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Subtractive gene expression profiling of articular cartilage and mesenchymal stem cells: serpins as cartilage-relevant differentiation markers

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Summary

Objective: Mesenchymal stem cells (MSCs) are a population of cells broadly discussed to support cartilage repair. The differentiation of MSCs into articular chondrocytes is, however, still poorly understood on the molecular level. The aim of this study was to perform an almost genome-wide screen for genes differentially expressed between cartilage and MSCs and to extract new markers useful to define chondrocyte differentiation stages.

Methods: Gene expression profiles of MSCs ($n = 8$) and articular cartilage from OA patients ($n = 7$) were compared on a 30,000 cDNA-fragment array and differentially expressed genes were extracted by subtraction. Expression of selected genes was assessed during *in vitro* chondrogenic differentiation of MSCs and during dedifferentiation of expanded chondrocytes using quantitative and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Protein secretion was measured by enzyme-linked immunosorbent assay.

Results: Eighty-seven genes were differentially expressed between MSCs and cartilage with a more than three-fold difference. Sixty-seven of them were higher expressed in cartilage and among them 15 genes were previously not detected in cartilage. Differential expression was confirmed for 69% of 26 reanalysed genes by RT-PCR. The profiles of three unknown transcripts and six protease-related molecules were characterised during differentiation. SERPINA1 and SERPINA3 mRNA expression correlated with chondrogenic differentiation of MSCs and dedifferentiation of chondrocytes, and SERPINA1 protein levels in culture supernatants could be correlated alike.

Conclusions: cDNA-array analysis identified SERPINA1 and A3 as new differentiation-relevant genes for cartilage. Since SERPINA1 secretion correlated with both chondrogenesis of MSCs and dedifferentiation during chondrocyte expansion, it represents an attractive marker for refinement of chondrocyte differentiation.
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Key words: cDNA array, SERPIN, Protease inhibitor, Chondrocytes dedifferentiation, Chondrogenic differentiation.

Introduction

Mesenchymal stem cells (MSCs), which can be found in different adult organs, are broadly discussed as a cell population able to support cartilage regeneration. The potential of MSCs from bone marrow or adipose tissue to differentiate towards the chondrogenic lineage has been established[1,2,3] and various approaches are tested *in vitro* for the use of MSCs to generate tissue engineered cartilage[4]. Differentiation of MSCs into articular chondrocytes, spontaneous regeneration of injured cartilage and repair after surgical treatments like microfracture or autologous cell therapy (ACT) are, however, still poorly understood on the molecular level. To support a better understanding of cartilage differentiation and regeneration, there is a need for multiple marker genes allowing a precise characterisation of distinct cell differentiation stages.

The cartilage transcriptome has been estimated to represent between 13,200 and 15,800 genes[5]. A few studies have compiled repertoires of gene expression in cartilage by sequencing foetal cartilage cDNA libraries[6,7] or adult osteoarthritic (OA) and healthy cartilage cDNA libraries[8]. This provided an overview on a high amount of genes expressed in the concerned tissue but only weak indications on the expression levels of genes. More quantitative microarray-based approaches allow comparative gene expression analyses. These approaches have been used for comparison of healthy with OA cartilage[9], or for the comparison of different types of cartilage-like hyaline cartilage and fibrocartilage[10]. Several studies compared cartilage to dedifferentiated chondrocytes in order to identify chondrogenesis associated genes[11,12]. On the other hand, expression profiles of MSCs have been well characterised and compared between different sources like human bone marrow-derived stem cells (BMSCs)[13] or adipose tissue-derived stromal cells (ATSCs)[14]. Comparative gene expression analysis has also been undertaken for BMSCs and fibroblasts[15], while differentiation-relevant evaluation of gene expression profiles of MSCs in comparison to hyaline cartilage is lacking.

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A direct comparison of MSCs with mature cartilage would allow finding new markers relevant for differentiation of progenitor cells into functional chondrocytes in permanent articular cartilage. The aim of this study was to identify such new marker genes for characterisation of chondrocyte differentiation. We performed an almost genome-wide screen (30,000-gene fragments) for transcripts expressed in seven cartilage and eight MSC samples and extracted possible marker genes by bioinformatic subtraction. Potential candidate genes were then assessed for their relevance as differentiation markers during chondrogenesis of MSCs and dedifferentiation of chondrocytes expanded in monolayer culture.

Material and methods

TISSUE SAMPLES

Healthy cartilage samples were obtained from the knees from five patients undergoing amputation. OA cartilage samples were obtained from 10 patients undergoing total knee arthroplasty (age from 54 to 82). Cartilage chips were carefully removed from the tibial plateau and condyles and washed with phosphate buffered saline (PBS) to avoid contamination by other cells. Bone marrow samples for isolation of mesenchymal stem cells (BMSCs) were obtained from five patients (age from 26 to 53) undergoing total hip replacement or iliac bone graft harvest. ATSCs ($n=5$) were isolated from lipoaspirates generated during elective liposuction procedures or from adipose tissue samples obtained from patients undergoing total hip arthroplasty (age from 21 to 53). The studies were approved by the local ethics committee and informed consent was obtained from all individuals included in the study.

CELL ISOLATION AND CULTIVATION

MSCs were isolated from fresh bone marrow samples as described previously[3]. 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[16] containing 2% foetal calf serum (FCS), with 10 ng/ml recombinant human epidermal growth factor (Strathmann Biotech, Hamburg, Germany) and 10 ng/ml recombinant human platelet-derived growth factor BB (Sigma–Aldrich, Deisenhofen, Germany). After 24–48 h, cultures were washed with PBS to remove non-adherent material. During expansion, medium was replaced twice a week.

ATSCs were isolated according to the method described by Hauner *et al.*[17]. Briefly, minced tissue or lipoaspirates were digested with Krebs–Ringer solution buffered with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (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-μm nylon mesh. Erythrocytes were removed using erythrocyte lysis buffer (0.154 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM ethylenediamine tetraacetic acid (EDTA). The remaining cells were seeded in culture flasks and maintained under conditions identical to those for BMSCs.

Human chondrocytes were isolated from cartilage by digestion with collagenase B (1.5 mg/ml) (Roche Diagnostics, Mannheim, Germany) and hyaluronidase (0.1 mg/ml) (Serva, Heidelberg, Germany), as described previously[12]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin, and were maintained at 37°C in a humidified atmosphere and 6% CO_2. During expansion, medium was replaced twice a week. Cells were seeded in order to achieve confluence after six population doublings (pd6) and counted for determination of population doublings achieved. Chondrocytes were harvested directly after collagenase/hyaluronidase digestion (pd0) as well as after two (pd2) or six (pd6) population doublings.

INDUCTION OF OSTEOGENIC AND ADIPOGENIC DIFFERENTIATION OF BMSCs AND ATSCs

MSCs were seeded in six-well plates at $1-3 \times 10^4$ cells/cm^2. For osteogenic induction, medium was switched to MSCs' growth medium (Poietics; BioWhittaker, Taufkirchen, Germany) supplemented with 0.1 μM dexamethasone, 0.05 mM ascorbic acid-2-phosphate, and 10 mM β-glycerophosphate for 2 weeks. Adipogenic medium composed of DMEM high glucose (DMEM-HG) containing 10% FCS, 0.01 mg/ml insulin, 1 μM dexamethasone, 0.2 mM indomethacin, 0.5 mM 3-isobutyl-1-methyl xanthine, 100 units/ml penicillin, and 100 μg/ml streptomycin was applied for 2 weeks. After fixation of washed cells in 4% paraformaldehyde, they were stained with von Kossa or Oil red O as described previously[3].

INDUCTION OF CHONDROGENIC DIFFERENTIATION OF BMSCs AND ATSCs

For induction of chondrogenesis at high cell density in three-dimensional culture, spheroids of $4-5 \times 10^5$ cells were formed by centrifugation at 300 g in 1.5 ml microcentrifuge tubes (Eppendorf, Hamburg, Germany). After incubation at 37°C, 6% CO_2 for 4 days, spheroids were transferred to 96-well cell culture plates. Cells were kept in induction medium for 14, 28 or 42 days. Chondrogenic medium consisted of DMEM-HG (DMEM containing 4.5 mg/l glucose) supplemented with 5 μg/ml insulin, 5 μg/ml tranferrin, 5 μg/ml selenous acid, 0.1 μM dexamethasone, 0.17 mM ascorbic acid-2-phosphate, 1 mM sodium pyruvate, 0.35 mM proline, 1.25 mg/ml BSA and 10 ng/ml transforming growth factor β_3 (TGF β_3; Sigma–Aldrich). Medium for ATSCs was additionally supplemented with 10 ng/ml BMP6 (R&D systems).

RNA EXTRACTION

Total RNA was isolated from cultured cells using a standard guanidinium thiocyanate/phenol extraction protocol (peqGOLD RNAPure™; Peqlab, Erlangen, Germany). Polyadenylated mRNA was then isolated with oligo-d(T)-coupled magnetic beads (Dynabeads; Invitrogen) according to the manufacturer's instructions. For the isolation of RNA from tissue samples of cartilage, shock-frozen tissue was pulverised mechanically and consecutively dissolved in lysis/binding buffer for direct poly(A)$^+$-mRNA isolation using oligo-d(T)-coupled beads (Dynabeads; Invitrogen).

cDNA-ARRAY ANALYSIS

The human Unigene Set – RZPD1 array (RZPD, Berlin, Germany) with 31,488 clones was used for hybridisation. mRNA isolated from 10 OA cartilage samples and from 10 MSC ($n=5$ ATSCs, $n=5$ BMSCs) samples was reverse-transcribed to ^{33}P-labeled cDNA probes according to the manufacturer's protocol (SuperScript II, Invitrogen).

The labelled cDNAs were denatured and hybridised to cDNA arrays in 5 × Denhardt's solution, 6 × Saline-sodium citrate (SSC)/0.25% sodium dodecyl sulfate (SDS) and 50 μg/ml salmon sperm DNA overnight at 65°C. Arrays were washed 3 × 30 min in 0.04 M sodium phosphate buffer, pH 7.2/1% SDS at 65°C. Arrays were exposed to a phosphor imaging plate for up to 72 h. Images were captured on a Bio-Imaging Analyser BAS-1800 II using BAS Reader 2.26 beta software (Fuji/Raytest, Straubenhardt, Germany). The hybridisation with one of the cartilage samples was of poor quality and rejected for that reason.

Spots were quantified with the Arrayvision software (Imaging Research Inc., St. Catharines, Ontario, Canada). Background-corrected intensity values were normalised and transformed to generalised log ratios through the variance stabilising method[18]. The normalised data are presented in Supplementary Table 2. The comparison between cartilage and stem cell probes was performed using the Significance Analysis of Microarrays software package (SAM[19]). Background-corrected signal intensities normalised to the mean signal of each array were used for the calculation of fold changes. The normalised signal intensities were evaluated for homogeneity on scatter plots by pair-wise comparison of all hybridisations from one group. Two cartilage and two stem cell samples showed a bimodal configuration of the scatter plot when compared to all the other samples, indicating divergent expression profiles. These samples were excluded from further analysis and evaluation was based on seven cartilage and eight stem cell samples.

SEQUENCE ANALYSIS

The clones identified on the cDNA arrays were purchased from RZPD. Plasmid DNA was prepared with the Qiagen plasmid miniprep kit (Qiagen), and given for insert sequencing to MWG Biotech (Ebersberg, Germany). Basic local alignment search tool (BLAST) algorithms were used to screen for DNA and protein sequence similarities in public databases at the National Center for Biotechnology Information (NCBI).

DESIGN OF A CUSTOMISED cDNA ARRAY COMPRISING 230 GENE FRAGMENTS

The 87 genes identified by cDNA-array analysis as well as further 139 genes selected by literature search and four housekeeping genes were chosen for the design of a customised cDNA array (Supplementary Table 1). cDNA fragments were amplified by polymerase chain reaction (PCR) from corresponding clones and were spotted on nylon membranes using a Biogrid spotter (Genomic solutions). The expression levels of the four housekeeping genes (β-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), RPL13A and RPS9) were used for the normalisation of signals.

RT-PCR

Reverse transcription was performed with poly(A+)-mRNA by using reverse transcriptase Superscript II (Invitrogen) and oligo-d(T) primers. The first strand cDNA was subjected to PCR. Oligonucleotide sequences used for PCR and Light-Cycler analysis are listed in Table I. Oligonucleotides were obtained from MWG Biotech (Ebersberg, Germany) and Thermo Electron (Ulm, Germany). PCR was performed with 1 μl template in a total volume of 50 μl containing 200 nM of each primer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1× PCR buffer and 1.5 U Taq-polymerase (Invitrogen). PCR conditions consisted of 4 min initiation at

Table I
List of primers used for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
ACTB	GTGGCATCCACGAAACTACC	GTACTTGCGCTCAGGAGGAG
C5orf4	TAACTTCATCTCTCGCTACC	ATAGAGAGAGATCACGCCAAT
CALU	TGTGGTGGGAAGAGTTAGG	CAACCAAACTCATAACTAATACT
CCDC95	GACGGCGAAGTGGACTAC	TCATCCACGTTCTCGTACTG
CLU	TTCTACTTCTGGATGAATGGT	GGGCTGTGGAAGTGGATG
COL2A1	TGGCCTGAGACAGCATGAC	AGTGTTGGGAGCCAGATTGT
FLJ21986	GGCTATGAAGTAGTTGACAC	GGCTCCTAGACCATTGTCC
GAPDH	CCACCCATGGCAAATTCCATGGCA	TCTAGACGGCAGGTCAGGTCCACC
HTRA1	CAAGGATGTGGATGAGAAAG	GTGTTAATTCCAATCACTTCAC
IFI44L	CTGTGTGGCTTATGTCTTAG	AGCATAATTTCCAACCATCAAA
KIAA0746	CTAGCAGTTATAAGGCACCT	CTTCCTCTTCCTCTCTAACA
LGALS1	TGGACTCAATCATGGCTTGT	ACATTTGATCTTGAAGTCACC
LOC112868	CTTGTTGCGATTCTTCTGTG	GCAATCGTCATGTTTAGAGAA
LOC25845	GGAAGCGTCAGCCACACA	CGCTCCTGCTCTGTTCTC
LOC285733	TCCTCCTCATCGCCTACTT	CTGCTGTTATACTGAGTCTCT
M96	ATGGGAAAGCATATTGGGAC	AAGCCCACCCAATACTACAA
MMP15	CTCTCAGCCCTCACACAC	GTGGAAGGCTCTTCCTAAG
MMP3	TTTTGATGGACCTGGAAATG	CTCACGGTTGGAGGGAAAC
PCSK1N	GTGTGAAACGCCTAGAGAC	TTGCTTCAGATCATGTTTATTG
PDE4DIP	AGAACGAGTCACACAGCAG	AGTGTTCCGCTTGTAGATGT
PLA2G2A	TGGTGAATTTCCACAGAATGA	ACACTCACACAGTTGACTTCT
SERPINA1	GACACCGAGGAAGAGGAC	GAGGAGCGAGAGGCAGTT
SERPINA3	TGAGGCAGAAATTCACCAGA	GTCGTTGATGAGCTTCTTAG
SERPINF1	TAGACCGAGAACTGAAGACC	CTTCGTGTCCTGTGGAATCT
SETD5	AGATATGAACATGGCTTAATGA	ATCCACCTACTGCTGATATTC
SPRN	ACAGGAGAGACATGGAGTG	GGGTGCTGGTCTTGACTTT
SURF4	GTCTTGCTGGTTCTGATGTT	GACTGAGCCATCGATTCTC
TAGLN	GTCCTTCCTATGGCATGAG	TCTGGAACATGTCAGTCTTG
TIEG2	GTTGCCGGAAGACCTACTT	AGCCTGGGATCTTCTTGGT

94°C followed by cycles of 30 s denaturation at 94°C, 30 s annealing at 58°C and 45 s extension at 72°C; and a final 5 min extension at 72°C. Depending on signal intensities, 25 to 35 cycles were performed. For templates to be compared, GAPDH was used as reference to adjust the template cDNA concentrations.

REAL-TIME RT-PCR

To quantify the mRNA levels with the Light-Cycler (Roche Diagnostics, Mannheim, Germany) aliquots of first-stranded cDNAs were amplified using gene-specific primer sets (Table I) and real-time fluorimetric intensity of CYBR green I was monitored. The Cycling parameters were optimized according to the Light-Cycler protocol from Roche (Light-Cycler Operator's Manual, Version 3.5, October 2000). The concentration of the housekeeping gene β-actin (ACTB) was used as the control reference gene. It was detected at the same level in all cell types. The ACTB concentration was determined once for each cDNA sample and used to normalize all other genes tested for the same cDNA sample. Melting curves and agarose gel electrophoresis of the PCR products were used as quality controls.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

For ELISA of supernatants from expanded chondrocytes, 5×10^5 cells were seeded in six-well plates and supplemented with 4 ml DMEM medium. For ELISA of supernatants from differentiating BMSCs, spheroids (5×10^5 cells) were supplemented with 200 μl chondrogenic medium. For both types of cell culture, supernatants were collected after 4 days. SERPINA1 (or α_1-antitrypsin) protein was detected with the α_1-antitrypsin ELISA kit (Immundiagnostik, Bensheim, Germany) according to the manufacturer's instructions. Supernatants were diluted in DMEM medium in order to achieve concentrations in the range of the corresponding standard curve.

WESTERN BLOT ANALYSIS

5×10^6 freshly isolated chondrocytes were expanded for 3 days in 20 ml of DMEM with 10% FCS. Medium was then switched to serum-free and the cells conditioned for 3 days. Concentrated medium (Vivaspin 20 centrifugal filter devices; 10,000 MWCO; Vivascience, Stonehouse, UK) was subjected to Western blotting. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose (Hybond C super; Amersham Pharmacia Biotech, Freiburg, Germany). The filter was blocked with 2% BSA in Tris-buffered saline (TBS)/Tween (25 mM Tris/HCl pH 7.4; 145 mM NaCl, 2.7 mM KCl, 0.1% Tween 20) overnight and incubated for 2 h with rabbit anti-SERPINA1 antibody (1:1000; Assaypro, St Charles, USA). After washing, filters were incubated with an alkaline phosphatase-conjugated secondary goat anti rabbit antibody (1 h, 1:10,000; Dianova, Hamburg, Germany) and stained with nitro-blue-tetrazolium/5 bromo-4-chloro-3-indolylphosphate. Human plasma α1-antitrypsin (Sigma–Aldrich, Germany) was used as positive control.

STATISTICAL ANALYSIS

Mean and standard deviation were calculated for all values. The Mann–Whitney *U* test was chosen to evaluate differences in gene expression levels between cartilage and MSCs assessed by real-time RT-PCR. This non-parametric two-tailed test is not based on assumptions about the distribution of expression values or the equality of variance. For the real-time RT-PCR data of differentiating BMSCs, Monte-Carlo one-tailed *P* values were calculated based on the Wilcoxon rank sum test. For the analysis of protein secretion levels for SERPINA1 in dedifferentiating chondrocytes, analysis of variance (ANOVA) was chosen after verification of normal distribution and equality of variance. For all tests a P value < 0.05 was considered significant. Data analysis was performed with SPSS for Windows (SPSS, Chicago, USA).

Results

DEFINITION OF THE MSC CULTURES FROM BONE MARROW AND ADIPOSE TISSUE

MSCs from two different origins, BMSCs and ATSCs, were chosen for this analysis in order to avoid the identification of genes specifically related to osteoblast or adipoblast progenitor cells. Expanded BMSCs and ATSCs were checked for the expression of MSC surface markers like CD29, CD44 or CD105. All cultures were negative for CD34. The adipogenic, osteogenic and chondrogenic potential of analysed MSCs was confirmed by histology (Fig. 1).

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES BY cDNA-ARRAY ANALYSIS

The expression levels of 31,188 transcripts in seven cartilage tissue samples and eight undifferentiated MSC samples were assessed. Four hundred six spots with significantly differential signals between cartilage and MSC samples were identified by the SAM software when the false discovery rate was set at 3.3%. Out of them, 105 showed a more than three-fold difference in expression levels between cartilage and MSC and were selected for further analysis. Four of the corresponding clones were not available from the provider; the others were purchased and sequenced. Since 14 genes were found to be represented twice in this list, 87 sequences with significantly different expression levels were finally identified by this analysis.

GENES DIFFERENTIALLY EXPRESSED BETWEEN CARTILAGE AND MSCs

Sixty-seven genes showed higher expression levels in cartilage than in MSCs. They are listed in Table II, sorted according to the cellular localisation of the gene products. The genes with the highest mean expression levels in cartilage were clusterin, TGFβ-inducible-early-growth-response 2 (TIEG2), serine (or cysteine) proteinase inhibitor clade A member 1 (SERPINA1) and the ADP-ribolysation-like factor 6 interacting protein 2 (ARL6IP2). A high amount of secreted proteins, membrane-bound proteins or proteins of the extracellular matrix was found (45%). Fourteen genes corresponded to proteins with unknown localisation (21%) and 23 genes represented intracellular and nuclear products (34%). According to the iCartiGD database[20], 15 of these genes were so far not described to be expressed in cartilage.

Twenty genes showed higher expression levels in MSCs than in cartilage (Table III). Of them only four genes corresponded to secreted products (20%). Three genes associated to the cytoskeleton, and no genes associated to

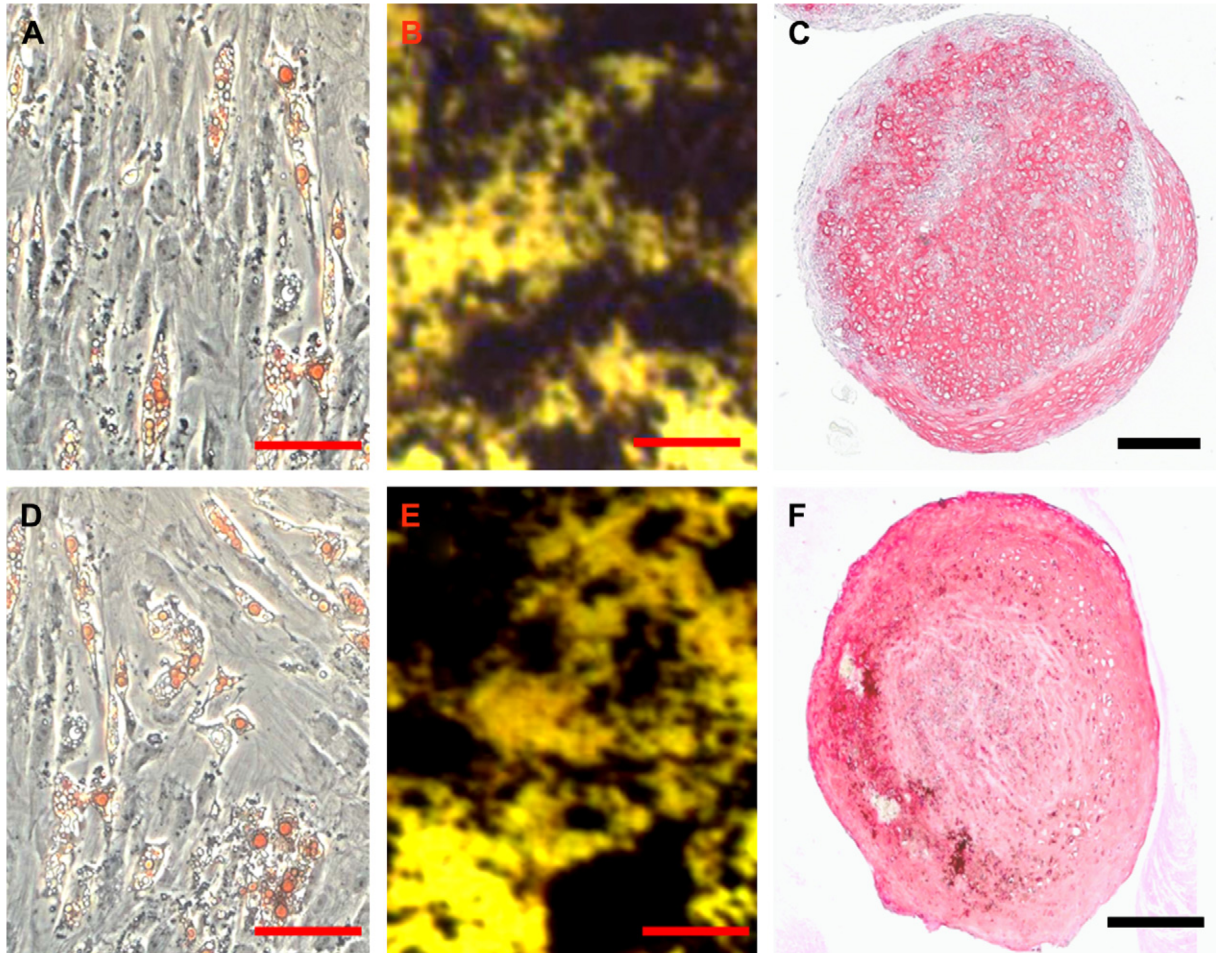

Fig. 1. Multilineage differentiation potential of BMSCs (A–C) and ATSCs (D–F). Oil red O staining of adipogenic cultures shows cell bodies filled with numerous lipid vesicles (A and D). Osteogenic cultures deposited mineralised extracellular matrix, as indicated by von Kossa staining (B and E). After chondrogenic differentiation in spheroid cultures a cartilage-like matrix was synthesised, as indicated by immunostaining of paraffin sections for type II collagen (C and F).

extracellular matrix (ECM) were found. High ratios between MSCs and cartilage were evident for transgelin and galectin 1 which, thus, deserve further attention as possible markers for undifferentiated cells. Further, two unknown transcripts with higher expression levels in MSCs were identified.

A customised cDNA array comprising all differentially expressed genes together with known cartilage or stem cell relevant gene fragments was produced for further analysis (total 230 genes listed in Supplementary Table 1). Gene expression profiles of the cartilage and MSC samples used for the genome-wide screening were assessed on this customised cDNA array for quality control.

CONFIRMATION OF GENE EXPRESSION RESULTS BY RT-PCR

The cDNA array results were further revaluated by quantitative RT-PCR as a second method using four independent cartilage and BMSC samples, respectively. Focus was on genes with highly differential expression levels and transcripts of unknown genes. Altogether 26 genes were revaluated and differential expression was confirmed for 18 (69%) of them (details in Tables II and III).

Several proteinases and proteinase inhibitors were confirmed to be higher expressed in cartilage by RT-PCR [Fig. 2(A)]: SERPINA1, SERPINA3, matrix metalloproteinase 3 (MMP3), the proprotein convertase inhibitor PCSK1N and the protease HTRA1. Serine (or cysteine) proteinase inhibitors (SERPIN) are a superfamily of proteins functioning mainly as inhibitors of proteinases of the chymotrypsin family[21]. While SERPINA1 and SERPINA3 showed higher expression in cartilage vs MSC (356-fold and 37-fold, respectively, according to real-time RT-PCR; $P < 0.05$; data not shown), the slightly higher expression levels of SERPINF1 in MSC (2.3-fold) did not reach significance. MMP3 mRNA levels were 467-fold higher in cartilage than in MSC ($P < 0.05$). PCSK1N could not be amplified by real-time RT-PCR, whereas for HTRA1 seven-fold higher expression levels were found in cartilage ($P < 0.05$).

Out of 11 tested transcripts of unknown genes or so far poorly described genes (as SPRN, PDE4DIP), seven were confirmed to be differentially expressed between MSCs and cartilage according to RT-PCR [Fig. 2(B)]. Real-time PCR demonstrated that the hypothetical protein KIAA0746 was higher expressed in MSC than in cartilage (20-fold), while the genes LOC25845, SETD5, C5orf4, PDE4DIP and SPRN all showed a four- to five-fold higher expression in cartilage ($P < 0.05$; data not shown). All

Table II
Classification of genes expressed in cartilage at significantly higher levels (>three-fold) compared to MSCs

Localisation	Mean cartilage	Mean MSCs	Fold	Symbol	Gene name	Accession
Secreted	193.3	2.8	68.0	CLU	Clusterin*	BC019588
	24.2	0.5	46.6	PLA2G2A	Phospholipase A2, group IIA*	NM_000300
	35.4	0.8	45.3	SERPINA1	Serine (or cysteine) proteinase inhibitor, clade A, member 1*	BC015642
	3.2	0.5	6.7	SERPINA3	Serine (or cysteine) proteinase inhibitor, clade A, member 3*	NM_001085
	6.1	1.1	5.6	CTGF	Connective tissue growth factor	NM_001901
	3.3	0.7	4.6	AEBP1	AE (adipocyte enhancer) binding protein 1 (ACLP)	NM_001129
	1.7	0.4	4.3	ANGPTL2	Angiopoietin-like 2	NM_012098
	2.2	0.6	3.7	PCSK1N	Proprotein convertase subtilisin/kexin type 1 inhibitor*	NM_013271
	1.7	0.5	3.6	MMP3	MMP3*	X05232
	40.6	11.7	3.5	HTRA1	HtrA serine peptidase 1 (PRSS11)*	NM_002775
	2.7	0.8	3.3	CFH	Complement factor H	NM_000186
	1.9	0.6	3.2	CFB	Complement factor B	NM_001710
Extracellular matrix	27.6	0.8	33.1	PRELP	Proline arginine-rich end leucine-rich repeat protein	U29089
	13.1	0.6	20.8	FMOD	Fibromodulin	U05291
	13.7	0.8	16.5	AGC1	Aggrecan 1	NM_013227
	34.0	2.4	14.2	DCN	Decorin	BC005322
	8.0	0.6	13.8	COL2A1	Collagen, type II, alpha 1	X16158
	3.9	0.6	6.8	COL11A1	Collagen, type XI, alpha 1	J04177
	4.4	0.7	6.6	LUM	Lumican	U18728
	3.7	0.6	6.4	TNXB	Tenascin XB	NM_019105
	2.8	0.6	5.0	CHI3L2	Chitinase 3-like 2 (YKL-39)	U58514
	2.2	0.5	4.6	HAPLN1	Hyaluronan and proteoglycan link protein 1 (cartilage linking protein 1)	U43328
	3.5	0.8	4.2	SCRG1	Scrapie responsive protein 1	NM_007281
Cell membrane	6.3	0.5	13.8	MMP15	MMP 15 (membrane-inserted)†	NM_002428
	3.5	0.5	6.6	CD74	CD74 antigen	BC018726
	2.4	0.4	5.4	GYPA	Glycophorin A	NM_002099
	3.1	0.7	4.7	CLSTN1	Calsyntenin 1	BC033902
	3.2	0.7	4.4	ITGA10	Integrin, alpha 10	NM_003637
	2.7	0.6	4.3	KEL	Kell blood group	NM_000420
	1.6	0.5	3.4	FGFR3	Fibroblast growth factor receptor 3	NM_022965
Intracellular	17.2	0.8	21.9	SURF4	Surfeit 4†	NM_033161
	23.3	1.1	20.5	PDE4DIP	Phosphodiesterase 4D interacting protein (myomegalin)*	AB042558
	31.3	1.9	16.8	ARL6IP2	ADP-ribosylation-like factor 6 interacting protein 2	BC053508
	10.3	0.7	15.6	H19	H19 gene	BC053637
	10.9	0.8	12.8	NEDD4	Neural precursor cell expressed, developmentally down-regulated 4	NM_006154
	11.8	1.2	9.6	HIBADH	3-hydroxyisobutyrate dehydrogenase	NM_152740
	4.3	0.6	7.6	WWP2	Nedd-4-like ubiquitin-protein ligase	NM_199424
	8.0	1.3	6.3	RAB24	RAB24, member RAS oncogene family	BC021263
	2.5	0.5	5.4	S100B	S100 calcium binding protein, beta	NM_006272
	5.5	1.3	4.3	SLK	STE20-like kinase (yeast)	U28250
	1.9	0.5	4.3	FXYD6	FXYD domain containing ion transport regulator 6	NM_022003
	2.1	0.5	4.0	CDO1	Cysteine dioxygenase, type I	BC024241
	1.7	0.5	3.5	MAP3K7IP1	Mitogen-activated protein kinase kinase kinase 7 interacting protein 1	NM_006116
	2.1	0.7	3.1	TSC22	Transforming growth factor beta-stimulated protein TSC22	BC016867
Cytoskeleton	3.6	1.2	3.1	ARHGAP5	Rho GTPase activating protein 5	NM_001173
Nuclear	58.4	1.2	48.9	KLF11	Kruppel-like factor 11*	NM_003597
	20.6	0.7	30.3	M96	M96 protein*	AF073293
	6.8	0.7	9.2	CRIP1	Cysteine-rich protein 1	NM_001311
	8.4	1.6	5.1	GLE1L	GLE1 RNA export mediator-like (yeast)	BC030012
	2.1	0.5	4.4	MGA	MAX gene associated	AB011090
	4.3	1.1	3.8	EGR1	Early growth response 1	NM_001964
	2.1	0.6	3.5	MYB	v-myb myeloblastosis viral oncogene homolog	AJ616235
	1.9	0.6	3.4	CRIP2	Cysteine-rich protein 2	NM_001312
Not defined	21.6	1.0	21.4	SPRN	Shadow of prion protein*	BC040198
	33.2	2.9	11.3	SETD5	SET domain containing 5*	BC020956
	9.7	1.0	9.5	ANKMY2	Ankyrin repeat and MYND domain containing 2	BC035353
	4.9	0.9	5.7	DCDC2	Doublecortin domain containing 2 (KIAA1154 protein)	AB032980
	2.1	0.5	4.7	SULF2	Sulfatase 2	NM_018837
	1.9	0.4	4.4	SAP30L	SAP30-like	BC009829
	2.7	0.8	3.3	TMEM112	Transmembrane protein 112	BC036550

(*continued on next page*)

Table II (*continued*)

Localisation	Mean cartilage	Mean MSCs	Fold	Symbol	Gene name	Accession
Unknown genes	10.9	0.6	19.6	C5orf4	Chromosome 5 open reading frame 4*	BC004506
	12.6	0.7	18.0	LOC25845	Hypothetical protein LOC25845*	XM_376366
	10.9	0.8	12.8	LOC285733	Hypothetical protein LOC285733†	XM_379432
	3.8	0.7	5.8	LOC112868	Hypothetical protein LOC112868*	AL390134
	2.9	0.7	4.0	FLJ11151	Hypothetical protein FLJ11151	BC006289
	1.4	0.4	3.5	C16orf69	Chromosome 16 open reading frame 69	NM_153261
	2.4	0.7	3.3	LOC91316	Similar to bK246H3.1	AK056875

*Differential expression was confirmed by quantitative or semi-quantitative RT-PCR ($P < 0.05$). †Differential expression could not be confirmed in all donors ($n = 4$).

confirmed molecules, thus, represented attractive candidates for further analysis as differentiation markers.

EXPRESSION DURING *IN VITRO* CHONDROGENESIS OF MSC

The relevance of the selected genes as markers for chondrogenesis was evaluated during chondrogenic induction of BMSC spheroids *in vitro*. Expression profiles were assessed by real-time RT-PCR analysis in two to five donors at days 0, 14, 28 and 42 after induction (Fig. 3). Consistent with successful chondrogenesis, the expression levels of COL2A1 were strongly induced over time. SERPINA1 was up-regulated during induction, reaching significantly higher expression levels compared to the control after 4 and 6 weeks. Mean expression levels of SERPINA3 increased alike (Fig. 3), yet at much lower levels of expression than SERPINA1. The expression profile of MMP3 reached highest mean expression levels after 14 days. Due to higher donor variability, no significance was reached for SERPINA3 and MMP3. HTRA1 expression was up-regulated in one donor while for the second donor expression was relatively stable during differentiation. For unknown genes like C5orf4 (Fig. 3), LOC25845 and SETD5 (data not shown), only low changes in gene expression were found during chondrogenesis.

EXPRESSION DURING DEDIFFERENTIATION OF CHONDROCYTES IN MONOLAYER CULTURE

Good differentiation markers would be expected to be down-regulated during dedifferentiation of chondrocytes in monolayer culture. Therefore, we analysed the expression levels of the selected genes also in cartilage tissue, freshly isolated chondrocytes and cells expanded for pd2 and pd6. SERPINA1 and SERPINA3 were expressed in healthy cartilage as well as in OA cartilage. SERPINA1 was 2.06-fold significantly higher expressed in healthy cartilage than in OA cartilage, whereas for SERPINA3 no differences were found [Fig. 4(A)]. Both genes were expressed in freshly isolated chondrocytes as well and their mRNA levels diminished during dedifferentiation in monolayer culture, parallel to the decreasing expression levels of collagen type II [Fig. 4(B)]. MMP3 mRNA was expressed at a low level in cartilage but strongly up-regulated in freshly isolated

Table III

Classification of genes expressed in MSCs at significantly higher levels (>three-fold) compared to cartilage

Localisation	Mean cartilage	Mean MSCs	Fold	Symbol	Gene name	Accession
Secreted	4.2	58.8	14.0	LGALS1	Lectin, galactoside-binding, soluble 1 (galectin 1)*	BC020675
	1.2	10.2	8.5	DF	D component of complement, adipsin	NM_001928
	1.0	8.3	8.3	SERPINF1	Serine (or cysteine) proteinase inhibitor, clade F, member 1*	BC000522
	0.9	4.0	4.4	ADM	Adrenomedullin	NM_001124
Intracellular	1.7	22.7	13.4	CALU	Calumenin†	NM_001219
	4.4	17.1	3.9	MTRNR2	Mitochondrion	AF382013
	6.4	23.0	3.6	RPSA	Ribosomal protein SA, 67 kDa (laminin receptor 1)	BC008867
Cytoskeleton	1.7	56.3	33.1	TAGLN	Transgelin*	BC024296
	1.4	13.4	9.6	TMSB10	Thymosin, beta 10	NM_021103
	2.3	20.0	8.7	TPM2	Tropomyosin 2 (beta)	BC011776
Nuclear	1.8	10.6	5.9	POLR2L	Polymerase (RNA) II (DNA directed) polypeptide L	NM_021128
	0.6	3.5	5.8	COPS8	COP9 constitutive photomorphogenic homolog subunit 8 (Arabidopsis)	NM_006710
	3.5	19.9	5.7	NIPBL	Nipped-B homolog (IDN3 protein)	NM_133433
Not defined	1.9	16.1	8.5	CCDC95	Coiled-coil domain containing 95†	BC047712
	6.5	41.9	6.4	API5	Apoptosis inhibitor 5 (FGF2-interacting factor, XAGL protein)	BC017709
	1.2	6.3	5.3	IFI44L	Interferon-induced protein 44, C1orf29†	NM_006820
	4.2	18.6	4.4	S100A11	S100 Calcium binding protein A11 (Calgizzarin)	NM_005620
	6.4	23.0	3.6	WDR45L	WDR45-like	BC000974
Unknown genes	0.9	27.1	30.1	FLJ21986	Hypothetical protein FLJ21986†	NM_024913
	1.4	6.9	4.9	KIAA0746	KIAA0746 protein*	BC009945

*Differential expression was confirmed by quantitative or semi-quantitative RT-PCR ($P < 0.05$). †Differential expression could not be confirmed in all donors ($n = 4$).

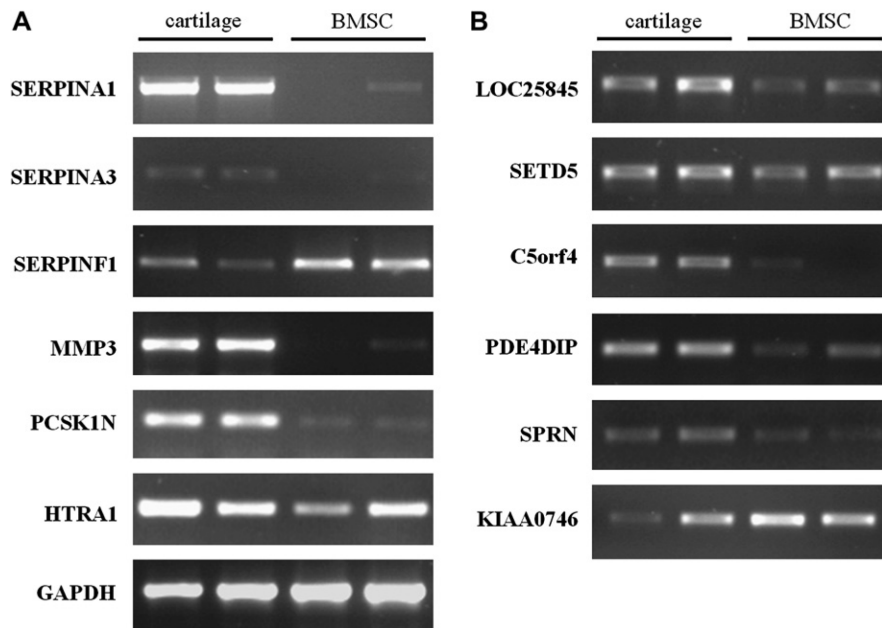

Fig. 2. RT-PCR analysis of the expression of proteases and protease inhibitors (A) and of unknown transcripts and poorly described genes (B) in cartilage and BMSCs. PCR was conducted with cDNA samples from cartilage and expanded BMSCs from two donors each. The concentration of templates used in the reactions was adjusted according to GAPDH signals in order to achieve comparable signals. PCR products were visualised by agarose gel electrophoresis.

chondrocytes. Its expression then decreased progressively during monolayer culture. During dedifferentiation, the expression of PCSK1N was down-regulated in cells from two out of three donors, whereas expression of HTRA1 seemed to be donor-dependent. In sum, SERPINA1 and A3 mRNA levels correlated best to COL2A1 expression during dedifferentiation in culture. As SERPINA1 achieved higher expression levels and as, it was consistently down-regulated during dedifferentiation and consistently up-regulated during *in vitro* chondrogenesis, it appeared as the most interesting candidate for evaluation on the protein level.

CORRELATION OF SERPINA1 SECRETION WITH CHONDROGENIC DIFFERENTIATION OF BMSCs AND DEDIFFERENTIATION OF CHONDROCYTES IN MONOLAYER CULTURE

Cell culture medium conditioned for 4 days with one spheroid from three distinct donors was assessed for the secretion of SERPINA1 protein at weeks 5, 7, 9 and 11 after start of chondrogenic induction [Fig. 5(A)]. The SERPINA1 concentration assessed by ELISA rose from a mean of 4.5 ng/ml at day 0 (plain medium) to a mean of 50.6 ng/ml/4days after 11 weeks of chondrogenic induction. Since the DNA content of the spheroids decreased over that time to about 70% of initial values, this up-regulation is attributed to cell differentiation rather than cell expansion. In parallel, the SERPINA1 secretion by chondrocytes dropped during expansion in monolayer culture in all five donors analysed [Fig. 5(B)]. Levels in cell culture media conditioned with 5×10^5 freshly isolated chondrocytes for 4 days decreased from a mean of 288 ng/ml SERPINA1 to 27−42% of the initial levels in all donors ($P < 0.05$) when cells from pd6 were assessed. Western blot analysis [Fig. 5(C)] shows that the secreted SERPINA1 protein corresponds to a blurred single band close to the 54 kDa band of human plasma SERPINA1, possibly indicating a different glycosylation status of the protein secreted by chondrocytes *in vitro*[22].

Discussion

Although the differentiation potential of MSCs towards the chondrogenic lineage has long been established in various approaches[1,2], the hallmarks of MSCs' differentiation into articular chondrocytes remain only partially defined. For molecular analysis of cartilage regenerates the knowledge of cartilage-relevant differentiation and dedifferentiation markers is demanded. In order to identify such marker molecules, a 30,000-gene fragment array was used, allowing an almost genome-wide screening for differential gene expression in cartilage and MSCs. On this array 31,488 cloned gene fragments were represented and data analysis selected 105 differentially expressed genes with a more than three-fold difference in expression between cartilage and MSC. After sequence verification 87 differentially expressed genes have finally been identified. Differential expression was validated by real-time PCR for 18 genes, representing a validation rate of 69%, which is in line with the literature[23]. Since OA cartilage was used for cDNA analysis, the expression of identified candidate genes was also evaluated by RT-PCR in healthy cartilage.

Among the genes identified, a large number of known cartilage-relevant genes such as collagen type II, aggrecan, decorin, connective tissue growth factor (CTGF), or integrin alpha 10 was found within the cartilage tissue but not within the MSC group, which is an evidence for the high quality of our data analysis. We identified SERPINA1 as an attractive new differentiation marker since its gene expression and protein secretion correlated consistently with chondrogenic differentiation of MSCs and dedifferentiation of expanded chondrocytes in culture. To the best of our

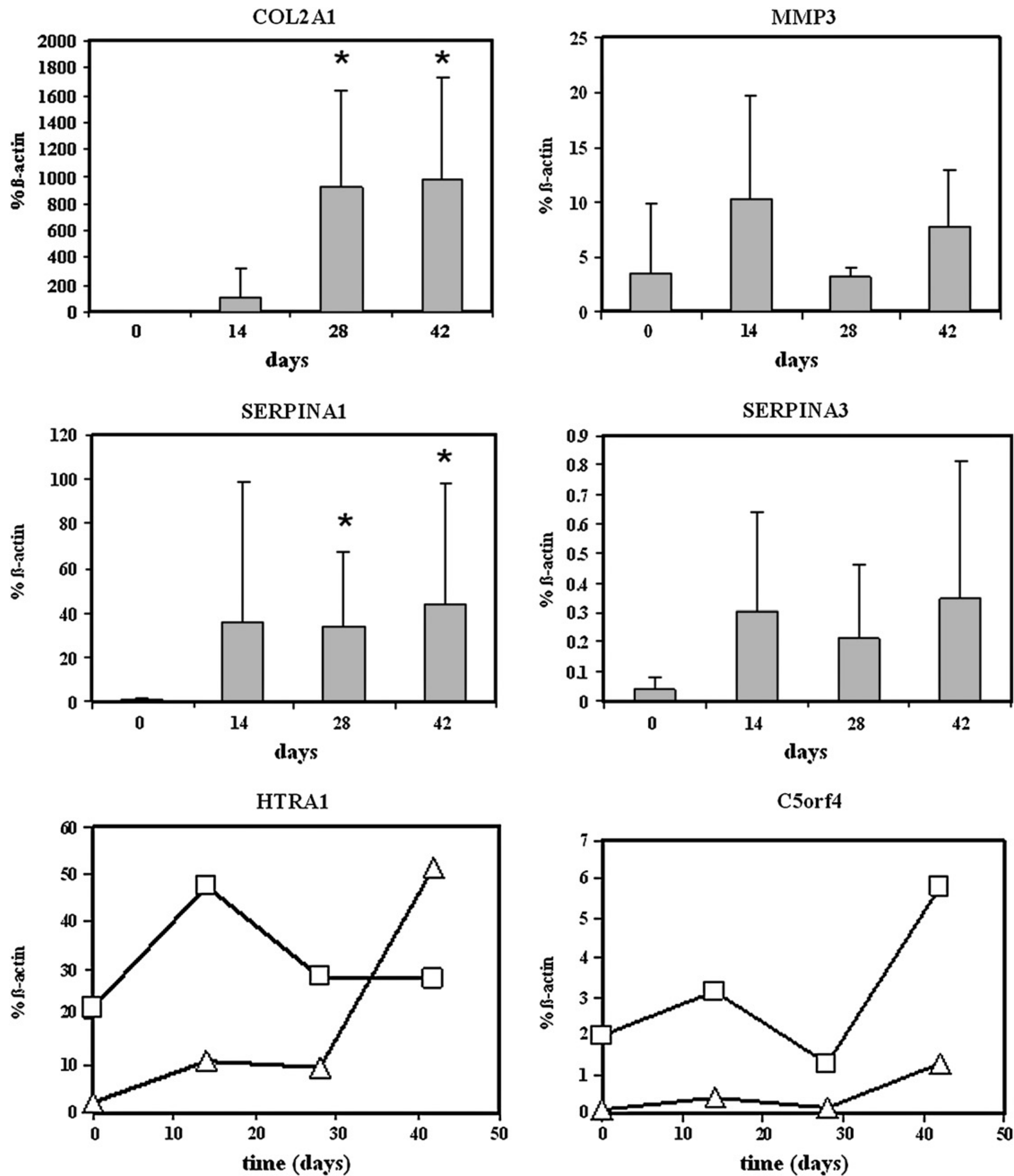

Fig. 3. Quantitative gene expression analysis during the chondrogenic differentiation of BMSCs. Expression levels of COL2A1, MMP3, SERPINA1, A3 ($n = 5$), HTRA1 and C5orf4 ($n = 2$) were assessed by real-time RT-PCR at days 0, 14, 28 and 42 after chondrogenic induction of BMSC spheroid cultures with TGF β_3 (□ and △ represent two donors). For each cDNA sample, the concentration of the reference housekeeping gene β-actin (ACTB) was used to normalise all genes tested. For COL2A1, MMP3, SERPINA1 and A3, mean values were calculated. *Significant differences to time point 0 ($P < 0.05$).

knowledge, this is the first study, which correlates the amount of a secreted marker protein in conditioned culture supernatant to the differentiation stage of cultured MSCs and chondrocytes.

The serpins are a superfamily of proteins comprising at least 35 human members which are involved in various biological processes[21]. They are characterised by the unique suicide substrate-like inhibitory mechanism they employ. Most serpins inhibit serine proteinases of the chymotrypsin family, but some cross-class inhibitors have been identified and several members no longer function as proteinase inhibitors. Serpins can be inactivated by various MMPs[24]. SERPINA1 and A3 in particular have been shown to be inactivated by MMP3[25]. For this reason, it is tempting to speculate that the two dedifferentiation markers SERPINA1 and MMP3 may be counter actors in the homeostasis of cartilage matrix.

SERPINA1 has been shown to be expressed in cultured human chondrocytes and up-regulated after IL-6 treatment[26]. It is also expressed in human cartilage, where decreased constitutive levels in OA cartilage as compared to normal cartilage have been noted[27] [Fig. 4(A)]. SERPINA1 can inhibit the action of plasmin, which may be a key activator of the pro-enzymes proMMP1 and proMMP3 in

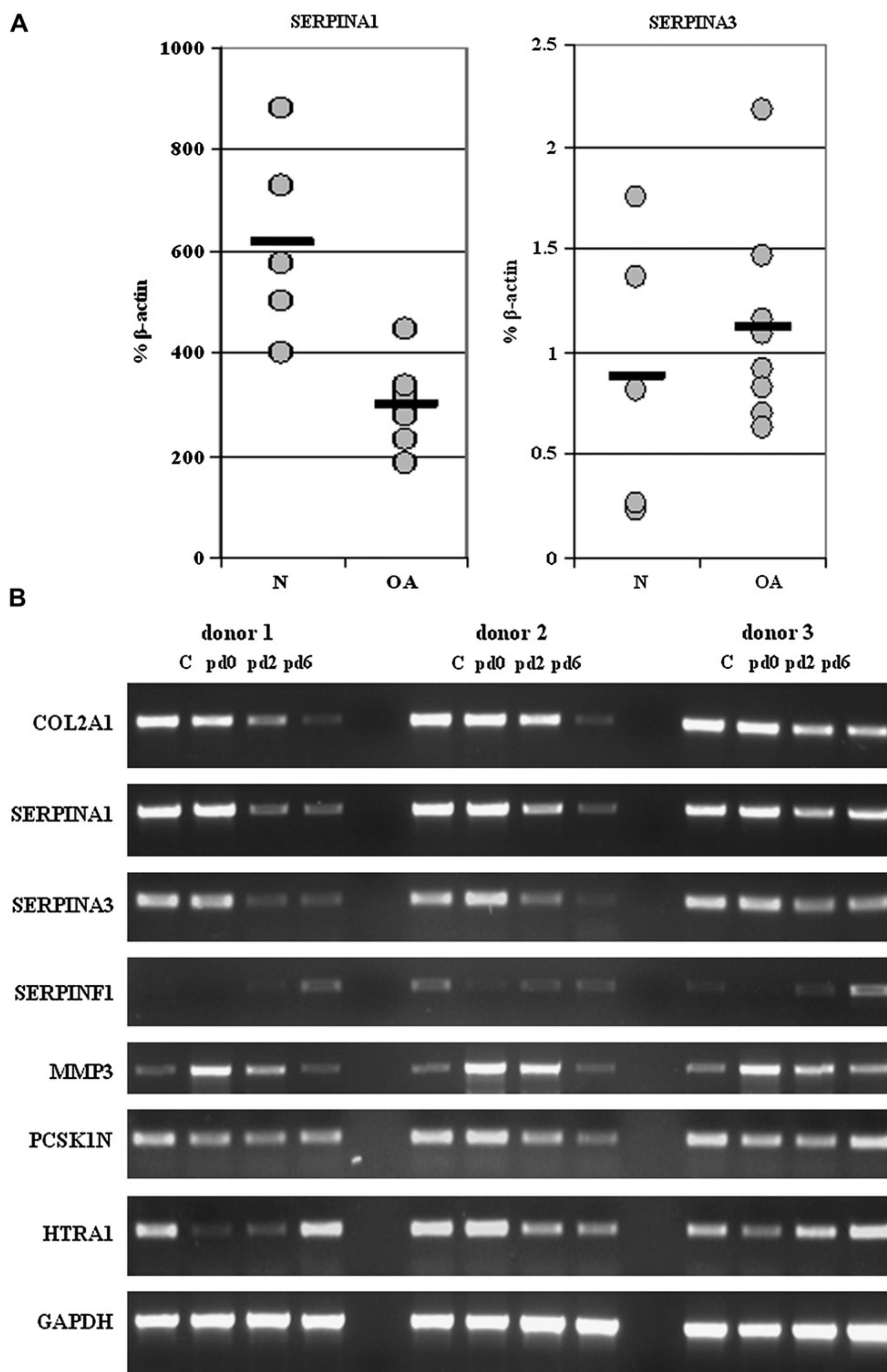

Fig. 4. (A) Expression of SERPINA1 and A3 in normal (N) cartilage ($n = 5$) and OA cartilage samples ($n = 8$). Expression levels were assessed by real-time RT-PCR. For each cDNA sample, the concentration of the reference housekeeping gene β-actin (ACTB) was used to normalise expression. (B) Expression of COL2A1, SERPINA1, A3, F1, MMP3, PCSK1N and HRTA1in cartilage and in dedifferentiating chondrocytes. RT-PCR was conducted with cartilage (C) and corresponding chondrocytes after digestion of cartilage (pd0), after pd2 and after pd6 from three donors. The concentrations of templates used in the reactions were adjusted according to GAPDH signals in order to achieve comparable signals. PCR products were visualised by agarose gel electrophoresis.

cartilage and blocks the interleukin1 (IL-1) and plasminogen dependent glycosaminoglycan (GAG) release of bovine cartilage explants[28] as well as the IL-1/oncostatin-dependant collagen degradation in bovine nasal cartilage explant cultures[29]. These effects of SERPINA1 have been attributed to a blockage of the activation of procollagenases and suggest a protective role for serine proteinases in the activation cascades of collagenases that initiate the breakdown of cartilage matrix. Most remarkably we now demonstrate that this inhibitor can serve as a marker of

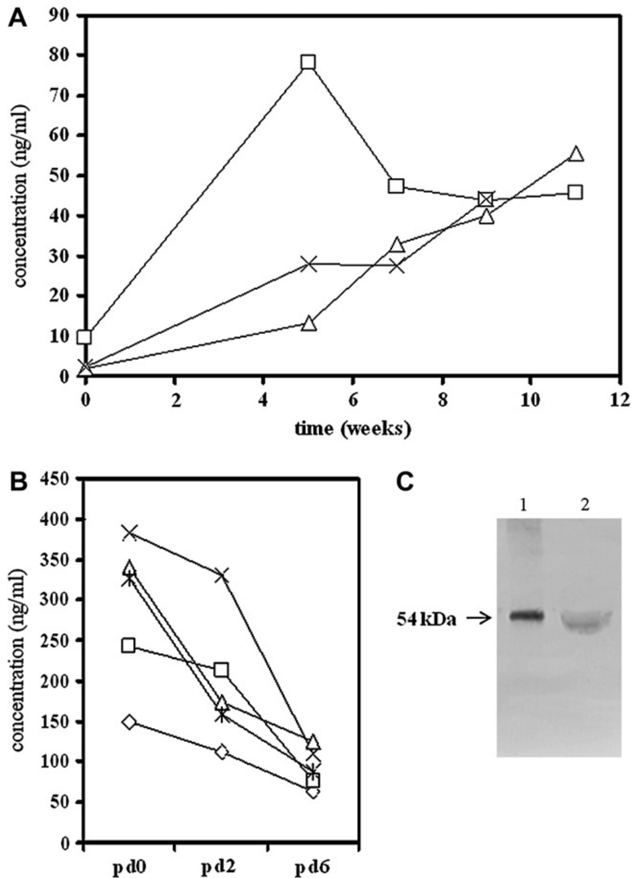

Fig. 5. Secretion of the SERPINA1 protein during the chondrogenic differentiation of BMSCs and the dedifferentiation of chondrocytes. Protein concentrations were assessed by ELISA in cell culture media conditioned for 4 days. (A) Supernatants from spheroids with 5×10^5 cells were measured after 5, 7, 9 and 11 weeks of chondrogenic induction for three donors. (B) Supernatants from 5×10^5 chondrocytes after digestion of cartilage (pd0), after pd2 and after pd6 were measured for five donors. SERPINA1 concentration after pd6 was significantly lower than from freshly isolated cells ($P < 0.05$). (C) Serum-free cell culture medium of freshly isolated chondrocytes conditioned for 3 days was used for immunoblotting. Western blot analysis of concentrated medium revealed a single band close to 54 kDa (lane 2). In lane one, 1 μg human plasma SERPINA1 was blotted as positive control.

chondrogenesis in MSCs and of dedifferentiation in cultured chondrocytes.

According to our data other proteases and protease inhibitors expressed in cartilage could further be used as differentiation markers. Among them is another serpin, SERPINA3, whose expression profile was similar to that of SERPINA1, but showed much lower mRNA expression levels. It was identified to be expressed in adult, healthy or OA cartilage[8] as well as in foetal cartilage[7]. For a better evaluation of SERPINA3 as a differentiation marker a quantitative protein assay is needed to assess its protein expression and secretion by chondrocytes.

A further potentially interesting inhibitor is the proprotein convertase subtilisin/kexin type 1 inhibitor (PCSK1N, also termed proSAAS), which was only weakly down-regulated during dedifferentiation of chondrocytes. The protein is cleaved in several smaller peptides which may function as endogenous inhibitors of prohormone convertase 1 (PC1). A role in cell–cell signalling has also been proposed[30,31].

For the HtrA serine peptidase 1 (HTRA1) no regulation was found during dedifferentiation and published data indicate that it may rather be associated to hypertrophic differentiation[32]. Several compounds of cartilage ECM have been identified as substrates for HTRA1: aggrecan, decorin, fibromodullin, collagen type II and fibronectin[33,32]. Interestingly HTRA1 has been shown to form a stable complex with SERPINA1 and to be inhibited by this protease inhibitor[34] indicating that an interacting network of proteases (HTRA1, MMP3) and inhibitors may be regulated during differentiation.

Besides the genes analysed in more detail, other potentially interesting markers may arise from the 87 genes identified in this study, like TSC22, potentially playing a stimulatory role in chondrogenesis[35]; calsyntenin, potentially implicated in cell adhesion[36] or the antiangiogenic factor SERPINF1, identified in MSCs.

Altogether, comparative cDNA-array analysis between MSCs and cartilage revealed new transcripts, genes with established or putative function in chondrogenesis as well as new candidate molecules relevant for chondrocyte differentiation. The expression of distinct proteases and protease inhibitors correlated to the differentiation of MSCs and the dedifferentiation of chondrocytes during monolayer expansion and expression of SERPINA1, both on the mRNA and the protein level, could be used as an indicator for the differentiation stage of the cells. Most attractively SERPINA1 is released into the medium of cultured cells which will allow correlation of the concentration of this factor to functional parameters like ectopic cartilage formation capacity of chondrocytes in future studies. This could yield attractive quality control parameters for chondrocytes used in cell therapy and tissue engineering approaches. Most interestingly, protease inhibitors like SERPINA1 may play a protective role against matrix degradation not only during cartilage development and regeneration but also under inflammatory and degenerative conditions found in joint diseases.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.joca.2007.05.008.

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