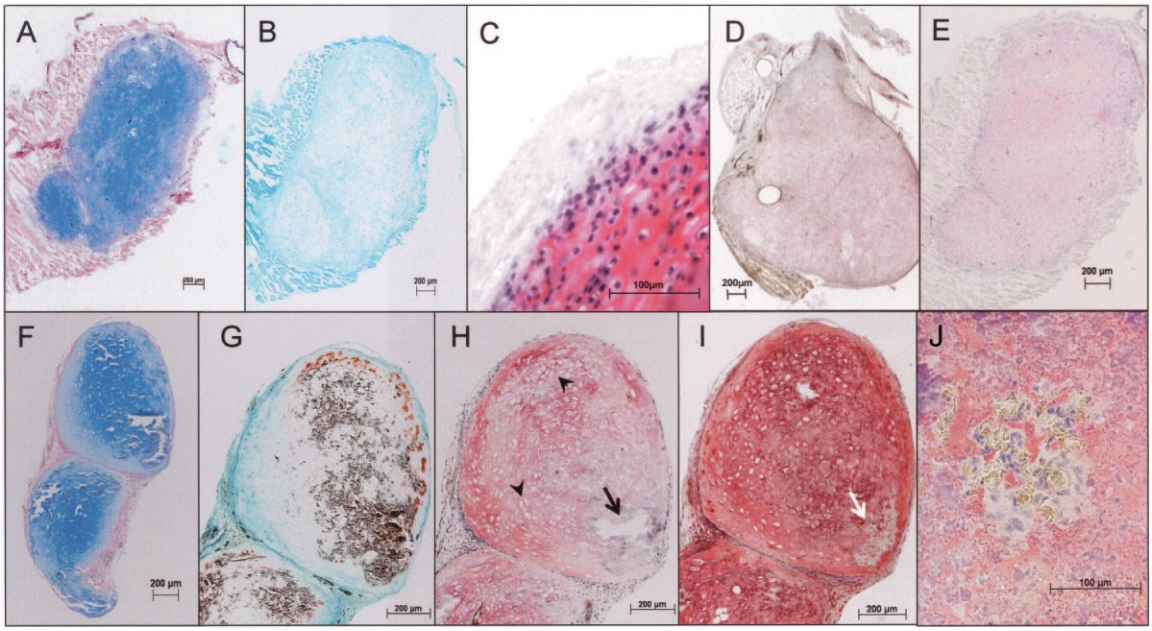

Figure 5. Histologic evaluation of ectopic cartilage-like transplants. Human articular chondrocyte (HAC) and mesenchymal stem cell (MSC) pellets were formed by centrifugation of 1×10^6 freshly isolated chondrocytes **(A–E)** or 5×10^5 MSCs (passage 3) **(F–J)**. Before transplantation, MSC pellets were subjected to 7 weeks of chondrogenic induction in the presence of transforming growth factor β. Four identical pellets per animal were transplanted subcutaneously in the backs of SCID mice and explanted 4 weeks later. Serial paraffin sections were stained for accumulation of proteoglycans (Alcian blue) **(A** and **F)** or deposition of calcium (alizarin red/fast green) **(B** and **G)** or were subjected to immunohistochemical analysis for type II collagen **(C** and **H)**, type X collagen **(D** and **I)**, and type I collagen **(E** and **J)**. Parallel sections stained without primary antibody were negative. While all chondrocyte-derived pellets formed stable ectopic cartilage in vivo, MSC-derived pellets underwent hypertrophy **(I)**, matrix calcification **(G)**, and vascular invasion **(J)**. Note the increase in cell volume **(arrowheads** in **H)** and deposition of minute ossicles **(arrows** in **H** and **I)**. In situ hybridization for human Alu genomic repeats on sections prestained for type II collagen demonstrated that HAC pellets **(C)** and MSC pellets (results not shown) consisted of cells of human origin (black nuclei).

tiation potential and their ability to form cartilage-like tissue upon chondrogenic induction in vitro (10,36). Since functional suitability and phenotypic stability of the transplanted cells are crucial traits for the clinical application of MSCs in cartilage repair, the recapitulation of natural differentiation programs during chondrogenesis of MSCs and the adoption of a stable chondrocyte phenotype are desired.

Natural chondrogenesis is a well-coordinated developmental differentiation program that leads to permanent articular cartilage in the joints or to transient cartilage during endochondral bone formation. The results of this study demonstrate that during in vitro chondrogenesis of adult MSCs, neither stable articular chondrocytes nor the well-synchronized differentiation cascade of endochondral ossification in which type II collagen expression and chondrocyte formation precedes type X collagen detection and hypertrophy of cells (32) was induced. The most evident aspect of these deficiencies was the very early onset of hypertrophic markers, such as COL10A1, within 1–2 days after chondrogenic induction of MSCs and well before the COL2A1 signal became significantly elevated. This phenomenon was equally observed on the protein level. A second aspect was the concomitant induction of a broad panel of hyaline cartilage molecules together with markers for terminal differentiation (MMP13) and osteoblasts (osteopontin and IBSP), which was followed by up-regulation of alkaline phosphatase enzyme activity. A third aspect was a terminal differentiation stage at 6 weeks in vitro, which combined features of fibrous, hyaline, and hypertrophic cartilage. On the basis of these observations, we had to reject the generally assumed hypothesis that natural differentiation cascades of chondrogenesis can be recapitulated by MSCs in this in vitro model.

Heterogeneity is a well-known phenomenon of MSC populations with multilineage capacity (37–39). However, although the differentiation stages sequentially adopted by MSCs clearly differed from known natural chondrogenic differentiation cascades, the triggered program and chronologic order of events were

highly reproducible and constant between MSCs from many donors in cDNA array analysis, although absolute mRNA levels after induction differed between donors according to RT-PCR analysis. This allowed us to define a 3-stage differentiation model based on gene expression analysis, which may be complemented by a fourth differentiation stage that is characterized by up-regulation of alkaline phosphatase activity. We have no evidence to support the idea that every cell in the pellet undergoes only these "mixed," unconventional differentiation stages, all in an identical manner, since this could only be elucidated on the single-cell level. We therefore need to consider the possibility that the final profile may be the summary of a simultaneous differentiation of subpopulations of cells into different lineages, such as chondroblasts plus osteoblasts plus some fibroblasts or even other cells.

Results in the present study support the latter view. First, we observed a speckled staining for type X collagen at 7 and 14 days after induction (Figures 3G and H), which might indicate secretion by only part of the cells. Since matrix molecules such as type X collagen and proteoglycans tend to spread in the extracellular matrix, this dotted staining is lost in the later stages. However, type X collagen expression might be initiated simultaneously in an increasing number of cells. A second argument is that, similar to the findings of other studies (10,11,36), we observed pellets in which only a part of the cells differentiated; only subregions of the pellet stained positive for type II collagen, even at late time points after induction. Analysis of serial sections of ectopic fibrocartilage-like transplants derived from MSCs demonstrated that calcification occurred predominantly in regions with strong proteoglycan and type II collagen deposition. This indicates that a program related to endochondral ossification, but less strictly coordinated than in the growth plate, was induced in MSC pellets. No evidence was obtained to indicate that stable cartilage tissue was induced side by side with direct osteoblast formation, as occurs in intramembranous ossification (Figure 5).

Most remarkaby, no aspects of hypertrophy and terminal differentiation were induced when monolayer-expanded HACs were subjected to identical culture conditions. Consistent with these findings, we achieved formation of stable ectopic cartilage after subcutaneous implantation of HAC-derived pellets, in contrast to the observations with in vitro–differentiated MSC pellets, which underwent extensive calcification and vascular invasion in vivo. This demonstrates that although HACs may down-regulate cartilage differentiation markers during monolayer culture (6), it appears that they do not "forget" their history as stable articular chondrocytes and are not prone to hypertrophic differentiation during expansion (40) or in the presence of TGFβ as an inhibitor of hypertrophy of chondrocytes (41). This suggests that HACs, in contrast to in vitro–differentiated MSCs, acquire an intrinsic and quite stable arrest before hypertrophy, which is independent of systemic factors and may make HAC pellets resistant to vascular invasion and calcification. Developmental memory may, for example, be mediated by epigenetic phenomena such as DNA methylation, which is associated with the silencing of genes and may be able to lock in an inactive state of genes (42,43). Whether the differences between MSCs and HACs described herein are of consequence for the success of autologous cell transplantation needs to be evaluated in appropriate cartilage-defect models in larger animals.

To the best of our knowledge, this study is the first to provide evidence that human adult MSCs derived from bone marrow can be programmed to produce ectopic fibrocartilage rich in proteoglycans and types I, II, and X collagen, and to undergo calcification and vascular invasion consistent with a program related to endochondral ossification. Remarkably, this sequence occurred in the absence of a 3-dimensional carrier and a growth factor depot, and without genetic manipulation of the cells.

When synovial membrane–derived MSC pellets were subjected to in vitro chondrogenesis for 3 weeks before subcutaneous implantion in nude mice, only fibrous transplants were retrieved after 1 week, and cells had disappeared or transdifferentiated by 2 weeks in this and in an alternate in vivo cartilage formation assay in muscle (44). From the previous mouse study (44), the authors concluded that the chondrogenic phenotype induced in MSCs in vitro was unstable in vivo and was not sufficient to guarantee stable lineage and a restriction of differentiation. Consistent with such findings, we found that 3 weeks of preinduction was an insufficient period of time to recover ectopic cartilage-like tissue at later stages. Prolonged in vitro induction of chondrogenesis and careful handling by an attached suture, however, allowed us to recover stable ectopic pellets in the absence of inflammatory reactions. Furthermore, consistent with the premature hypertrophic differentiation cascade initiated by the TGFβ-driven protocol, extension of the in vitro induction up to 7 weeks did not prevent calcification and vascular invasion.

In previous studies, type X collagen expression occurring independent of any chondrogenic induction

and in the absence of type II collagen was repeatedly detected in expanded adult MSC populations (11,45). Findings in human costal cartilage demonstrated that type X collagen expression can be independent of alkaline phosphatase activity, enlargement of cells, and mineralization of the matrix (46). This indicates that markers of the chondrogenic differentiation phases can occur independent of each other, and therefore the static concept of a differentiation program may be inappropriate when MSCs are manipulated in vitro. It is likely that a single-step induction protocol for chondrogenesis, as used currently in in vitro models, will be far too simple to imitate the natural conditions for permanent cartilage differentiation or the feedback loops between distant cell clusters in endochondral ossification (47). Thus, for cartilage repair studies, appropriate means to induce permanent articular chondrocytes are needed.

Before our results are used to question MSCs as a source for cartilage repair, we suggest that common protocols of in vitro chondrogenesis be improved so that permanent chondrocyte differentiation can be induced in the absence of hypertrophy. Second, we wish to emphasize that the subcutaneous environment in mice is not representative of a cartilage repair situation, and therefore further studies should address the performance of MSCs, with and without prior chondrogenic induction, after transplantation in cartilage defects in a joint environment. The combined evaluation of cells and tissue by gene arrays and marker protein analysis will be a valuable approach to improve in vitro models for chondrogenic differentiation of adult MSCs, and will help to develop quality control parameters for tissue engineering, cell therapy, and cartilage repair studies in the future.

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