

# Chondrogenic Differentiation of Human Bone Marrow-Derived Mesenchymal Stem Cells in Self-Gelling Alginate Discs Reveals Novel Chondrogenic Signature Gene Clusters

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We have used a disc-shaped self-gelling alginate hydrogel as a scaffold for *in vitro* chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells. The comparison of monolayer cells and alginate embedded cells with or without differentiation medium allowed us to perform a detailed kinetic study of the expression of a range of genes and proteins known to be involved in chondrogenesis, using real-time polymerase chain reaction, fluorescence immunohistochemistry, and glycosaminoglycan measurement in the supernatant. mRNA encoding type II collagen (COL2), COL10, aggrecan, and SOX5, 6, and 9 were greatly elevated already at day 7, whereas COL1 and versican mRNA were gradually reduced. COL2 and aggrecan were dispersed throughout the extracellular matrix at day 21, whereas COL10 distribution was mainly intra/pericellular. COL1 seemed to be produced by only some of the cells. SOX proteins were predominantly localized in the nuclei. Then, using microarray analysis, we identified a signature cluster of extracellular matrix and transcription factor genes upregulated during chondrogenesis similar to *COL2A1*, and clusters of genes involved in immune responses, blood vessel development, and cell adhesion downregulated similar to the chemokine *CXCL12*. Analysis of the signature chondrogenic clusters, including novel potential marker genes identified here, may provide a better understanding of how the stem cell fate could be directed to produce perfect hyaline cartilage implants.

## Introduction

ARTICULAR CARTILAGE IS CHARACTERIZED by its limited capacity for spontaneous regeneration after injury or degeneration. The treatment of these clinical manifestations is as yet insufficient. Techniques such as microfracture or autologous chondrocyte implantation show some promising clinical effects, but fail to totally restore durable hyaline cartilage.<sup>1,2</sup> Because of these limitations cartilage has become an attractive candidate for tissue engineering approaches.

Chondrocytes expanded during *in vitro* culture will de-differentiate, leading to the production of fibrous instead of hyaline extracellular matrix (ECM).<sup>3</sup> Although chondrocytes from monolayer culture can be redifferentiated to build structural cartilage for generative surgery of the ear or nose,<sup>4,5</sup> there are no successful systems for the production of hyaline cartilage in the joint yet. In addition, the removal of a biopsy from healthy cartilage to obtain cells for *in vitro* expansion may lead to donor-site morbidity.<sup>6,7</sup> Because of these

complications, the use of human mesenchymal stem cells (hMSCs) has become an interesting alternative for tissue engineering of hyaline cartilage.<sup>8</sup> These cells may easily be obtained in the autologous setting, and they proliferate rapidly in culture. Using hMSCs, the best chondrogenic differentiation is obtained in three-dimensional (3D) culture systems. For this a variety of scaffolds have been described, including alginate hydrogels.<sup>9,10</sup> However, the process of *in vitro* chondrogenesis is not yet totally understood, and the composition of the tissue obtained remains suboptimal for clinical use. Typical problems include inadequate expression of hyaline ECM molecules, persistence of fibrous ECM molecules, and the expression of markers of embryological chondrocyte hypertrophy, such as mRNA encoding type X collagen (COL10; gene name *COL10A1*), matrix metalloproteinase 13 (MMP13; *MMP13*), alkaline phosphatase (ALPL; *ALPL*), or runt-related transcription factor 2 (RUNX2; *RUNX2*).<sup>11,12</sup> To optimize the cell characteristics of differentiated MSCs for cell-based therapies, it is therefore crucial to

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understand the underlying processes and regulatory mechanisms.

The aim of this study was to establish a novel model system for 3D chondrogenic differentiation of hMSCs. It is based on self-gelling alginate, which allows even distribution of the cells within a disc the size of potential lesions of hyaline cartilage. This hydrogel permits the deposit of hyaline ECM molecules throughout the intercellular space, which is a prerequisite for the production of a cartilage implant. We show, for the first time, a detailed account of the kinetics of expression of a range of hyaline ECM molecules at the gene and protein level. In addition, mRNA microarray analysis revealed clusters of signature genes specifically altered by the effect of the differentiation cocktail on hMSCs in 3D. We believe that these may be tools that will enable us to better understand the regulatory networks and identify novel potential key components of chondrogenesis.

## Materials and Methods

### Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

### Cultivation and expansion of human bone marrow derived MSCs

The mononuclear cell fractions were isolated from human bone marrows of three healthy voluntary donors (age 24–50). The study was approved by the Regional Committee for Medical Research Ethics, Southern Norway, Section A. Forty to 50 mL of bone marrow was aspirated from the iliac crest and the mononuclear cells were isolated using density gradient centrifugation (Lymphoprep; Fresenius Kabi). The cells were seeded in 175 cm<sup>2</sup> tissue culturing flasks (Nunc) and cultured in the expansion medium containing Dulbecco's Modified Eagle Medium (DMEM)-F12 (Gibco), 1% penicillin/streptomycin (PS), 2.5 µg/mL amphotericin B, and 10% fetal bovine serum (FBS; Cambrex) in 5% CO<sub>2</sub> at 37°C. After 48 h, the nonadherent cells were removed by medium exchange. The adhering cells were expanded in monolayer culture with medium change twice a week until colonies reached 70% confluence. Cultures were passaged using trypsin/EDTA and reseeded at 5000 cells/cm<sup>2</sup>. After the first passage, amphotericin B was removed from the culture medium. For practical purposes, the cells were frozen at passage 2 in DMEM-F12 containing 20% FBS and 5% dimethyl sulfoxide. After thawing, cells were reseeded at a density of 5000 cells/cm<sup>2</sup> and passaged when 70% confluent.

### Validation of cells as MSCs

Before using the cells for differentiation experiments in passage 3 or 4, they were validated as MSC using flow cytometry and differentiation procedures. The cells were labeled with the following mouse anti-human monoclonal antibodies: CD19-APC, CD105-APC, and HLA-DR-APC (Diatec); CD34-FITC, CD44-FITC CD45-PE, and CD73-PE (BD Biosciences); and CD90-PE (AbD Serotec) and CD14-FITC. Irrelevant control monoclonal antibodies (Diatec) were

included for all fluorochromes. Analysis was performed using a FACSCalibur flow cytometer (BD Biosciences).

The multipotency of the MSCs was validated by differentiation to osteogenic and adipogenic lineages as described previously.<sup>13</sup> For osteogenic differentiation cells were seeded at 3000 cells/cm<sup>2</sup> and differentiated using DMEM/F12 medium containing 10% FBS, 10 mM β-glycerophosphate, 100 nM dexamethasone, and 0.05 mM L-ascorbic acid-2-phosphate. The medium was changed every 3–4 days. After 3 weeks, the cells were washed with phosphate-buffered saline (PBS), then fixed for 1 h with 1% paraformaldehyde, and rinsed with PBS. Mineralization was observed by staining with 40 mM Alizarin Red S (pH 4.2) for 5 min. For adipogenic differentiation cells were seeded at 50,000 cells/cm<sup>2</sup> and cultured for 24 h using DMEM/F12 containing 10% FBS and 1% PS. The confluent cultures were then differentiated by adding 10 µg/mL insulin (Novo Nordisk), 0.5 µM 1-methyl-3 isobutylxanthine, 1 µM dexamethasone, and 100 µM indomethacin (Dumex-Alpha). The medium was changed every 3–4 days. After 3 weeks the cells were washed with PBS and then fixed for 1 h with 1% paraformaldehyde and washed in 50% isopropanol. To observe lipid droplets, the cells were incubated for 10 min with Oil-Red O, washed in isopropanol, and subjected to nuclear staining with hematoxylin. As a negative control 3000 cells/cm<sup>2</sup> cells were seeded and cultured using DMEM/F12 containing 10% FBS and 1% PS. The medium was changed every 3–4 days and after 3 weeks stainings for Alizarin Red S, Oil-Red O, and hematoxylin were performed.

### In vitro chondrogenic differentiation

The self-gelling alginate system was obtained from NovaMatrix. It consists of a lyophilized and milled calcium-alginate particle mixture, which slowly releases calcium ions upon contact with soluble alginate solutions.<sup>14,15</sup> PRONOVA-LVG alginate and calcium-alginate particles from the self-gelling system were prepared to a final alginate concentration of 1% using a 4.6% mannitol solution. MSCs were washed with DMEM to remove serum, and 4 × 10<sup>6</sup> cells were resuspended in 400 µL of the PRONOVA-LVG solution and transferred to 12-well cell culture plates (Nonstick; Nunc). Four hundred microliters of the calcium-alginate particle solution was added, and the cell/alginate suspension was thoroughly mixed. The gelling process was supported by washing with a 50 mM SrCl<sub>2</sub> solution. After stabilization of the alginate structure the disc, now with 5 × 10<sup>6</sup> cells/mL alginate, was washed three times with DMEM and normal MSC expansion medium was added for undifferentiated 3D controls, while chondrogenic differentiation was induced by high-glucose DMEM (4.5 g/L) supplemented with 1 mM sodium pyruvate (Gibco), 0.1 mM ascorbic acid-2-phosphate, 0.1 µM dexamethasone, 1% ITS (insulin 25 µg/mL, transferrin 25 µg/mL, and sodium selenite 25 ng/mL), 1.25 mg/mL human serum albumin (Octapharma), 500 ng/mL bone morphogenic protein-2 (InductOs; Wyeth Taplow), and 10 ng/mL recombinant human transforming growth factor-β1 (TGF-β1, R&D Systems, Minneapolis, MN). For the first 3 days the medium was changed daily, subsequently every 2–4 days. Samples were collected at day 7, 14, or 21. The viability was determined by acridine orange/ethidium bromide staining of the cells before and after degelling.

### Fluorescence immunohistochemistry

Primary antibodies and all working concentrations are specified in Table 1. Alexa488-conjugated goat anti-rabbit secondary antibody (used at 5 µg/mL) was purchased from Invitrogen and Cy3-conjugated donkey anti-mouse IgG (used at 2 µg/mL) and Cy3-conjugated donkey anti-rat IgG (used at 12.5 µg/mL) were purchased from Jackson Immuno Research. Formalin-fixed, paraffin-embedded samples of cells in alginate were sectioned and deparaffinized using standard laboratory procedures and postfixed for 10 min in 4% paraformaldehyde in PBS (Electron Microscopy Sciences). Tissue sections were boiled for 20 min in 0.05% citraconic anhydride in ddH<sub>2</sub>O (pH 7.4), incubated with primary antibodies diluted in PBS/1.25% bovine serum albumin with 0.1% saponin for permeabilization overnight at 4°C, followed by secondary reagents for 1.5 h at room temperature. Double staining with mouse and rabbit reagents was achieved by mixing the primary antibodies and the fluorochrome-conjugated reagents, respectively. Stained sections were mounted using ProLong Gold antifading reagent with DAPI (Invitrogen). Microscopy was performed with a Nikon Eclipse E-600 fluorescence microscope equipped with Nikon Plan-Fluor objective lenses and a Color View III digital camera controlled by Cell-B software (Olympus; www.olympus-global.com/en/). For reasons of homogeneity all protein stainings in Figures 2B and 5B are shown in red.

### Determination of sulfated glycosaminoglycan content

The supernatants of differentiated cells and the undifferentiated control cells were analyzed for sulfated glycosaminoglycan content using the Blyscan™ Sulfated

Glycosaminoglycan Assay kit (Biocolor Ltd.) following the company's protocol. At all time points the medium had been unchanged for 3 days.

### Total RNA extraction

For RNA isolation of undifferentiated monolayer MSCs, 10<sup>6</sup> cells were pelleted, dissolved in 1 mL TRIzol (Invitrogen), frozen in liquid nitrogen, and stored at -80°C until used. To obtain single cells for RNA isolation, the alginate discs were depolymerized by enzymatic digestion using G-Lyase (kindly provided by professor Gudmund Skjåk-Bræk). Briefly, gel slices were washed with PBS to remove serum. Discs were digested with 5 U/mL G-Lyase on a thermo shaker at 37°C for 30 min in a volume of 1 mL/disc. The cells were washed and counted. Cells were from now on treated like the monolayer control cells. RNA was isolated following the TRIzol protocol (Invitrogen). Total RNA was quantified using NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies).

### Quantitative real-time reverse transcription–polymerase chain reaction

After DNase I treatment (Ambion), reverse transcription (RT) was performed according to the manufacturer's protocol (Applied Biosystems) using 200 ng total RNA per 50 µL RT reaction. The cDNA samples were analyzed by quantitative real-time polymerase chain reaction (PCR) using primers from Applied Biosystems (Table 1). After verifying the stable expression of *GAPDH*, this gene was included as endogenous control. Calculation for relative expression

TABLE 1. PRIMERS USED IN REAL-TIME REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION AND ANTIBODIES USED IN IMMUNOHISTOCHEMISTRY

| Protein                                     | Gene symbol | Primer for RT-PCR (TaqMan assay no., Applied Biosystems) | Antibodies for immunohistochemistry |               |                    |
|---|-------------|--|-------------------------------------|---------------|--------------------|
|   |             |  | Designation (concentration)         | Specification | Company            |
| Type I collagen (COL1)                      | COL1A1      | Hs00164004_m1  | I-8H5 (1 µg/mL)                     | Mouse IgG2a   | MP Biomedicals     |
| Type II collagen (COL2)                     | COL2A1      | Hs00264051_m1  | II-4C11 (0.83 µg/mL)                | Mouse IgG1    | MP Biomedicals     |
| Type X collagen (COL10)                     | COL10A1     | Hs00166657_m1  | X53 (1:200)                         | Mouse IgG1    | Klaus von der Mark |
| SRY -box containing gene 5 (SOX5)           | SOX5        | Hs00374709_m1  | ab26041 (0.67 µg/mL)                | Rabbit        | Abcam              |
| SRY -box containing gene 6 (SOX6)           | SOX6        | Hs00264525_m1  | HPA001923 (1.25 µg/mL)              | Rabbit        | Sigma              |
| SRY -box containing gene 9 (SOX9)           | SOX9        | Hs00165814_m1  | AB5535 (0.2 µg/mL)                  | Rabbit        | Millipore          |
| Aggrecan (ACAN)                             | ACAN        | Hs00202971_m1  | 969D4D11 (4.55 µg/mL)               | Mouse IgG1    | BioSource          |
| Cartilage oligomeric matrix protein (COMP)  | COMP        | Hs00164359_m1  | —                                   | —             | —                  |
| Versican (VCAN)                             | VCAN        | Hs01007941_m1  | MAB3054 (2.5 µg/mL)                 | Rat IgG1      | R&D                |
| Matrix metalloproteinase 13 (MMP13)         | MMP13       | Hs00233992_m1  | —                                   | —             | —                  |
| Alkaline phosphatase (ALPL)                 | ALPL        | Hs00758162_m1  | —                                   | —             | —                  |
| Runt-related transcription factor 2 (RUNX2) | RUNX2       | Hs00231692_m1  | —                                   | —             | —                  |
| SRY -box containing gene 8 (SOX8)           | SOX8        | Hs00232723_m1  | H-95 (0.33 µg/mL)                   | Rabbit        | Santa Cruz         |

RT-PCR, reverse transcription–polymerase chain reaction.



changes were performed using the relative standard curve method (ABI Prism 7700 Sequence Detection System, User Bulletin 2; PE Applied Biosystems) and normalized to *GAPDH* expression.

#### Microarray hybridization procedure and bioinformatics analyses

Total RNA was analyzed by Illumina's beadarray technology at the Norwegian Microarray Consortium using the manufacturer's protocols. Briefly, biotin-labeled cRNA was synthesized from 415.8 ng total RNA using the Illumina® TotalPrep RNA Amplification Kit (Ambion). About 1.5 µg of cRNA was hybridized onto Illumina Human-6 v3 Expression Beadchips containing 48,000 transcripts (Illumina) at 58°C overnight. The hybridized chip was stained with streptavidin-Cy3 using FluoroLinkTMCyTM3 (Amersham) and scanned with an Illumina beadarray reader. The images were imported into Illumina GenomeStudio V2009.1, Gene Expression version 1.1.1 for extraction, quality control, and quintile normalization. The array annotation file "HumanWG6\_V3\_0\_R2\_11282955\_A.bgx" was used. The resulting data were exported to J-express 2009 (MolMine) for expression analysis. After mean normalization and log2 transformation of the data, we performed statistical analysis to find differentially expressed genes using feature subset selection.<sup>16</sup> As cut-off level for differentially expressed genes, we used >2-fold expression change (log2 ratio >1) and  $p < 0.05$ . To get a first overview of the functional changes during chondrogenic differentiation, we performed gene set enrichment analysis (GSEA) comparing monolayer cells to the different time points of differentiation ([www.broad.mit.edu/gsea/doc/GSEAUUserGuideFrame.html](http://www.broad.mit.edu/gsea/doc/GSEAUUserGuideFrame.html)).<sup>17,18</sup> Next we performed similarity search to detect common expression patterns in the dataset. We used Euclidean distance measurement and looked for the 5% most similarly changed genes of the differentially expressed genes, using *COL2A1* and *CXCL12* as index genes. Gene ontology (GO) annotation was performed on these similarity lists to analyze the functional characteristics of these signature clusters.

## Results

### 2D and 3D cultures

At the end of 2D expansion the cells were validated as MSCs. By flow cytometry they were verified to express CD105, CD73, CD90, and CD44, and were negative for CD34, HLA-DR, CD45, CD14, and CD19 (Supplementary Fig. S1; Supplementary Data are available online at [www.liebertonline.com/tea](http://www.liebertonline.com/tea)). The MSCs from all three donors were found to differentiate to osteogenic and adipogenic lineages (Supplementary Fig. S2). After the gelling procedure the cells were embedded within a disc-shaped hydrogel. The disc retained its shape, and the cells showed a rounded morphology and maintained high viability throughout the chondrogenic differentiation (Supplementary Fig. S3).

### Gene expression kinetics

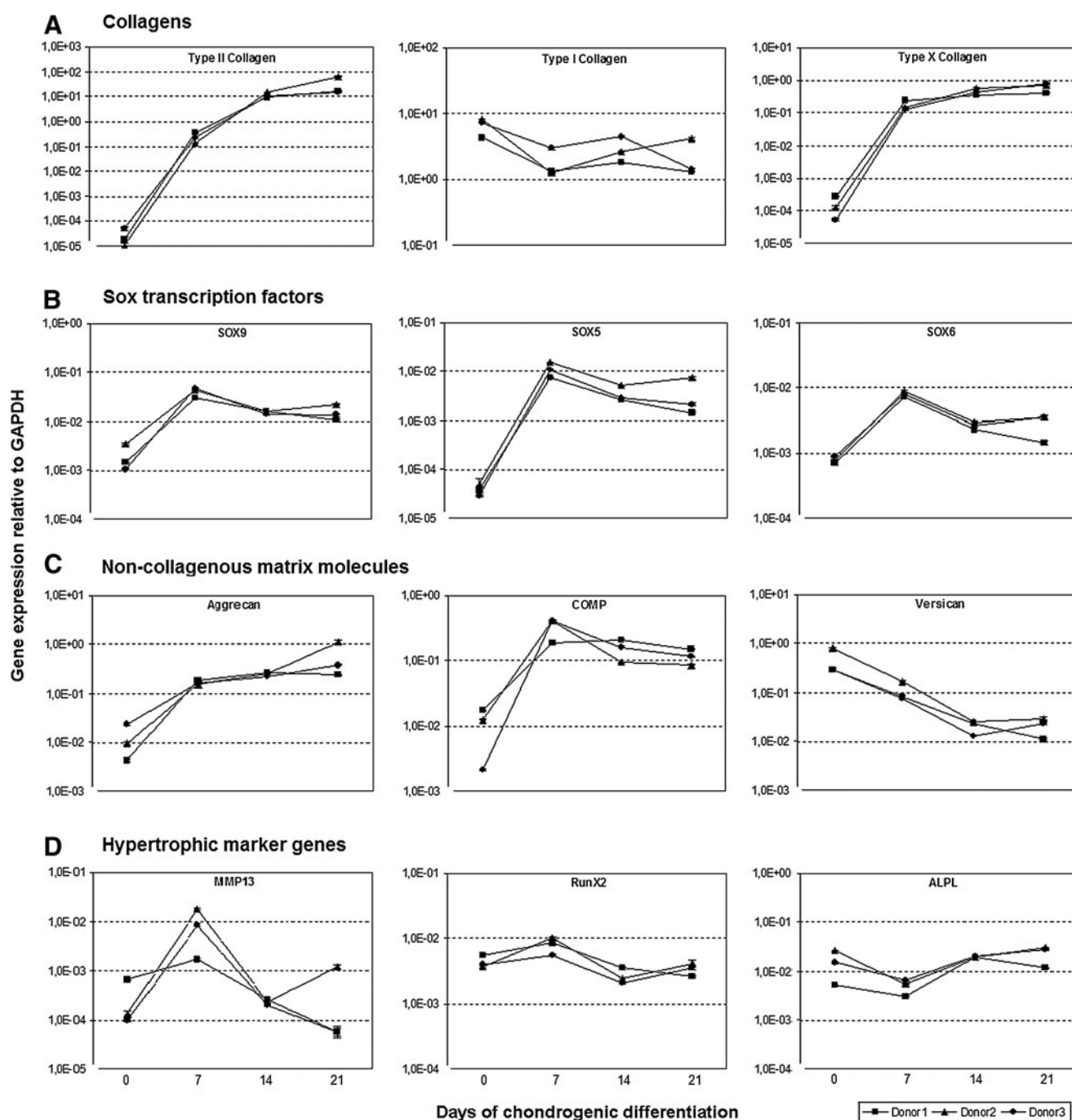
To characterize the kinetics of the changes in gene expression occurring as a consequence of chondrogenic differentiation in 3D hydrogel we performed quantitative

real-time RT-PCR of a number of well-known chondrogenic marker genes. The collagen profile (Fig. 1A) showed a rapid and sustained increase of COL2 and COL10 mRNA from background and near-background levels. On day 21 the *COL2A1/COL10A1* ratio was around 50. COL1 mRNA was expressed at high levels in monolayer MSCs, and fell slightly in the course of differentiation. The *COL2A1/COL1A1* ratio on day 21 was ~13. COL2 synthesis is thought to be regulated predominantly by the SOX transcription factor triad SOX5, 6, and 9. mRNAs encoding these factors increased from very low constitutive levels to peak at moderate levels on day 7, followed by a slight decrease through day 21 (Fig. 1B). For the noncollagenous matrix molecules associated with chondrogenesis (Fig. 1C), we detected an increase in mRNA levels of aggrecan (ACAN) and cartilage oligomeric matrix protein (COMP), whereas the mRNA level of versican (VCAN) decreased considerably over time. Substantial interest is focused on the importance of markers of chondrocyte hypertrophy during embryogenesis for *in vitro* chondrogenesis. In addition to *COL10A1* we examined the hypertrophic markers *MMP13*, *RUNX2*, and *ALPL* (Fig. 1D). *MMP13* peaked on day 7, whereas *RUNX2* and *ALPL* remained stable or were slightly downregulated. From day 14 onward none of the three marker genes reached mRNA expression levels much above those observed for monolayer control cells.

### Protein expression analysis

To characterize kinetics at the protein level, we performed co-staining of COL2 and SOX9 on days 7, 14, and 21 of differentiation (Fig. 2A). SOX9 could be detected in approximately half of the cells on day 7 and showed strict nuclear localization. Throughout this time line there were always some SOX9 negative nuclei. Around 2 weeks of differentiation the cells started producing COL2, and on day 21 COL2 was detected as a strong, diffusely distributed extracellular signal around most of the cells. We saw no intracellular COL2 staining. Many of the cells were positive for both signals, but not all of the COL2-producing cells were SOX9 positive, and a strong SOX9 signal was not necessarily associated with COL2 expression.

To further characterize our differentiation system we examined the protein expression of chondrogenic markers at day 21 of differentiation (Fig. 2B). Staining for COL1 showed an extracellular signal around less than half of the cells. COL10 gave a strong predominantly intracellular cytoplasmic signal in a majority of the cells but was not detected in the matrix at this time. On day 21 of differentiation SOX5 was expressed by almost all the cells and showed a nuclear localization as well as a distinct perinuclear, cytoplasmic signal. SOX6 was also expressed in a majority of cells but showed an exclusively nuclear staining similar to SOX9. Like COL2, aggrecan was widely distributed in the extracellular space. However, COL2 was seen exclusively in the extracellular space, whereas aggrecan was detectable also inside the cells. Versican was predominantly detected in the cytoplasm of a great majority of cells, with an occasional extracellular halo. We detected high levels of glycosaminoglycans in the culturing medium, increasing with state of differentiation, whereas the undifferentiated control cells in alginate did not produce glycosaminoglycans (Fig. 3).

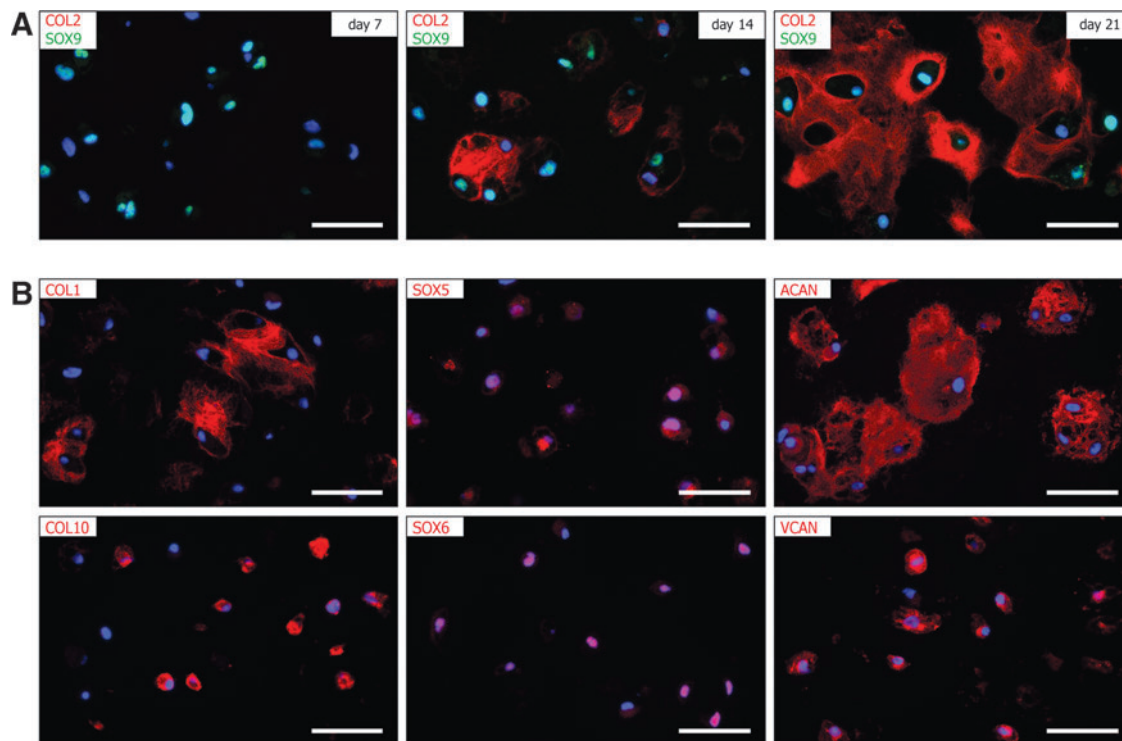


**FIG. 1.** Quantitative real-time reverse transcription–polymerase chain reaction analysis of genes expressed in human bone marrow (hBM)–mesenchymal stem cells (MSCs) undergoing chondrogenic differentiation in self-gelling alginate scaffold. Samples from three different donors were gathered at days 0, 7, 14, and 21. Shown values are the mean and SD of triplicate determination of each single donor, normalized to the expression of the housekeeping gene *GAPDH*. The mRNA expression profiles shown are for (A) type II, I, and X collagen; (B) the chondrogenesis-associated transcription factors SOX9, SOX5, and SOX6; (C) the noncollagenous matrix molecules aggrecan (ACAN), cartilage oligomeric matrix protein (COMP), and versican (VCAN); and (D) the hypertrophic marker genes matrix metalloproteinase 13 (MMP13), runt-related transcription factor 2 (RUNX2), and alkaline phosphatase (ALPL).

*Transcriptional profiling of differentiated cells by mRNA microarray analysis allows identification of chondrogenic signature clusters*

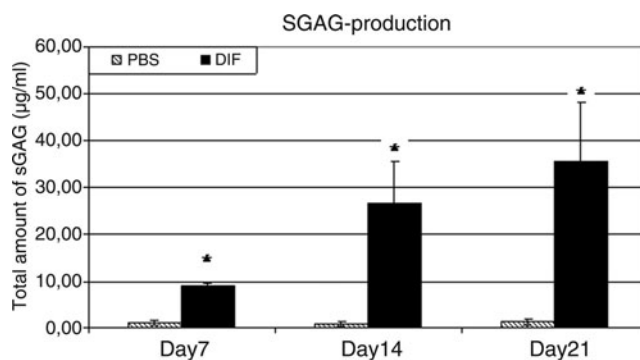
mRNA microarray analysis revealed 1969 genes that were differentially expressed ( $p < 0.05$ ;  $> 2$ -fold change) between

monolayer MSCs and chondrogenic cells at least at one of the examined time points, 1072 downregulated and 898 upregulated. GSEA in differentiated cells compared with monolayer MSCs revealed a significant upregulation of genes associated with skeletal system development and ECM at all time points (Supplementary Fig. S4). Enriched gene sets in



**FIG. 2.** Fluorescence immunohistochemical analysis of protein expression in chondrogenically differentiated hBM-MSCs. **(A)** Co-staining of SOX9 (green) and COL2 (red) in samples derived after 7, 14, and 21 days of chondrogenic differentiation of hBM-MSCs in alginate. **(B)** Expression on day 21 of chondrogenic differentiation of the COL1 and COL10, SOX5, and SOX6 transcription factors and the noncollagenous matrix molecules ACAN and VCAN at day 21 of differentiation. Nuclei are counterstained with DAPI (blue). Scale bars = 50  $\mu$ m.

MSCs depended on the time point during differentiation that was used for comparison. Genes associated with, for example, blood circulation or activation of leukocytes were significantly enriched in MSCs compared with early differentiation time points, whereas genes associated with mitosis or cell division dominated the list of enriched genes in MSCs

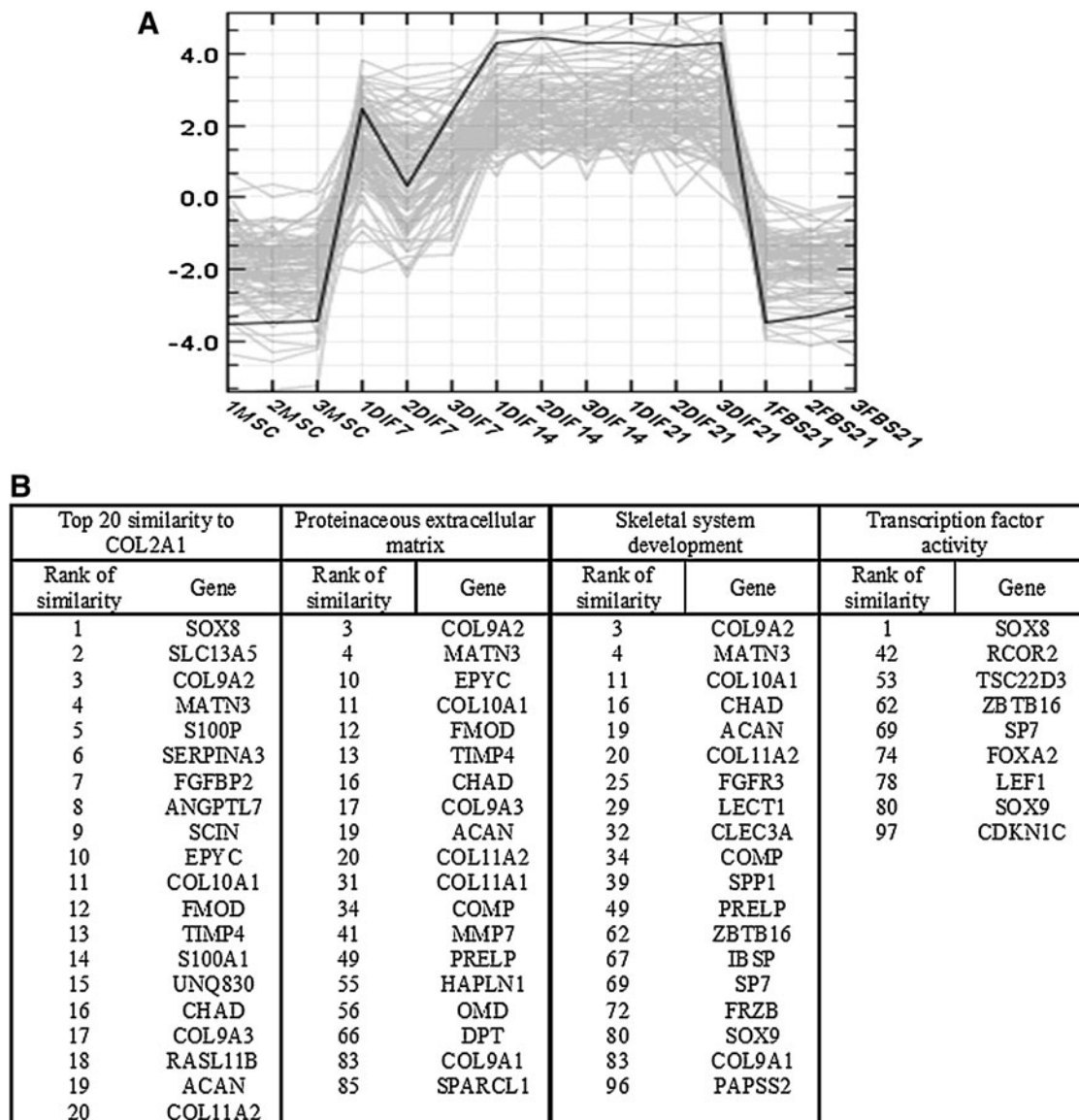


**FIG. 3.** Synthesis of sulfated glycosaminoglycans (sGAG) during chondrogenic differentiation of hBM-MSC in alginate discs. sGAG concentrations were measured in supernatants from days 7, 14, and 21 in cultures of hBM-MSC in alginate discs maintained in the culture medium (hatched bars) and in the chondrogenic differentiation medium (dark bars). The concentration of synthesized sGAG in the supernatant was measured after 3 days without changing the medium. Data presented are mean values for the three donors, each measured in triplicate. \* $p < 0.05$ .

compared with later differentiation time points (Supplementary Fig. S4). This gave us a first idea of the main functional changes during chondrogenesis: the cells undergo morphological changes and lose their MSC-specific properties, gradually reaching a resting, nonproliferative stage while upregulating chondrogenic genes, especially those involved in matrix production.

To further characterize chondrogenic signature gene clusters and identify novel potential regulating molecules, we performed a similarity search of differentially expressed genes and looked for expression patterns most similar to the chondrocyte marker *COL2A1* and the MSC marker *CXCL12*, which encodes a chemokine secreted by MSCs and their offspring to promote homing of hematopoietic cells to their bone marrow niches.<sup>19</sup> The *COL2A1* cluster, like the real-time RT-PCR analysis of *COL2A1*, showed a rapid increase in expression from monolayer to day 7, and a moderate increase from day 7 to 14, where the expression stabilized (Fig. 4A). The figure also shows that the changes within this gene cluster are entirely induced by the combination of 3D and the differentiation cocktail, and not by 3D alone. GO terms significantly upregulated within this cluster included ECM, skeletal system development, and transcription regulator activity (Fig. 4B). Among the individual genes within this cluster we found genes encoding collagens *COL9A2*, *COL10A1*, *COL9A1*, *COL9A3*, *COL11A1*, *COL11A2*, as well as other well-known hyaline matrix molecules such as *ACAN*, *COMP*, and *HAPLN1*, which encodes the link protein. A number of genes encoding proteins with a modifying effect on chondrogenesis were upregulated, including epiphysean



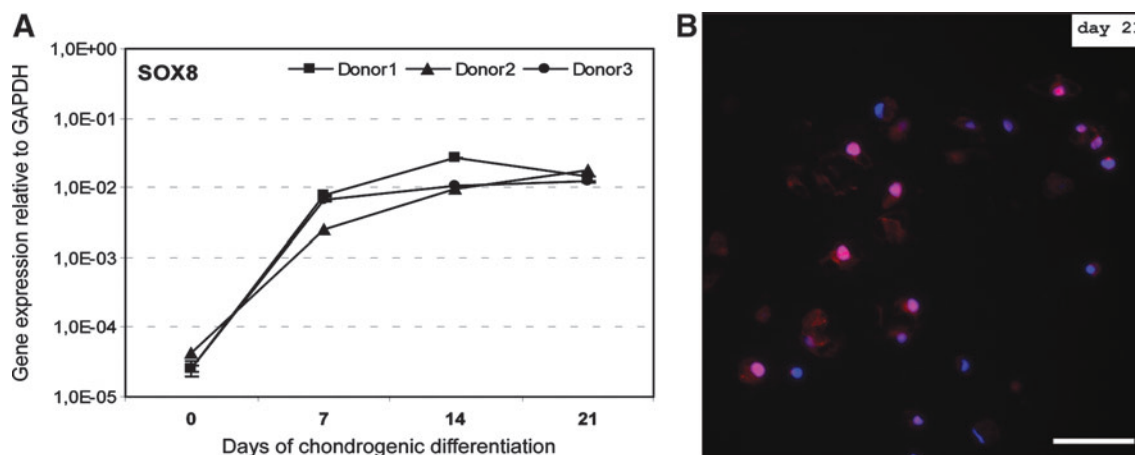


**FIG. 4.** (A) Expression profile of genes obtained from *COL2A1* similarity search. Data were obtained from three donors under five different conditions: undifferentiated monolayer cells (MSC), 7 days of chondrogenic differentiation (DIF7), 14 days of chondrogenic differentiation (DIF14), 21 days of chondrogenic differentiation (DIF21), and undifferentiated three-dimensional control cells, which were cultured in alginate for 3 weeks using 10% fetal bovine serum (FBS; FBS21). The 98 shown genes (shown in gray) are the 5% most similar differentially expressed genes ( $>2$ -fold change,  $p < 0.05$ ) compared to expression of *COL2A1* (shown in black). Relative expression levels ( $\log_2$  ratios) after mean normalization are shown. (B) The 20 most similar genes (left column) and genes within the 5% most similar differentially expressed genes belonging to three selected gene ontology (GO) terms are shown together with their similarity rank number relative to *COL2A1* expression. All of these GO terms were significantly upregulated in the similarity cluster ( $p < 0.001$ ).

(*EPYC*), matrilin 3 (*MATN3*), fibromodulin (*FMOD*), TIMP metalloproteinase inhibitor 4 (*TIMP4*), chondroadherin (*CHAD*), and dermatopontin (*DPT*). *PAPSS2*, which also belongs to this category, is of particular interest because this gene is found in proliferating chondrocytes in embryogenesis, but is dramatically downregulated in hypertrophic chondrocytes.<sup>20</sup> Among the most similarly expressed genes, we also found *UNQ830*. The protein has not yet been functionally identified, but shows a very high similarity to *COL2* and other matrix proteins and might therefore be involved in ECM organization. Heatmap and detailed gene list is given in Supplementary Figure S5.

Among a number of genes encoding transcription factors upregulated in this cluster, we found, as expected, *SOX9*. However, we also found *SOX8*, which has not yet been associated with regulation of chondrogenesis. To verify this unexpected observation, we performed real-time RT-PCR analysis, which confirmed a pattern of upregulation very similar to that observed for *COL2A1*, although slightly less dramatic (Fig. 5A). At the protein level, *SOX8* was found exclusively in the nuclei of a majority of differentiating cells on day 21 (Fig. 5B).

*CXCL12* was greatly downregulated in differentiating cells, but was unaffected in MSCs cultured in 3D without



**FIG. 5.** (A) Upregulation of SOX8 mRNA during chondrogenesis was validated by quantitative real-time reverse transcription–polymerase chain reaction. Data shown were obtained from three different donors, each measured in triplicate, at different time points of chondrogenesis. Day 0 corresponds to undifferentiated monolayer MSCs. Expression levels are relative to GAPDH expression. (B) To characterize the protein expression of SOX8, we performed fluorescent immunohistochemical staining at day 21 of differentiation. Nuclei are counterstained with DAPI (blue). Scale bar = 50  $\mu$ m.

differentiation cocktail (Fig. 6A). Among the GO terms, most significantly downregulated in the *CXCL12* similarity cluster were blood vessel development, which most likely reflects loss of genes associated with the MSCs known function as pericytes,<sup>21</sup> and immune responses (Fig. 6B). This suggests that the chondrocytes obtained after differentiation have lost the known ability of MSCs to affect immune responses.<sup>22</sup> On scrutiny of the gene list, some of the genes involved in vasculogenesis were *VCAM* (CD106, vascular cell adhesion molecule), *CD248* (endosialin), *EPAS1* (also known as *HIF2A*, hypoxia inducible factor 2 $\alpha$ ), neuropilin-1 (*NRP1*; vascular endothelial cell growth factor 165 receptor), and thrombospondin (*THBS2*). Among the genes involved in immune responses we observed interleukin 6 (*IL6*), *CCL2* (chemokine, CC motif, ligand 2), *CXCL16*, TGF- $\beta$  receptor 3 (*TGFBR3*), human leucocyte antigen DR (*HLA-DR*), and complement factor B (*CFB*). In addition, we see a significant downregulation of genes associated with cell adhesion (Fig. 6B). Heatmap and detailed gene list is given in Supplementary Figure S6.

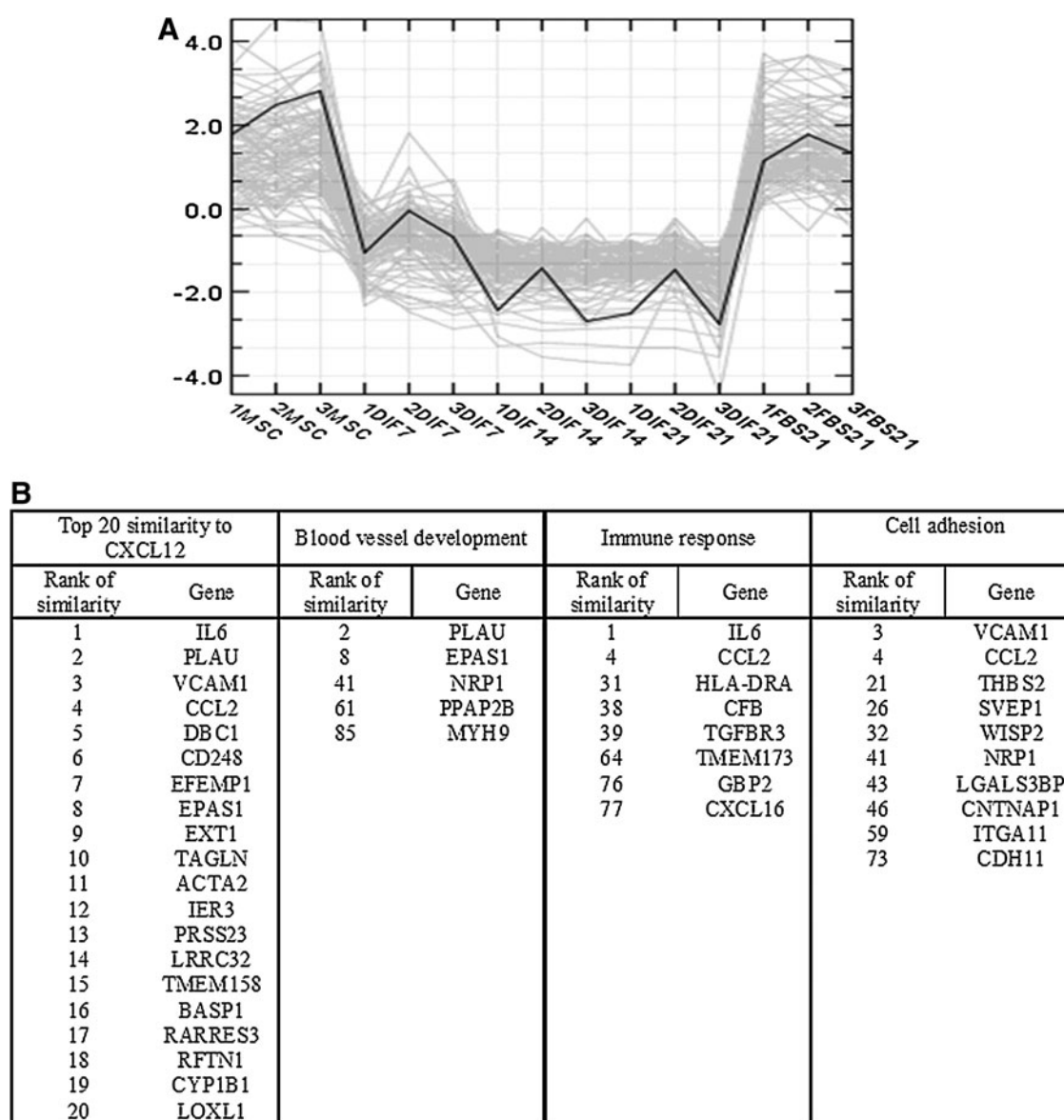
## Discussion

In this study we have used a disc-shaped self-gelling alginate hydrogel as a scaffold for *in vitro* chondrogenic differentiation of hMSCs. The long-term aim of this strategy is to produce hyaline autologous cartilage tissue, which may be implanted in cartilage lesions. When the ECM produced by the differentiating MSCs can be made of the right composition and sufficiently strong, the alginate may be removed by chelating agents or enzymatic digestion. Here we present two aspects of this strategy: first, a detailed kinetic study of the expression of a range of genes known to be involved in chondrogenesis and the production and distribution of their corresponding proteins; second, the composition of signature gene clusters derived from kinetic microarray analysis of *in vitro* chondrogenesis. To date, very few studies have performed analysis of genome-wide gene expression during

chondrogenic differentiation of MSCs in 3D scaffold.<sup>10,23</sup> We conclude that our cell/scaffold-based strategy for tissue engineering of hyaline cartilage is a promising *in vitro* system and that the signature gene clusters may provide insight to help us improve the strategy toward production of a cartilage implant.

The real-time RT-PCR gene expression analysis supplemented with data from the *COL2A1* similarity search shows that we can document a high level of upregulation of a large number of genes associated with hyaline chondrogenesis. At the protein level, COL2 and aggrecan were diffusely distributed in the extracellular space already on day 21, and the SOX triad of transcription factors was expressed as expected. It may be that this differentiation cocktail, when it acts on hMSCs in 3D, starts a differentiation program that leads to the expression of all the genes required for hyaline chondrogenesis. However, it also upregulates some unwanted genes. With *RUNX2*, *MMP13*, and *ALPL*, expression of *COL10A1* is known to be a marker for chondrocyte hypertrophy, apoptotic death, and replacement by bone during embryological chondrogenesis.<sup>24,25</sup> Although *COL10A1* was greatly upregulated in our differentiating MSCs, we believe that they are not destined for hypertrophy, osteogenesis, or death, because *RUNX2*, *MMP13*, and *ALPL* expression stayed low at day 21, whereas *PAPSS2*, which is greatly downregulated in hypertrophic chondrocytes, was actually strongly upregulated in our cells. However, COL10 is a network-forming, not a fibril-forming, collagen, and as such not desirable in the ECM of hyaline cartilage. Although it seems to be made in much smaller quantities than COL2, it would be valuable to be able to downregulate *COL10A1* in an improved differentiation protocol. *COL1A1* was not upregulated, rather maintained, and the expression relative to *COL2A1* was similar to that found in uncultured articular chondrocytes (Karlsen *et al.*, submitted). COL1 is known to be synthesized by chondrocytes in the superficial zone in hyaline cartilage,<sup>26</sup> but in our cell/scaffold constructs COL1-producing cells





**FIG. 6.** (A) Expression profile of genes obtained from CXCL12 similarity search. Data were obtained from three donors under five different conditions: undifferentiated monolayer cells (MSC), 7 days of chondrogenic differentiation (DIF7), 14 days of chondrogenic differentiation (DIF14), 21 days of chondrogenic differentiation (DIF21), and undifferentiated three-dimensional control cells, which were cultured in alginate for 3 weeks using 10% FBS (FBS21). The 98 shown genes (shown in gray) are the 5% most similar differentially expressed genes ( $>2$ -fold change,  $p < 0.05$ ) compared to expression of CXCL12 (shown in black). Relative expression levels (log2 ratios) after mean normalization are shown. (B) The 20 most similar genes (left column) and genes within the 5% most similar differentially expressed genes belonging to three selected GO terms are shown together with their similarity rank number relative to CXCL12 expression. All of these GO terms were significantly upregulated in the similarity cluster ( $p < 0.001$ ).

were scattered throughout the scaffold. Thus, an improved differentiation stimulus would be one that quickly down-regulates also COL1A1 in hMSCs.

A number of statistical analyses may be performed on the long list of genes found to be differentially expressed between MSCs in monolayer and 3D chondrogenic differentiation. We have chosen to focus on the genes upregulated similar to COL2A1, and downregulated similar to CXCL12. For the COL2A1 signature cluster, again a number of associations may be made, but we decided to highlight the role of genes involved in the ECM, as well as transcription factors

possibly involved in ECM synthesis. The large number of genes on this list known to be involved in hyaline chondrogenesis serves as a quality control for this kind of analysis and our differentiation strategy. In addition, a number of unexpected genes appeared of which, to us, SOX8 was the most surprising. The gene was quickly and greatly upregulated upon differentiation, which was verified by the observation of the protein in most of the nuclei on day 21. Sox8 is an early marker of limb chondrogenesis, and has been shown to be a negative regulator of osteoblast differentiation, but has never been implicated in *in vitro* chondrogenesis.<sup>27,28</sup>

Clearly, it will be important to determine the role played by SOX8 in this differentiation process.

The most interesting aspect of the *CXCL12* similarity search was the observation that the MSCs lose some of their characteristic functionalities during chondrogenesis. MSCs are found to be identical to perivascular cells that support blood vessels in multiple organs.<sup>21</sup> As such, they are likely to interact with endothelial cells and other vascular cells during vessel homeostasis and neovascularization. Not surprisingly, this functionality is turned off at the gene expression level when these cells differentiate to become chondrocytes, which reside in an avascular tissue. Another functionality that is turned off is the ability of MSCs to modulate immune responses. This too is not surprising, as it would be unexpected to find the active maintenance of immunosuppressive properties in chondrocytes, which reside in a biologically inert tissue. The indicator molecule, *CXCL12*, also acts to ensure homing and maintenance of hematopoietic stem cells in their niche,<sup>29</sup> clearly an unwanted functionality in cartilage. Thus, our genome-wide analysis of gene expression during chondrogenesis has identified logical changes in gene clusters, and may provide better understanding of how the stem cell fate could be directed to produce perfect chondrocytes for cell therapy of cartilage lesions.

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### Disclosure Statement

None of the authors have financial interests that could create an actual or potential conflict of interest with regard to this work. However, Jan Egil Melvik is an employee of the firm NovaMatrix/FMC, the producer of the alginate used in the hydrogel discs.

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