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Gene expression during redifferentiation of human articular chondrocytes

Tommi Tallheden B.Sc.†*, Camilla Karlsson M.Sc.†, Andreas Brunner‡, Josefine van der Lee M.Sc.†, Rupert Hagg Ph.D.‡, Roberto Tommasini Ph.D.‡ and Anders Lindahl M.D., Ph.D.†

†*Department of Clinical Chemistry and Transfusion Medicine, Sahlgrenska University Hospital, Gothenburg, Sweden*

‡*Millenium Biologix AG, Wagistrasse 23, CH-8952 Zurich-Schlieren, Switzerland*

Summary

Objective: The aim of the present study was to investigate gene expression during the *in vitro* redifferentiation process of human articular chondrocytes isolated from clinical samples from patient undergoing an autologous chondrocyte transplantation therapy (ACT).

Method: Monolayer (ML) expanded human articular chondrocytes from four donors were cultured in a 3D pellet model and the redifferentiation was investigated by biochemistry, histology, immunohistochemistry and microarray analysis.

Results: The culture expanded chondrocytes redifferentiated in the pellet model as seen by an increase in collagen type II immunoreactivity between day 7 and 14. The gene expression from ML to pellet at day 7 included an increase in cartilage matrix proteins like collagen type XI, tenascin C, dermatopontin, COMP and fibronectin. The late phase consisted of a strong downregulation of extracellular signal-regulated protein kinase (ERK-1) and an upregulation of p38 kinase and SOX-9, suggesting that the late phase mimicked parts of the signaling processes involved in the early chondrogenesis in limb bud cells. Other genes, which indicated a transition from proliferation to tissue formation, were the downregulated cell cycle genes GSPT1 and the upregulated growth-arrest-specific protein (gas). The maturation of the pellets included no signs of hypertrophy or apoptosis as seen by downregulation of collagen type X, Matrix Gla protein and increased expression of caspase 3.

Conclusion: Our data show that human articular chondrocytes taken from surplus cells of patient undergoing ACT treatment and expanded in ML, redifferentiate and form cartilage like matrix *in vitro* and that this dynamic process involves genes known to be expressed in early chondrogenesis.

Key words: Articular chondrocytes, Redifferentiation, Microarray, Autologous cell transplantation, Chondrogenesis.

Introduction

Articular cartilage is an avascular tissue with limited or no potential of self-repair. Lesions in adult cartilage caused by trauma will eventually lead to erosion of the cartilage surface and development of osteoarthritis (OA). In contrast to the adult cartilage, the embryonic cartilage has the ability to repair[1]. Based on these findings one strategy in the promising field of tissue engineering related to cartilage regeneration is to try to mimic the embryonic events of chondrogenesis during joint formation by applying specific growth factors, progenitor cells[2] or combinations thereof.

The use of autologous culture expanded chondrocytes for repair of cartilage lesions in adults was first reported from our laboratory in 1994[3]. In this method, mature chondrocytes are released from their surrounding matrix and proliferated in culture dishes under the influence of serum. During this expansion the cells dedifferentiate and gradually change their shape as well as their metabolic state and program of matrix biosynthesis[4–6]. Generally, downregulated syntheses of cartilage-specific proteoglycans and protein markers, such as collagen type II, are seen as signs of chondrocyte dedifferentiation[4].

The dedifferentiated chondrocytes are able to redifferentiate and upregulate the synthesis of cartilage matrix proteins when placed in 3D cultures such as "pellet" cultures[7–9]. The pellet system has also been used within chondrogenic differentiation studies of embryonic limb bud cells[10] and mesenchymal stem cells (MSC s)[11] isolated from bone marrow[12,13].

More recently, microarray technology has been increasingly applied during *in vitro* cartilage and bone formation to decipher the signals that trigger and control specific molecular events of differentiation[14–19]. Considering the enormous complexity of such differentiation pathways involving a large number of genes, simultaneous gene expression profiling of hundreds to thousands of genes by means of microarrays is well suited to better understand the underlying mechanisms[20].

Thanks to microarrays and other cutting edge technologies, the old concepts of linear and irreversible stem cell differentiation have to be revisited and replaced these days by new evolving concepts of stem cell plasticity[21]. We[22] and

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* Address correspondence and reprint requests to: Tommi Tallheden, B.Sc., Department of Clinical Chemistry and Transfusion Medicine, Sahlgrenska University Hospital, Gothenburg University, 41345 Gothenburg, Sweden. Tel: 46-31-3422445; Fax: 46-31-412250; E-mail: tommi.tallheden@medic.gu.se
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others[23,24] have recently shown that even adult human articular chondrocytes show some phenotypic plasticity. Once dedifferentiated in monolayer (ML), it was demonstrated that multiclonal populations of adult human chondrocytes could be differentiated into the chondrogenic, osteogenic, and adipogenic lineage.

In the present study we asked the question whether culture expanded chondrocytes express embryonic genes during redifferentiation *in vitro*, which would further support the hypothesis of the existence of progenitor cells within adult articular cartilage.

Material and methods

CELL CULTURE

Surplus chondrocytes from patients (age 32–41) undergoing autologous chondrocyte transplantation (ACT)[3] were cultured in a 3D pellet culture system as described earlier[12,13]. Briefly, 2.0×10^5 cells from passage 1 (maximum 7 cell doublings), were placed into polypropylene conical tubes with 0.5 ml of defined medium, consisting of DMEM-HG (PAA Laboratories, Linz, Austria) supplemented with ITS-G (Life Technologies, Sweden), 5.0 μg/ml linoleic acid (Sigma–Aldrich, Stockholm), 1.0 mg/ml human serum albumin (HSA [Equitech-Bio, TX, USA]), 10 ng/ml TGF-β1 (R&D Systems, UK), 10^{-7} M dexamethasone (Sigma), 14 μg/ml ascorbic acid (Sigma) and Penicillin–Streptomicin (PEST [PAA lab.]). The cells were centrifuged at 500 *g* for 5 min and maintained at 37°C in 7% CO_2/93% air. The medium was changed twice a week.

HISTOLOGY OF PELLETS

On day 7 and 14 the pellets were fixed in Histofix™ (Histolab products AB, Sweden), dehydrated in increasing concentrations of ethanol and embedded in paraffin. Five micrometer sections were cut and placed onto microscope slides (Superfrost Plus, Menzel-Gläser, Germany), deparaffinized and stained with Alcian blue/van Gieson and Safranin-O or immunoassayed with anti-type I and II collagen, as described below. The stained sections were analyzed by a Nikon microscope.

IMMUNOHISTOCHEMISTRY OF PELLETS

Sections were deparaffinized with decreasing amounts of ethanol, digested with hyaluronidase, 8000 units/ml (Sigma) in 0.1 M phosphate buffered saline (PBS) for 60 min at 37°C, blocked with 3% BSA (Sigma) in PBS for 5 min. The primary monoclonal antibodies (anti-collagen type I and II (ICN Biomedicals, Aurora, OH)) were diluted 1:200 in PBS containing 3% BSA and incubated with the sections for 1 h at room temperature. The secondary antibody (FITC conjugated goat anti-mouse), diluted 1:100, was incubated for 1 h and after rinsing the sections were mounted with fluorescence mounting medium. The sections were then analyzed with Nikon fluorescence microscope and digital pictures were taken with the ACT-1 software. By using Adobe Photoshop, the pictures of collagen type I staining were changed into a pseudo color and mounted together with the collagen type II picture.

BIOCHEMICAL ANALYSIS OF PELLETS

On day 7 and 14, pellets were digested with papain (Sigma) solution (0.3 mg papain/ml sodium phosphate buffer [20 mM] with 1 mM EDTA and 2 mM dithiothreitol) for 60 min at 60°C. The digested pellets were then mechanically dissolved by vortex and further analyzed for DNA, glycosaminoglycan (GAG) and hydroxyproline content.

The amount of DNA was measured with CyQuant cell proliferation assay kit (Molecular Probes, Eugene, OR), according to the manufacturer's instruction.

The GAG content was measured spectrophotometrically at 515 nm using dimethylmethylene blue assay[25], with chondroitin sulphate as a standard, and normalized to the DNA amount in the pellet.

Hydroxyproline, as a measurement of total collagen in the digested pellet, was analyzed with a modified method as described earlier[26]. Briefly, digested pellets were hydrolyzed over night with 6 N hydrochloric acid at 127°C and then dissolved in assay buffer ([3:2:10] *n*-propanol, deionized water and stock buffer, pH 6.1 (0.24 M citric acid, 0.88 M sodium acetate trihydrate, 0.88 M anhydrous sodium acetate, 0.21 M acetic acid and 0.85 M sodium hydroxide)). To the dissolved samples chloramine-T reagent (0.282 g chloramine-T [Sigma], 1 ml deionized water, 1 ml *n*-propanol and 8 ml stock buffer) and DMBA reagent (2 g dimethylaminobenzaldehyde [Sigma], 1.25 ml *n*-propanol, and 2.75 ml perchloric acid) were added. Hydroxyproline content was measured spectrophotometrically at 550 nm with reference at 650 nm and normalized to the DNA content in the pellet. As a standard, hydroxyproline (Sigma) was used. All samples were made in duplicates.

RNA ISOLATION

Total RNA was extracted from cells cultured in ML (1×10^6) or from pellets at day 7 and 14. The pellets were collected in 1.5 ml micro tube (Greiner) containing RLT buffer and disrupted by sonication. To shear genomic DNA a QIAshredder column (Qiagen) was used prior to RNA cleanup. Total RNA was extracted by using RNeasy Mini Kit (Qiagen) according to the protocol provided by the manufacturer.

MICROARRAYS

Two micrograms of total RNA was used for RNA amplification and labeling by the methods of Eberwine *et al.*[27] and Baugh *et al.*[28] with some modifications[29]. Labeled and fragmented amplified RNA (aRNA) was diluted in 800 μl hybridization solution and hybridized over night onto CARTCHIP™ 300 microarray (Millenium Biologix AG) in a provided hybridization chamber at 42°C according to the protocol provided by the manufacturer[29]. The hybridization chamber used within the current studies has been designed to fit into a PCR thermal cycler in order to provide minimal temperature variations and optimal heat transfer during the hybridization process.

Subsequently microarray slides were washed and dried for data acquisition in a 418 Microarray Scanner (Affymetrix).

DATA ANALYSIS

Data analysis and data normalization were performed based on the method described by Quackenbush *et al.*[30]. Quantitative spot intensity was determined for all 12 samples by adding up the pixel intensities within a gene representing a spot. For background correction the mean intensity of the spot surrounding area was subtracted for each spot (local area background). Data sets were normalized by dividing the mean intensity value of every spot's duplicate by sum of all

spot intensities within a sample to eliminate experimental or data acquisition variations. Normalized data were directly used to calculate gene expression level ratios between different culture stages. Two-fold change threshold was set to exclude unspecific ratios.

For hierarchical gene cluster analysis ratios were calculated for every gene to the median value of all samples within a distinct gene and performed according to the method of Eisen *et al.*[31]. Genes as well as expression profiles for all samples were clustered with uncentered correlation.

STATISTICS

The biochemical analyses of the pellets were made in duplicates from all four donors. The values are presented as mean $\pm$ SD. Changes in GAG/DNA and HP/DNA from day 7 to 14 were analyzed with the Wilcoxon paired signed rank test. Values of $P < 0.05$ were considered to indicate statistically significant differences.

Results

BIOCHEMICAL ANALYSIS AND HISTOLOGY

Redifferentiation of the expanded chondrocytes [Fig. 1(A)] was performed by pelleting the cells into micromass cultures in polypropylene tubes in serum free culture medium. Immediately after centrifugation the cells aggregated into a flat disk that gradually expanded in size into a rounded pellet [Fig. 1(B)]. The expansion in size of the pellet during the culture period was accompanied with accumulation of increasing amounts of cartilage matrix as demonstrated by biochemical analysis of total proteoglycans and collagens [Fig. 2(A and B)]. In addition, maturation of the pellets was shown by decreasing cell density and increasing Safranin-O staining of proteoglycans and

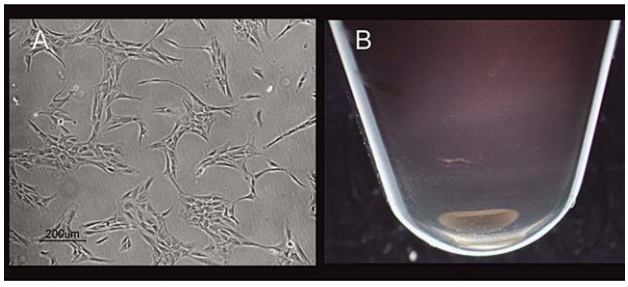

Fig. 1. Monolayer (2D) and pellet (3D) culture of human articular chondrocytes. (A) Phase contrast picture of passage 1 human articular chondrocytes and (B) macroscopic picture of day 6 pellet in 15 ml polypropylene tubes.

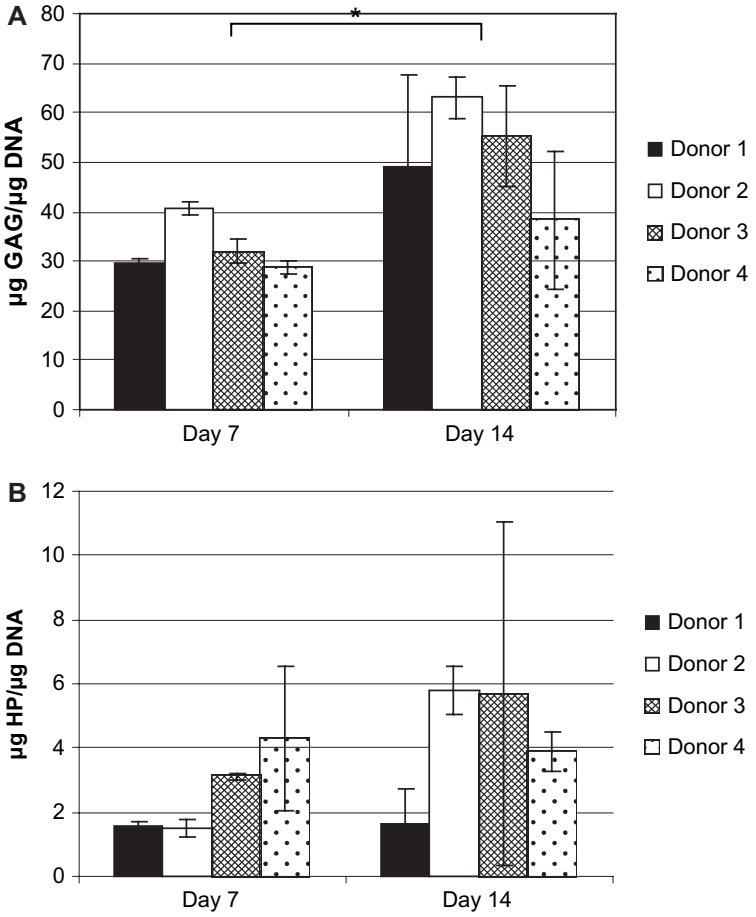

Fig. 2. Glycosaminoglycan (GAG) and hydroxyproline (HP) content normalized to the DNA amount of pellets from donors 1 to 4 at day 7 and 14. (A) The amount of GAG/DNA increased in all donors from day 7 to 14. (B) The amount of HP/DNA as a measurement of total collagen secretion per cell increased in donors 2 and 3 between day 7 and 14, while in donors 1 and 4 it remained at a constant level. An asterisk (*) indicates statistically significant difference between day 7 and 14.

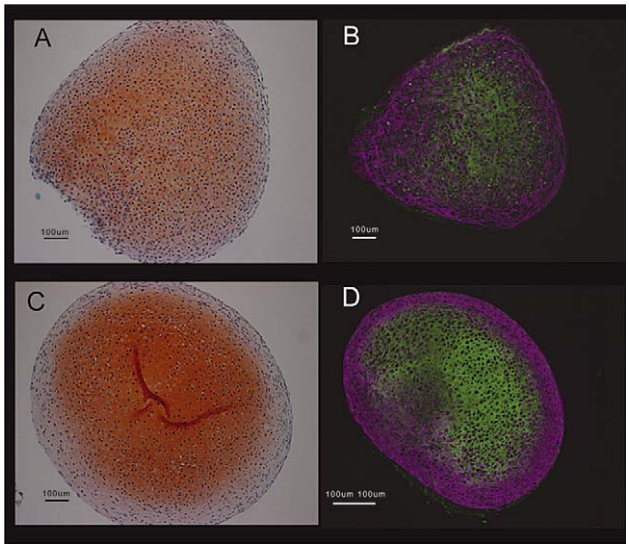

Fig. 3. Histological sections of pellets from Donor2 at day 7 and 14. The Safranin-O staining for glycosaminoglycan (GAG) showed increasing amounts from day 7 to 14 (A,C). The increase in matrix proteins production resulted in a lower cell density in the *in vitro* formed tissue. The increase in GAG from day 7 to 14 was accompanied by an increase in immunoreactivity collagen type II (green) (B,D) and a decrease in collagen type I (pink) (B,D).

with antibodies raised against collagen type I and type II. The presence of high amounts of collagen type II and a strong staining with Safranin-O as shown in Fig. 2 provide evidence that the chondrocytes have redifferentiated into a cartilage like phenotype.

The change in pattern of collagen type I staining from a homogenous staining at day 7 into a perichondreal like staining surrounding the type II rich matrix, further indicate an ongoing maturation of the pellets towards a cartilage like tissue [Fig. 3(A–D)]. Assays of total DNA showed no difference between day 7 and 14, indicating that the process of redifferentiation took place without change in cell number (data not shown).

MICROARRAYS

Two major dendogram branches were found when gene expression profiles of all four donor samples from different time points were clustered with uncentered correlation [Fig. 4(A)]. Group 1 contained expression profiles of proliferated chondrocytes as well as chondrocytes cultured for 7 days in pellet culture, whereas group 2 consisted of expression profiles of chondrocytes cultured for 14 days. Interestingly one sample profile from Donor2 ML was clustered in a separate branch indicating a different gene expression profile with respect to the proliferated sample from the other donors. The other profiles of Donor2 (day 7 and 14) were clustered in group 1 and group 2, respectively.

The current gene expression analysis revealed that approximately half of the 336 genes provided on the microarray were either induced or repressed. Fig. 4(B) shows a selected subset of 98 genes out of 336 representing similarities in gene expression pattern within different samples. Genes (rows) were clustered with uncentered correlation. The samples (columns) remained unclustered to point out consistencies and differences between all donors.

The majority of the expressed genes in Fig. 4(B) marked with bars **a** and **b** show a characteristic gene expression pattern for corresponding time points from the different donors, indicating maturation process relevant genes. Major changes were found from day 7 to 14 (late phase), whereas only few expression changes can be seen between ML and day 7 (early phase). Most of the selected genes shown in the matrix in Fig. 4(B) are either up- or downregulated during the late phase, but also regions that do not show any obvious changes within all samples can be seen. Outside the marked regions **a** and **b** are shown in Fig. 4(B), some differentially expressed genes may be associated with a patient specific expression pattern.

When the gene expression ratios of individual genes were calculated between the pellets and ML cultures for all 336 genes, 133 showed a significant change in at least 3 out of 4 donors. Table I shows 97 of these genes, selected by extended literature review to be described in relation to cartilage or chondrocytes. In the table the genes are functionally grouped accordingly, from one time point to the other, providing all the gene expression ratios for each time point including genes that did not change during a previous or later time point. From this table either a specific expression profile for a certain maturation phase can be deduced.

In order to identify specific genes for the early and late phases, genes from Table I, which were significantly changed in either of the phases, were selected into early and late genes and plotted against each other and displayed in a scatter plot [Fig. 5(A and B)]. The early phase contained 9 genes which were specifically and significantly changed while the late phase included 48 genes, illustrating larger similarities in gene expression between ML and day 7 pellets than day 7 and 14 pellets, confirming the results from the cluster analysis.

Discussion

Autologous culture expanded chondrocytes, isolated from full depth biopsies have been used in our lab since 1987 for repair of cartilage defects with promising long term results[32]. In this method, a mixed population of cells is transplanted into an *in vivo* bioreactor, formed by a periosteal flap and the surrounding cartilage, within the joint. In this environment the cells aggregate, attach and start to build matrix[33,34]. The high number of cells used for implantation ($1\times10^6/cm^2$) promotes chondrogenic redifferentiation, since they support cell–cell interactions[35]. In this study, by using microarray technology, we have shown that expanded human chondrocytes redifferentiate in an *in vitro* model and form stable cartilage like constructs. During this dynamic process, the cells express many of the genes associated with early chondrogenesis indicating that even differentiated cells are capable of reinitiating the process. This has an important implication with respect to the clinical use of cell based therapies for regeneration of cartilage defects.

Because of the genetic variability among individuals, many investigators have chosen to pool RNA samples from different donors before use in a cDNA array analysis[36]. Other studies have evaluated RNA samples separately and used either average changes in gene expression or changes in gene expression found in the majority of their samples[37]. In our study we decided to analyze the RNA samples from four individuals that underwent ACT treatment separately. By using this approach we were able to study both similarities and differences between the donors in the

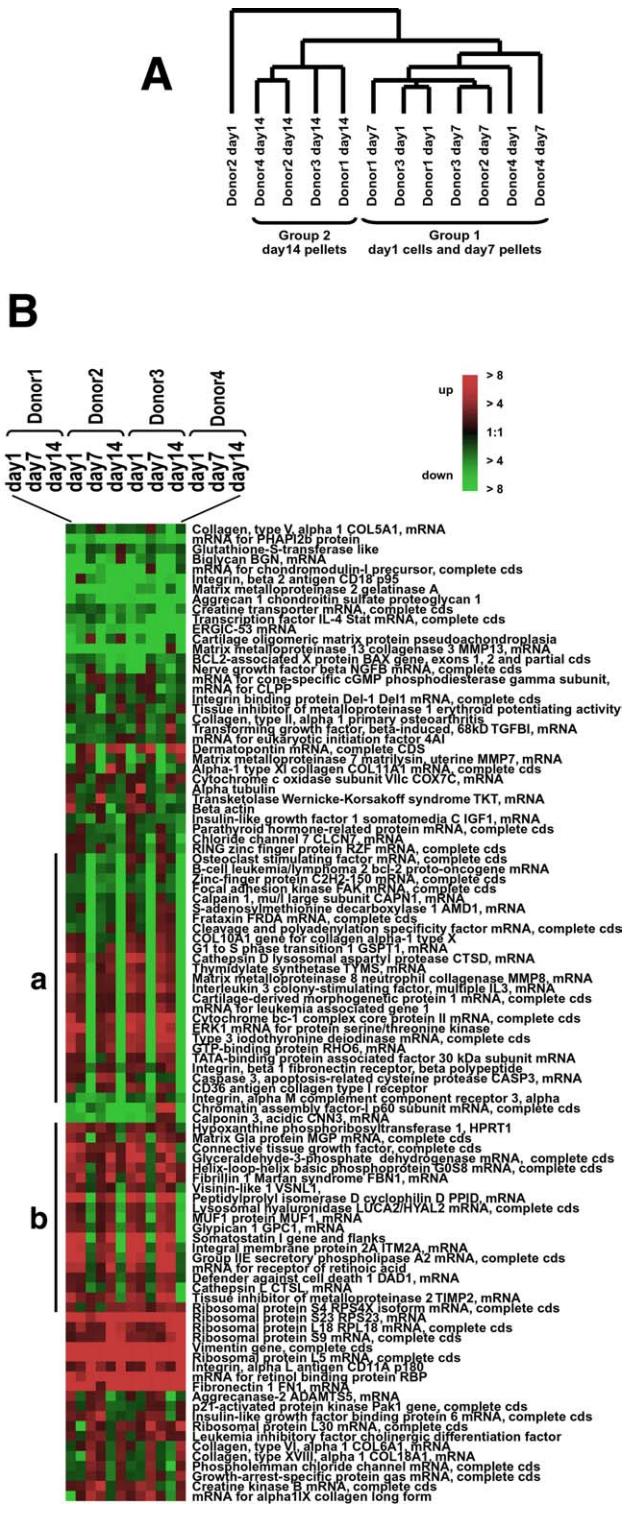

Fig. 4. Variation in expression of 336 genes in 12 experimental samples. Data are presented in a matrix format: each row represents a single gene, and each column an experimental sample. In each sample, the ratio of the abundance of transcripts per gene to the median abundance of the gene's transcript among all the different cell samples is presented by the color of the corresponding matrix. Green squares, transcript levels below the median; black squares, transcripts levels equal to the median; red squares, transcript levels greater than the median. Color intensity/saturation reflects the magnitude of the ratio relative to the median for each set of samples (see scale, top right). (A) Dendogram showing clustered samples according to their gene expression profile. Samples with similar gene expression profiles are located in a specific branch. In general, two main branches can be seen. Group 1 including ML and day 7 samples and group 2 consisting of samples from pellets cultured for 14 days. Only one sample (Donor2 ML) was clustered in a separate branch. (B) Gene expression matrix representing similarities in gene expression patterns between different experimental samples. A representative subset of 98 genes is shown. Genes with similar gene expression levels were clustered together. Expression profiles remained unclustered to illustrate differences in gene expression within donors. Two specific gene clusters showing differences between ML cells, day 7 and 14 pellets are indicated as bar **a** and **b**.

gene expression profiles during the redifferentiation process.

Regarding the performed microarray analysis within the current study we do not believe that the differences in gene expression ratios between the donors are caused by technical shortcomings. The oligonucleotides used within the current microarrays are spotted in duplicates and this drastically reduces the risk of false-positive signals caused by fluorescent particles. Additionally, the use of a controlled environment allowing a streamlined and accurate sample processing is indispensable for reproducible and reliable hybridization experiments. For this reason the amplified RNA samples were hybridized in a T-Gradient PCR thermal cycler (Whatman Biometra) in a hybridization chamber (Millenium Biologix) specially developed to ensure precise temperature and volume control. We also used one single round of amplification which has been suggested to be generally used for all RNA specimens prior to microarray-based gene expression analysis to improve data quality and detection of differentially expressed genes[38]. In order to validate the amplification protocol for our microarrays, we performed amplification reactions under different conditions. We found that the amplification of mRNA could be performed in a very robust manner with low experimental variations for varying RNA starting amounts as well as different microarray slides[39]. For this purpose, 0.5 up to 2 μg of total RNA was amplified and hybridized onto two microarray slides. Spot intensity ratios of both arrays were calculated as described before and correlated with a correlation coefficient of 0.9. These results are in accordance with the literature where similar experiments with even lower amounts of starting RNA have been used and compared[28]. The described protocol thereof, offers a reasonable reproducibility when the amount of RNA starting material is limited, especially when isolated from pellet cultures.

During the embryonic formation of the skeleton, the differentiation of the chondrogenic mesenchyme can be divided into three sequential phases: epithelial–mesenchymal interaction, condensation and differentiation, each phase consisting of specific cellular events and molecular characteristics. Similar phases could be observed during the redifferentiation of chondrocytes in the pellet model used within the current study. While expanded chondrocytes showed a flat fibroblast looking morphology, cells cultured as pellets turned into a round matrix secreting cell population [Figs. 1(B) and 3]. After the initial morphogenesis, the chondrocytes started to express collagen proteins and at day 7 high expression of collagen type I was observed in the whole pellet. This expression was changed into collagen type II at day 14 (Fig. 3). Furthermore, the collagen type I expression was at day 14 limited to the flat cells surrounding the collagen type II matrix, imitating a formation of a

Table I

*Gene expression ratios of ML vs day 7 pellets and day 7 vs day 14, of 97 selected genes. The genes are grouped into functional groups and the ratios are shown for all donors (upregulated=italics and downregulated=normal). Genes with specific changes only during the "early" phase are indicated with **E** and markers of the "late" phase are indicated with **L***

Name		ML vs day 7				ML vs day 14				Marker
		Don 1	Don 2	Don 3	Don 4	Don 1	Don 2	Don 3	Don 4	
Matrix proteins										
XM_042153	Biglycan (BGN)	*1,06*	*1,23*	*1,65*	*1,29*	*3,25*	*4,27*	*2,36*	*2,99*	L
X60382	Collagen, type X, alpha-1 (COL10A1)	1,26	*1,15*	1,47	1,40	4,14	6,21	6,57	19,25	L
J04177	Collagen type XI alpha-1 (COL11A1)	*1,59*	*11,02*	*2,88*	*5,76*	*1,16*	*4,26*	*2,46*	*11,61*	
XM_036175	Collagen, type XVIII, alpha 1 (COL18A1)	1,86	1,63	1,27	1,73	*3,51*	*1,59*	*2,10*	*2,45*	L
NM_002160	Hexabrachion (tenascin C, cytotactin) (HXB)	*5,99*	*9,49*	*2,54*	*2,14*	*9,34*	*12,70*	*2,31*	*1,50*	
XM_009336	Cartilage oligomeric matrix protein (COMP)	*2,32*	*2,77*	*4,16*	*11,02*	*8,82*	*6,01*	*1,68*	*3,95*	
Z22865	Dermatopontin	*6,26*	*7,49*	*16,88*	*34,40*	*11,37*	*8,32*	*26,57*	*60,33*	
XM_055254	Fibronectin 1 (FN1)	*6,60*	*31,20*	*3,57*	*8,58*	*5,02*	*44,80*	*1,53*	*3,79*	
NM_005506	CD36 antigen (collagen type I receptor) (CD36L2)	3,17	*1,19*	1,19	1,18	7,61	5,51	10,57	6,36	L
XM_002321	Glypican 1 (GPC1)	1,03	*2,09*	1,49	1,27	2,80	2,11	3,31	7,72	L
NM_002379	Matrilin 1 (MATN1)	1,06	1,74	1,00	2,58	*1,62*	*3,08*	*2,43*	*2,54*	L
XM_029245	Collagen, type I (COL1A2)	1,69	6,67	1,32	1,04	*2,36*	*2,95*	*3,68*	*1,62*	L
XM_054566	Collagen, type VI (COL6A1)	1,08	1,76	1,60	1,73	*1,97*	*2,43*	*3,04*	*4,72*	L
XM_037965	Chondroadherin (CHAD)	1,23	2,59	1,08	1,91	*1,55*	*2,04*	*2,76*	*2,30*	L
Metalloproteinases										
NM_002421	Matrix metalloproteinase 1 (MMP1)	1,16	3,44	1,08	1,64	1,10	2,56	*2,33*	*2,30*	L
XM_017384	Matrix metalloproteinase 7 (MMP7)	*6,89*	*2,82*	*3,27*	*6,73*	*9,74*	*4,67*	*10,10*	*23,27*	
NM_002424	Matrix metalloproteinase 8 (MMP8)	1,52	*1,06*	1,20	1,34	9,76	13,27	11,53	19,82	L
NM_004995	Matrix metalloproteinase 14 (MMP14)	*1,27*	4,09	*1,40*	1,64	*1,73*	1,56	1,64	*2,91*	
NM_001912	Cathepsin L (CTSL)	1,22	*1,86*	1,56	1,16	2,95	2,02	3,62	7,37	L
XM_006121	Cathepsin D (CTSD)	1,54	*1,15*	1,18	1,37	7,67	13,85	10,09	23,45	L
XM_085705	Tissue inhibitor of metalloproteinase 2 (TIMP2)	1,51	*1,43*	1,13	1,04	2,08	3,24	2,81	10,84	L
U09577	Lysosomal hyaluronidase (LUCA2/HYAL2)	1,51	*1,38*	1,23	1,27	3,55	2,78	3,87	6,55	L
NM_005186	Calpain 1 (mu/l) large subunit (CAPN1)	*1,11*	*1,34*	1,66	1,48	3,32	4,75	6,50	13,15	L
XM_035662	Cathepsin B (CTSB)*	1,88	4,20	1,99	1,88	*2,44*	*1,70*	*2,43*	*2,25*	
Integrins										
NM_000632	Integrin, alpha M (CD11b)	2,22	*1,30*	1,18	1,29	2,31	1,71	2,05	2,81	L
NM_002211	Integrin, beta 1 (fibronectin receptor, CD29) (ITGB1)	*1,00*	1,10	*1,09*	*1,96*	2,67	2,60	2,33	5,48	L
XM_003913	Integrin, alpha 2 (CD49B) (ITGA2)	1,17	5,16	1,08	1,64	*4,35*	1,34	*5,20*	*6,19*	L
XM_036107	Integrin, beta 2 antigen CD18 (p95) (ITGB2)	*5,96*	1,33	*2,17*	*4,70*	*2,48*	2,65	*3,06*	*3,31*	
L13616	Focal adhesion kinase (FAK)	1,03	*1,20*	1,09	1,32	2,69	4,53	3,51	4,93	L
U59289	H-cadherin	1,03	2,52	2,05	3,12	*2,06*	*1,33*	*1,85*	*1,02*	E
NM_080682	Vascular cell adhesion molecule 1 (VCAM1)	2,39	3,25	2,25	6,79	*1,01*	2,69	1,87	1,77	E
Cell cycle										
NM_002094	G1 to S phase transition 1 (GSPT1)	1,30	*1,97*	1,37	1,39	5,41	8,39	7,44	19,79	L
XM_042664	Nuclear autoantigenic sperm protein (NASP)	2,40	6,41	*2,30*	1,64	*1,18*	2,63	*3,09*	*3,23*	
NM_007306	Breast cancer 1, early onset (BRCA1)	1,30	3,97	*1,58*	1,64	*1,95*	4,11	*1,85*	*1,37*	
L13720	Growth-arrest-specific protein (gas)	1,07	2,31	1,07	1,96	*2,00*	*2,55*	*2,44*	*3,71*	L
Development										
X60188	ERK-1 for protein serine/threonine kinase	1,15	*1,14*	1,21	1,44	14,01	15,42	14,99	34,31	L
XM_039094	SRY (sex determining region Y)-box 9 (SOX9)	1,73	1,05	1,15	2,94	*4,84*	*1,54*	*3,54*	*4,30*	L
U92268	Mitogen activated protein kinase p38-2	1,82	2,73	1,33	1,89	*2,73*	*2,29*	*3,02*	*3,62*	L
M97676	Homeobox protein (region 7) (HOX7)	1,04	3,02	1,08	8,01	*1,61*	*1,98*	*2,62*	*2,48*	

Apoptosis										
XM_054686	Caspase 3, apoptosis-related cysteine protease (CASP3)	1,20	1,10	1,15	1,12	4,69	23,24	5,65	7,36	L
M13994	B-cell leukemia/lymphoma 2 (bcl-2) bcl-2-alpha protein	1,31	*1,21*	1,50	1,60	4,27	5,36	6,25	7,82	L
XM_017591	Annexin A6 (ANXA6)	1,68	3,28	2,14	9,32	*1,23*	2,13	*1,21*	1,36	E
Growth and cell stimulating factors										
U13660	Cartilage-derived morphogenetic protein 1 (CDMP-1)	1,33	1,02	1,20	1,34	6,87	10,46	7,91	15,63	L
XM_031221	Interleukin 1, alpha (IL1A)	1,52	3,23	*1,01*	1,64	*2,60*	*1,08*	*3,58*	*3,03*	L
XM_003752	Interleukin 3 (colony-stimulating factor, multiple) (IL3)	1,22	1,03	1,37	1,45	9,07	13,24	13,24	26,67	L
XM_031289	Interleukin 8 (IL8)	*1,38*	5,61	1,08	1,64	*1,74*	3,38	*2,86*	*2,89*	L
U63717	Osteoclast stimulating factor	1,16	*1,22*	1,39	1,54	3,40	2,08	4,08	11,03	L
M92934	Connective tissue growth factor	1,25	*1,26*	1,29	1,36	2,18	1,25	4,27	6,21	L
J00306	Somatostatin I gene and flanks	1,74	*2,76*	1,09	*1,05*	22,15	8,15	26,24	73,51	L
X00129	Retinol binding protein (RBP)	1,46	1,48	*1,24*	1,11	*6,20*	*4,45*	*3,54*	*3,52*	L
X06614	Receptor of retinoic acid	*1,02*	*3,19*	1,40	1,02	2,94	1,36	3,43	5,83	L
Metabolism										
AF189279	Group IIE secretory phospholipase A2	1,01	*1,33*	1,17	1,35	4,59	4,09	7,03	6,62	L
S79854	Type 3 iodothyronine deiodinase	1,65	*1,28*	1,05	1,01	9,89	6,63	12,20	24,49	L
U37012	Cleavage and polyadenylation specificity factor	*1,37*	*1,89*	1,35	1,11	1,85	5,96	4,23	15,15	L
U43747	Frataxin (FRDA)	1,13	*1,63*	1,41	1,32	3,44	5,97	5,36	14,27	L
X58957	Agammaglobulinaemia tyrosine kinase	*1,17*	2,02	2,31	3,65	*2,45*	*1,12*	*1,62*	*1,44*	E
XM_046765	Thymidylate synthetase (TYMS)	1,10	*1,82*	1,46	1,33	6,09	7,92	7,28	16,75	L
XM_048201	Metallothionein 1L (MT1L)	*8,69*	*1,63*	*3,15*	*3,91*	*6,81*	*3,12*	*8,01*	*12,02*	
XM_086368	MUF1 protein (MUF1)	1,33	*1,53*	1,23	1,14	4,36	3,35	5,51	7,74	L
K00065	Superoxide dismutase (SOD-1)	1,30	3,02	1,08	1,64	*1,45*	2,25	*5,38*	*5,44*	L
Transcription and signaling factors										
M58549	Matrix Gla protein (MGP)	1,43	*1,13*	1,16	1,31	8,04	2,64	5,82	1,78	L
AF037204	RING zinc finger protein (RZF)	1,03	2,27	1,04	1,22	1,62	2,71	2,49	2,57	L
D49835	DNA-binding protein	2,29	2,61	*1,86*	3,77	*1,34*	*1,23*	*3,51*	*1,60*	E
NM_014470	GTP-binding protein (RHO6)	1,78	*1,67*	1,15	*1,01*	11,19	8,57	12,49	24,56	L
U13991	TATA-binding protein, 30 kDa subunit (tafII30)	1,14	*1,68*	1,13	1,15	3,88	4,19	3,80	8,36	L
U38864	Zinc-finger protein C2H2-150	*1,05*	1,09	1,68	1,88	2,63	5,32	4,70	10,52	L
U45975	Phosphatidylinositol (4,5)bisphosphate 5-phosphatase	*1,10*	4,77	*1,77*	1,64	*1,21*	2,15	*2,96*	*4,10*	L

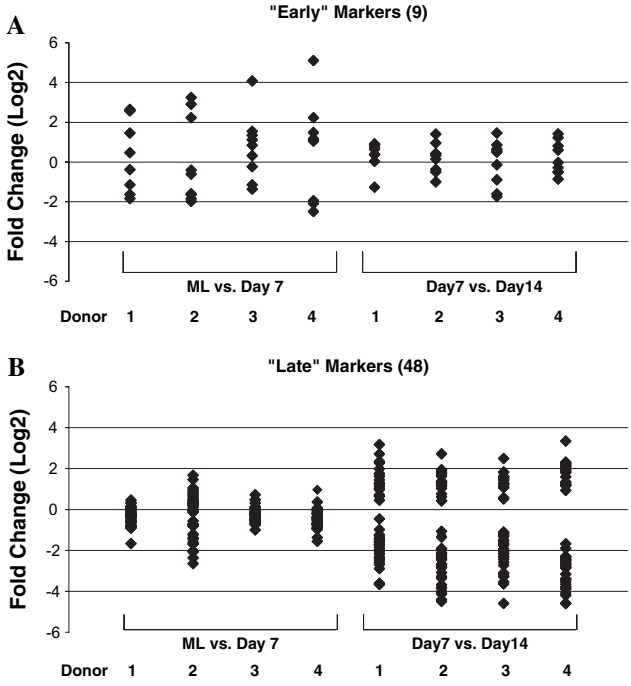

Fig. 5. Scatter plot analysis of genes expressed during the early (ML to day 7) and the late phase (day 7 to 14) of the *in-vitro* redifferentiation period. Early and late marker genes were defined as specific genes that showed at least a two-fold change in the gene expression level and a similar expression profile in at least 3 out of 4 donors (based on average values of patient to patient variation) exclusively in the defined time period. (A) Scatter plot of genes that were classified as early markers (9) and (B) as late markers (48) (for additional information see also Table I).

perichondrium like layer. A similar change or remodeling in cartilage matrix composition has been observed in biopsies taken from transplanted areas from patients treated with ACT[40,41]. This could indicate that the maturation process in the pellet model is representative for the *in vivo* conditions in the joint.

The changes between the early phase and the late phase that were seen at the protein level were also observed in gene expression by the cluster analysis. However, the change of gene expression levels from ML until day 7 included fewer genes than from day 7 until day 14 [see also Fig. 5(A and B)], which may indicate that the early phase in the pellet culture is a ''quiet phase'' and that the real redifferentiation takes place after day 7.

Genes which were upregulated in the early phase, were genes coding for matrix proteins like collagen type XI, dermatopontin, COMP, while glypican was especially upregulated for Donor2. The increased expression of dermatopontin has also previously been described during differentiation of MSCs towards the chondrogenic lineage[18]. The function of this recently described 22 kDa protein is relatively unclear but it has been reported to interact with decorin[42], to accelerate collagen fibrillogenesis and to enhance and modify the behavior of TGF-β[43].

Other genes that were upregulated in the early phase for some of the donors were genes that have been associated with the development of OA. Matrix metalloproteinase 7 (MMP-7) has been shown to be overexpressed in human OA cartilage[44] as well as tenascin-C[45] and fibronectin[46]. Tenascin that has been shown to be present in the dense mesenchyme surrounding developing epithelia, in tendon anlagen and in developing cartilage and bone[47], could indicate the start of the development of new tissue. Fragments of fibronectin have further been shown to enhance the catabolic activity of articular chondrocytes[48].

As already pointed out and shown in Fig. 5(B), the majority of genes changed their gene expression levels during the late phase from day 7 to 14. Among genes which increased in expression during the late phase were collagen types I, VI, XVIII, Matrilin1 and chondroadherin, while TIMP2 and MMP8 were strongly downregulated indicating dynamic regulations of proteases and inhibitors thereof[49]. Other genes involved in the modulation and maturation of cartilage matrix that showed differential regulation were the cystein rich proteolytic cathepsins. These enzymes have been shown to increase in ML cultures of articular chondrocytes[50] and to have differential distribution in articular cartilage during skeletal development[51]. Cathepsin D, which has further been immunolocalized in hypertrophic chondrocytes adjacent to the osteochondral junction[52] was together with cathepsin L downregulated in our *in vitro* experiment while cathepsin B was upregulated after day 7.

With respect to the cell cycle we found that genes like GSPT1, which controls G1 to the S phase transition[53], and growth-arrest-specific protein[54] were strongly up-respectively downregulated between 7 and 14 days, thus being in accordance with the transition from the proliferative phase in monolayer to a tissue forming process. Other signs of a change towards a more differentiated and organized structure, were the strong downregulation of the cell adhesion molecules FAK and the Integrin beta 1 (fibronectin receptor).

In the developing skeleton and during endochondral bone formation, the maturation of chondrocytes is characterized by hypertrophy, apoptosis, mineralization and bone formation. During this maturation a number of genes like collagen type X and Matrix Gla protein are expressed[55]. Our data show downregulation of the expression of the genes mentioned above, indicating that the maturation in the pellet system happened without any signs of hypertrophy.

The pellets did further mature without changes in cell numbers indicating potentially no involvement of necrosis and/or apoptosis as shown by the constant amount of DNA in the pellets (data not shown) and decreased expression of caspase 3.

Interestingly, the gene expression during the maturation process in the pellets mimicked the signaling processes involved in the early chondrogenesis shown in micromass cultures with cells isolated from embryonic limb buds. Especially we observed a significant decrease between day 7 and 14 of the expression of retinoic acid receptor (RAR) which proceeds the intracellular signaling processes, such as activation of p38 kinase and inhibition of extra cellular signal-regulated protein kinase (ERK-1), known to be involved in the regulation of SOX-9 expression[56]. While expression of the RAR has been shown to attenuate redifferentiation, SOX-9 plays an essential role in establishing the precartilaginous condensation and in initiating chondroblast differentiation[57]. Our results from the microarray analysis showed a strong downregulation of ERK-1 and upregulation of p38 kinase and SOX-9 suggesting that the signaling cascade is activated during the redifferentiation and maturation process of adult chondrocytes *in vitro*.

Other genes expressed during the embryonic limb formation are the HOX genes. These genes are involved in the formation of digits and are suggested to be regulated by retinoic acid via direct control of cdx1[58]. In our microarray analysis we studied two of the HOX genes of which the Msx-1 (formerly HOX-7) was upregulated during the late phase.

Regarding specific differences in gene expression levels between different donors we have found that for Donor2 the gene expression profile of ML cultures differed significantly from other cultures as shown in Fig. 4(A), while samples from day 7 and 14 were grouped accordingly with the other donors. Especially Collagen type I and XI, tenascin C and fibronectin were strongly upregulated on day 7, while RAR-responder-proteins were strongly downregulated compared to the other samples, potentially indicating an earlier predisposition to the redifferentiation process. Alternatively it may be postulated that either the cells were in a different state when isolated from the tissue (patient history unknown) or that the culture conditions like e.g., autologous serum had different effects on the cells resulting in a differentially expressed and characterized gene expression profile. Another possibility is that difference between the donors could be associated with the depth of the biopsy, reflecting different cell sources or populations that may be present after the tissue has been digested. The cells used in this study (surplus cells from ACT treatment) originate from full depth biopsies taken from non-weight bearing areas in the joint, but the techniques for obtaining biopsies could slightly differ even between experienced surgeons. The cultures may therefore contain a mixed population of cells representing the characteristics from the different layers of cartilage[59]. This could also be an explanation for the simultaneous production of collagen type II rich matrix and expression of both primitive and mature chondrogenic genes. The cells producing collagen type II may originate from the middle layers while the cells expressing primitive genes may originate from chondrogenic progenitors suggested to be found in the superficial layer of articular cartilage[60].

The use of autologous chondrocytes for repair of cartilage lesions will always result in individual differences in the cells used for transplantation. In our lab we have expanded cells from over 1000 biopsies without any failures in the cultures. However, we do not know if the cells from all patients have had the same chondrogenic potential. In the current study we have provided evidence, that by applying microarray analysis it may be possible to characterize the cell population used within an ACT treatment based on its gene expression profile and to assess the chondrogenic potential of the expanded cells. These findings may further be used as a quality control tool before delivering the cells to the patient and to ensure to provide cells that are strongly capable of reinitiating new cartilage tissue formation within the treated area. Additional information from the gene expression profiles may be deduced that could potentially allow us to better modify or treat expanded cell culture, in order to increase their chondrogenic potential before implantation.

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