OsteoArthritis and Cartilage (2006) **14,** 449–459

doi:10.1016/j.joca.2005.11.008

Osteoarthritis and Cartilage

International Cartilage Repair Society

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Comparison of gene expression profile between human chondrons and chondrocytes: a cDNA microarray study

Dr Z. Zhang M.D.†*, J. Fan Ph.D.‡, K. G. Becker Ph.D.§, R. D. Graff Ph.D.¶, G. M. Lee Ph.D.¶ and C. A. Francomano M.D.§

† *Department of Biomedical Engineering, Johns Hopkins University, 3400 N. Charles Street, Clark Hall 102, Baltimore, MD 21218, USA*

‡ *Division of Allergy and Clinical Immunology, Johns Hopkins University, School of Medicine, Baltimore, MD, USA*

§ *National Institute on Aging/NIH, Intramural Research Program, Baltimore, MD, USA*

¶ *Thurston Arthritis Research Center, University of North Carolina, Chapel Hill, NC, USA*

Summary

Objective: The chondron is a basic unit of articular cartilage that includes the chondrocyte and its pericellular matrix (PCM). This current study was designed to investigate the effects of the chondron PCM on the gene expression profile of chondrocytes.

Design: Chondrons and chondrocytes were enzymatically isolated from human articular cartilage, and maintained in pellet culture. Pellets of chondrons or chondrocytes were collected at days 1, 3 and 5 for cDNA microarray analysis.

Results: In comparison with chondrocytes alone, chondrons had 258 genes, in a broad range of functional categories, either up- or downregulated at the three time points tested. At day 1, 26 genes were significantly upregulated in chondrons and four downregulated in comparison to chondrocytes. At day 3, the number of upregulated chondron genes was 97 and the number downregulated was 43. By day 5, there were more downregulated genes (56) than upregulated genes (32) in chondrons. Upregulation of a group of heat shock proteins (HSPA1A, HSPA2 and HSPA8) in chondrons was validated by real time reverse transcription polymerase chain reaction (RT-PCR). Genes related to chondrocyte hypertrophy and dedifferentiation such as SSP1 and DCN were downregulated in chondrons as compared to the expression in chondrocytes.

Conclusion: The presence of the PCM in chondrons has a profound influence on chondrocyte gene expression. Upregulation of the heat shock protein 70 may contribute to the robustness and active matrix production of chondrons. The intact PCM may further stabilize the phenotype of chondrocytes within chondrons.

Key words: Cartilage, Chondrocytes, Chondrons, Extracellular matrix, Osteoarthritis.

Introduction

Given the fact that chondrocytes are individually embedded in the extracellular matrix (ECM), the interaction between chondrocytes and ECM is crucial for the functional regulation of chondrocytes and cartilage. Anatomically, the pericellular matrix (PCM) is the primary location for exchange of signals between chondrocytes and ECM. A chondron, including the chondrocyte and its PCM or microenvironment, is the basic anatomical, functional and metabolic unit of cartilage[1,2]. Type VI collagen is preferentially localized to the PCM and has been identified as a marker of chondrons[3]. The existence of the PCM in chondrons offers chondrocytes protection from physical stress. Chondrons have demonstrated resistance to compression[4] and increased Young's modulus when compared to chondrocytes[5]. The pericellular "capsule" represents about 60% of the cross-sectional area of chondrons, and is an osmotic buffer zone to the enclosed chondrocytes[6]. The greater physical properties of chondrons are associated with the integrity of molecular composition of the PCM, such as the presence of type VI collagen and keratan sulphate[4,6].

The establishment of enzymatic methods for chondron isolation enables the harvesting of a large number of viable chondrons *in vitro*[7]. Isolated chondrons are active in matrix deposition[8,9], a necessity of tissue engineering and cartilage repair.

Chondrons have been characterized with respect to their structural and physical properties. Enzymatically-isolated chondrons have also been shown to develop more ECM than isolated chondrocytes in pellet culture[9]. The influence of the intact PCM on gene expression of chondrocytes within chondrons has not been previously examined. In the current study, chondrons and chondrocytes were enzymatically isolated from human articular cartilage, and maintained in pellet culture. The gene expression profiles of chondrons and chondrocytes were compared using cDNA microarray analysis. A subset of genes that showed significant differences in cDNA microarray analysis, and with known relevance to chondrocyte phenotype and function,

*Address correspondence and reprint requests to: Dr ZiJun Zhang, Department of Biomedical Engineering, Johns Hopkins University, 3400 N. Charles Street, Clark Hall 102, Baltimore, MD 21218, USA. Tel: 1-410-516-5021; Fax: 1-410-516-8152; E-mail: zhangzj@jhu.edu

Received 12 July 2005; revision accepted 19 November 2005.

was further studied using real time reverse transcription polymerase chain reaction (RT-PCR).

Materials and methods

ISOLATION AND CULTURE OF CHONDRONS AND CHONDROCYTES

Adult human articular cartilage was obtained from three patients undergoing knee replacement surgery due to end-stage osteoarthritis with approval from the University of North Carolina Committee on the Protection of the Rights of Human Subjects. From each knee, all of the cartilage was collected, minced, and then divided into two parts for chondron and chondrocyte isolation. The cartilage from each knee was kept separate. According to a previously defined protocol[7], chondrons were enzymatically isolated by a 5-h digestion in 3.4 U/ml dispase (Life Technologies, Grand Island, NY) and 384 U/ml collagenase (Worthington Biochemical Corp., Freehold, NJ) in phosphate buffered saline (PBS) at 37°C. The obtained chondrons were morphologically comparable with chondrons originally described[1,7]. Chondrocytes were isolated by digestion with 1320 PUK/ml pronase in Dulbecco's modified Eagle medium (DMEM, Calbiochem, San Diego, CA) for 1 h followed by 0.4% collagenase (CLS-2, Worthington) in DMEM for 3 h. After straining through 70 μm nylon mesh, 6×10^5 chondrocytes as chondrons or chondrocytes were centrifuged at 400*g* for 10 min in 0.75 ml tubes in a 96 well format (Matrix Technologies Corp., Hudson, NH). The pellets were maintained in 400 μl Opti-MEM with GlutaMax (Life Technologies), a proprietary medium containing insulin, transferrin and selenium, and supplemented with 2.7 mM $CaCl_2$, 25 μg/ml ascorbate-2-PO_4 (Sigma), and with penicillin/streptomycin. Based on preliminary studies of optimum matrix synthesis, cultures were started in media with 2% fetal bovine serum and then fed with serum free media on days 2 and 4. Ten to 15 pellets were collected from each of the three human subjects, after 24 h, 3 days and 5 days in culture. RNAlater (Ambion, Inc., Austin, TX) was added to the individual culture tubes before storage at −80°C, pending RNA isolation.

cDNA MICROARRAY

Total RNA was isolated from 10 pellets of chondrocytes and chondrons from each human subject, using the TRIzol RNA isolation method (Invitrogen, Carlsbad, CA). Probe preparation was accomplished by radiolabelling (α[^{33}P]dCTP) 3–5 μg total RNA in a reverse transcription (RT) reaction using Superscript II (Invitrogen). Briefly, RNA was annealed in 10 μl H_2O, with 1 μg of 24-mer poly (dT) primer (Research Genetics, Huntsville, AL), by heating at 65°C for 15 min and cooling on ice for 5 min. The RT reaction was carried out by adding 8 μl of 5× first-strand RT buffer (Invitrogen), 4 μl of 20 mM dNTPs (without dCTP, Invitrogen), 4 μl of 20 mM DTT, 50 U of rRNasin (Promega, Madison, WI), 6 μl of 3000 Ci/mM α[^{33}P]dCTP (Perkin Elmer, Boston, MA) to the RNA/primer mix to a final volume of 40 μl. Superscript II reverse transcriptase was then added and the mix incubated at 42°C for 45 min followed by an additional 2 μl of Superscript II reverse transcriptase and another 45 min of incubation. The reaction was stopped by the addition of 5 μl of 0.5 M EDTA (ethylenediaminetetraacetic acid). Samples were then incubated at 65°C for 30 min after the addition of 10 μl of 0.1 M NaOH to hydrolyze and remove any remaining RNA. Samples were purified using Sephadex G-50 columns (Bio-Rad Laboratories, Hercules, CA) to remove unincorporated radioactivity.

Mammalian Genome Collection cDNA microarrays, with 9600 human genes (6385 unique), were developed by the Gene Expression and Genomics Unit, National Institute on Aging/NIH[10]. All cDNA spots are printed in duplicate. Due to the nature of the array construction several cDNAs are represented multiple times on the array. Such replicates are reported separately. Separate microarrays were done for chondrons and chondrocytes from each human subject and for each time point, 18 microarrays in total. Briefly, membranes were hybridized with α[^{33}P]dCTP labeled cDNA probes in 10 ml Ultrahyb hybridization solution (Research Genetics) containing 10 μl of 10 mg/ml polyA for 12 h at 60°C in a rotating hybridization oven. Hybridized arrays were washed in 10 ml of 2× sodium chloride/sodium citrate buffer (SSC) and 0.1% sodium dodecyl sulfate (SDS) twice at 60°C, followed by two to three more washes in 1× SSC and 0.1% SDS at 65°C. The microarrays were then exposed to phosphor imager screens for 12–24 h and scanned in a Molecular Dynamics Storm Phosphor Imager (Molecular Dynamics, Sunnyvale, CA) at 50 μm resolution. Scanned images were then processed using ImageQuant (Molecular Dynamics) and the raw intensity values were transferred into Microsoft Excel spreadsheets.

STATISTICAL ANALYSIS OF GENE ARRAY DATA

Raw intensity values were processed using *Z*-score transformation. *Z*-score transformation normalizes data within each hybridization array to permit pooling from multiple arrays[11]. Using *Z* transformation, each hybridized value for a given gene on the array is thereby expressed as a unit of standard deviation from the mean intensity of all genes on the array, normalized to zero. The value for each gene is an average of the two independent readings of duplicates. Briefly, the $\log_{10}$ of each spot intensity was adjusted to the mean and divided by the standard deviation of all the spot intensities. Changes in gene expression between chondrocytes and chondrons were then calculated by subtracting the average of experimental replicates ($n = 3$). This value is referred to as *Z* difference ($Z_{\text{diff}} = Z_{\text{chondrons}} - Z_{\text{chondrocytes}}$). Significance was defined as $P < 0.05$, $-2.0 > Z_{\text{diff}} > 2.0$, and average intensity > 0. Significant changes in gene expression calculated in this manner take into account the variation between replicates on a gene-by-gene basis. This method allows a rigorous statistical analysis of the microarray data taking advantage of multiple ($n = 3$) experimental replicates. The consistency of data was monitored by adding repeats of the same gene on the same array. For example, HSP1A1 was repeated at different locations on the array, and the upregulation of HSP1A1 was repeatedly found in the database.

Significant chondron genes were clustered using GeneSpring software (Silicon Genetics, Redwood City, CA) to assess gene expression patterns with K-mean analysis. The requested output was 10 sets of clusters. To facilitate functional analysis, PubMatrix (Gerontology Research Center, National Institute on Aging/NIH, Baltimore, MD), a gene and literature searching software[12] was also used. Gene Ontology (GO) data were downloaded from GO Consortium website: www.geneontology.org for gene functional classification.

RT-PCR

Based on microarray analysis, some genes were chosen from the list of significant differentially regulated genes in chondrons to be verified with real time RT-PCR. The criteria

for the selection of these genes were (1) genes with known functions that may affect the chondron biology and (2) genes related to chondrocyte phenotype. Most of them had significant differences on at least two time points of the culture period. Both up- and downregulated genes in chondrons were considered.

Using the SuperScript™ first-strand synthesis system for RT-PCR (Invitrogen), 2.5 μg of total RNA was reverse-transcribed with 50 U of SuperScript II RT at 25°C for 10 min, 42°C for 50 min, and finally at 70°C for 15 min. The products of RT were treated with RNase H before storage at −20°C. Real time PCR was performed on an ABI Prism 7700 sequence detector (PE Applied Biosystems, Foster City, CA). Using SYBR® green PCR core reagents (PE Applied Biosystems), each reaction mixture consisted of 2.5 μl of 10× SYBR PCR buffer, 3.0 μl of 25 mM $MgCl_2$, 2.0 μl of dNTP Blend, 0.6 U of AmpliTaq gold DNA polymerase, 0.25 μl of AmpErase UNG, 2.5 μl of 1:50 diluted reverse transcript product, optimized volume of 5 mM primers and diethylpyrocarbonate (DEPC)-treated water, for a total volume of 25 μl. No-template and no-reverse transcript reactions were included in each PCR plate as negative controls. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and succinate dehydrogenase complex, subunit A (SDHA) were used as internal standards in each PCR plate. Both GAPDH and SDHA were used to check the consistency of gene expression, and GAPDH was taken for quantification. After 10 min at 95°C, the PCR amplification was performed for 40 cycles; each cycle consisted of amplification at 95°C for 50 s and at 65°C for 30 s. Primers (for details see Table I) were supplied by Integrated DNA Technologies (Coralville, IA). The amplification cDNA was monitored by analysis of row data. The efficiency was calculated as $[10^{(-1/\text{slope})}] - 1$. An efficiency >90% is required for further processing of the data. At least five replicates of each reaction were performed, and duplicate plates were made for each set of reactions. The cycle at which the fluorescent level was statistically above the background was defined as the threshold cycle (Ct). The Ct values of the gene under investigation were normalized (ΔCt) by subtraction of the Ct value of GAPDH. The comparison of gene expression in chondrons and chondrocytes, ΔΔCt, was a subtraction of ΔCt of chondrons from the ΔCt of chondrocytes. Gene expression differences between chondrons and chondrocytes were expressed as $2^{\Delta\Delta Ct}$ (User bulletin #2, Applied Biosystem), where a value >1 means an upregulation and <1 for downregulation.

RT-PCR STATISTICAL ANALYSIS

The standard deviation of ΔCt, $s = (s_1^2 + s_2^2)^{1/2}$, where s_1 is the standard deviation for the gene of interest and s_2 is the standard deviation of GAPDH. ΔCt of chondrons and chondrocytes were evaluated with paired Student's t test. Significance was defined as $P < 0.05$. The standard deviation of ΔΔCt is equal to the standard deviation of $\Delta Ct_{chondron}$ (User bulletin #2, Applied Biosystem).

Results

MICROARRAY

The cDNA microarray data of gene expression in chondrons were directly compared with chondrocytes to determine an up- or downregulation. At day 1, there were 26 genes in chondrons significantly upregulated and four downregulated when compared to those from chondrocytes. On day 3, 97 genes were found to be upregulated in chondrons and 43 downregulated. There were more downregulated genes (56) than upregulated genes (32) in chondrons at day 5. All together, chondrons showed 258 genes, including the replicates of the same genes at different time points, which were either up- or downregulated as compared to chondrocytes under culture for comparable times. Of these, 90 genes were of unknown function. The remaining genes were classified into six functional categories (Table II). Included were genes involved in proliferation/cell cycle, metabolism, transcription factors, signaling, protein assembly/transport, and DNA repair. Signaling, protein assembly/transport, and metabolism were the best represented categories. The greatest difference between chondron and chondrocyte gene expression was seen at day 3.

K-MEANS CLUSTERING

K-means clustering was used to identify the intrinsic relations among the significant genes by expression patterns. Of the 10 output clusters, generated by an input of 258 significantly regulated chondron genes, three separate clusters with a reasonable number of genes had a distinct expression pattern. Cluster A included 42 genes (Table III). Most of the genes were expressed in the (linear, normalized) intensity range of 0−3. During the culture period, this cluster of genes in chondrocytes decreased in expression from day 1 to day 3, and to a slight degree from day 3 to day 5. In chondrons, their expression was upregulated at day 3 and maintained at this level through day 5 [Fig. 1(a)]. In comparison between chondrons and chondrocytes, the expression of these genes was statistically insignificant at day 1. At days 3 and 5, the expression of this cluster of genes in chondrons was significantly greater than that in chondrocytes [Fig. 1(b)]. Most of these genes are classified as genes involved in signaling and unknown function.

Cluster B consisted of 46 genes (Table IV). This cluster of genes showed steady expression in chondrocytes throughout the culture period, in the (linear, normalized) intensity range of 0−1. However, chondrons had an increase in expression at day 3 and maintained this elevated level of expression through day 5 [Fig. 2(a)]. Both chondrocytes and chondrons had similar expression levels of these genes at day 1. At days 3 and 5, these genes were more highly

Table I
Primers for real time RT-PCR

Gene name	Gene (accession no.)	Forward primer	Reverse primer
Heat shock 70 kDa protein 1A	HSPA1A (BC009322)	AGCAGGTGTGTAACCCCATC	GCAGCAAAGTCCTTGAGTCC
Heat shock 70 kDa protein 8	HSPA8 (BC009338)	GGAGGTGGCACTTTTGATGT	GTACGGAGGCGTCTTACAGC
Heat shock 70 kDa protein 2	HSPA2 (BC001752)	CACAGTGCAGTCGGATATGA	CTTGGTCTCCCCCTTGTACT
BCL2-associated athanogene 3	BAG3 (BC014656)	AGAAGTTTAACCCCGTTGCT	CCTCCAGTCCAGAGCTACAA
Secreted phosphoprotein 1	SPP1 (BC017387)	GCAACCGAAGTTTTCACTCC	ATTCAACTCCTCGCTTTCCA
Decorin	DCN (BC005322)	GCCCAGAAGTTCCTGATGAC	TCAGAACACTGGACCACTCG

Table II
Summary of significantly regulated chondron genes, in comparison to chondrocytes, in different functional categories

	Upregulation			Downregulation		
	Day 1	Day 3	Day 5	Day 1	Day 3	Day 5
Proliferation/cell cycle	1	8	3		6	7
Metabolism	3	13	1	2	9	9
Transcription factors	1	5	2		1	2
Signaling	8	10	8	1	12	18
Protein assembly/ transport	5	14	2	1	4	4
DNA repair		3	2		1	2
Unknown function	8	44	14		10	14
Total	26	97	32	4	43	56

expressed in chondrons than in chondrocytes [Fig. 2(b)]. Most of the genes in this cluster were found to be involved in metabolism, protein assembly/transport, or had unknown functions.

There were 13 genes in cluster C (Table V). Except for one gene, this cluster of genes was expressed in the (linear, normalized) intensity range of 0−1. The gene expression pattern in this cluster was not as uniform as clusters A and B. During culture, the expression of these genes in chondrocytes changed little. From day 1 to day 3, some were slightly upregulated and a few slightly downregulated. In chondrons, a significant decrease in the expression of this cluster of genes occurred at day 3. This decrease reversed at day 5 [Fig. 3(a)]. However, the expression in chondrons was still less than that in chondrocytes [Fig. 3(b)]. When compared with chondrocytes, the expression of this cluster of genes in chondrons was generally unchanged at day 1, but decreased from day 3 to day 5. This group of genes spreads over several functional categories, except transcription factors and DNA repair.

INDIVIDUAL GENES WITH VALIDATION BY REAL TIME RT-PCR

Real time RT-PCR was utilized as a means of verifying the up- or downregulation of chosen genes found in microarray (Table VI). The magnitude of the change was calculated by relative, rather than absolute, quantification (User bulletin #2, Applied Biosystem), and may not match well with the differences in microarray expression due to the differences in cDNA amplification procedure, the treatment of reference intensity, and mathematical process of the data in microarray and PCR.

Heat shock protein 70

A group of heat shock protein 70 genes was upregulated in chondrons. Among them, HSPA8 expression was increased at days 1 and 3 when compared to chondrocytes, which were in line with real time RT-PCR. The upregulation of HSPA1A in chondrons was at day 5. HSPA2 was also upregulated in chondrons at day 3. By real time RT-PCR, however, its expression was only slightly elevated in comparison with chondrocytes, although the difference of average ΔCt of chondrons and chondrocytes was statistically significant ($P < 0.001$).

BCL2-associated athanogene 3 (BAG3)

In chondrons, BAG3 expression increased at days 1 and 3 as compared to chondrocytes. A greater upregulation was seen at day 1.

Secreted phosphoprotein 1 (osteopontin, SPP1)

SPP1 expression was decreased in chondrons at day 1. In real time RT-PCR, SPP1 expression in chondrons was just 7% of that in chondrocytes.

Decorin (DCN)

DCN expression in chondrons decreased at days 3 and 5, in comparison to the expression in chondrocytes. The tendency of downregulation was consistent in both cDNA microarray and real time RT-PCR, that is, the expression level was lower at day 5 than at day 3.

Discussion

The abundant ECM in cartilage offers chondrocytes mechanical protection and regulates chondrocytes metabolic activities and phenotype[13]. In culture, chondrocytes are not only challenged by changes in oxygen tension[14] and osmolality[15], but also loss of 3-dimensional contacts with ECM. Gene expression by chondrocytes is instantly altered by the enzymatic isolation procedure[16]. In contrast, chondrocytes enclosed by PCM in chondrons have performed differently in mechanical strength and matrix production[4,6,8,9]. We hypothesized that the characteristics of chondrons result from the effects of PCM on the gene expression of chondrocytes within chondrons.

In the present study, cDNA microarray analysis revealed that the intact PCM in chondrons has profound effects on chondrocyte gene expression. The difference involves a large number of genes in a broad range of functional categories, and persists over an extended period of time. In culture, reconstruction of the ECM by isolated chondrocytes is a dynamic process. The activeness of isolated chondrocytes is evidenced in the first few days in culture, although complete restoration of ECM takes longer[17]. Significant amounts of PCM are deposited and, consequently, improvements in physical properties occur after only 3 days in culture[18]. By day 6, a complex PCM with integrity of both molecular composition and biomechanics is restored. The intact PCM of chondrons changes chondrocyte behavior in culture. There were 258 genes in chondrons that were differentially expressed at the designated three time points of this 5-day culture. Of them, 140 genes had altered expression at day 3. The three distinct K-mean clusters represent unique gene expression patterns in chondrons that are different from chondrocytes, and have a common starting point at day 3. The timing indicates that these genes may have roles in the reconstruction of ECM, and in particular, the PCM that is assembled around chondrocytes before it extends into interterritorial matrix[17]. Further studies on the functions of those genes are significant for manipulation of chondron properties for tissue engineering.

For chondrons, the gene expression patterns over time of clusters A and B were essentially the same, but exhibited different magnitudes. Together, clusters A and B represent 88 genes in chondrons with a unique expression pattern that increases expression after day 3. Equally important is cluster C in which the expression of those genes in chondrons was decreased at day 3. The influence of PCM on chondrocytes is continuous, but changes roles. At day 3, most of the significantly regulated genes in chondrons were in the functional categories of metabolism, protein assembly/transport and signaling. By day 5, the most prominent functional group was signaling.

Table III
Genes in cluster A

Symbol	GenBank	Name	Z_{diff}		
			D1	D3	D5
PC4	BC018189	Activated RNA polymerase II transcription cofactor 4	−0.2	2.4*	2.3*
MGC4771	BC004917	Hypothetical protein MGC4771	0.3	2.4*	2.2*
LOC120224	BC016153	Hypothetical protein BC016153	0.3	2.4*	2.0*
CHRNB1	BC011371	Cholinergic receptor, nicotinic, beta polypeptide 1 (muscle)	0.8	2.5*	1.8
MGC14126	BC007827	Hypothetical protein MGC14126	0.4	2.0*	1.5
LOC142937	BC008131	Hypothetical protein BC008131	0.4	2.5*	2.4
FLJ20013	BC017096	Hypothetical protein FLJ20013	0.5	2.1*	2
RPF1	BC016051	RNA processing factor 1	0	2	2.1*
CCBL1	BC013069	Cysteine conjugate-beta lyase	1.3	2.3*	2.2
DKFZP434H132	BC001762	DKFZP434H132 protein	0	1.9	2.0*
MB	BC014547	Myoglobin	−0.1	2	2.1*
MGC4278	BC020241	Hypothetical protein MGC4278	0.2	2.0*	1.6
SLC1A5	BC000986	Solute carrier family 1 (neutral amino acid transporter), member 5	−0.1	1.9	2.0*
C1QB	BC008983	Complement component 1, q subcomponent, beta polypeptide	−0.3	2	2.1*
NMB	BC008603	Neuromedin B	1.7	2.9**	2.1*
CCL5	BC008600	Chemokine (C−C motif) ligand 5	1.3	2.8**	2.2
SEDL	BC016915		0.2	2.3*	2.1*
S100A4	BC016300	S100 calcium binding protein A4	−0.1	2.2*	2.1*
MGC13007	BC017072	Hypothetical protein MGC13007	−0.3	2.2*	2
RABIF	BC018488	RAB interacting factor	−0.4	2.2*	2.2
CXCL6	BC013744	Chemokine (C−X−C motif) ligand 6	−0.2	2.1	2.0*
FLJ36666	BC018441	Hypothetical protein FLJ36666	−0.4	2.1	2.1*
RNAHP	BC015505	RNA helicase-related protein	−0.3	2.1	2.1*
MRPS25	BC003590	Mitochondrial ribosomal protein S25	−0.1	2.1*	1.9
TCEB1	BC013809	Transcription elongation factor B (SIII), polypeptide 1	−0.2	2.2*	2.1*
MYL3	BC009790	Myosin, light polypeptide 3, alkali	0	2.1*	2.0*
C20orf98	BC001963	Chromosome 20 open reading frame 98	−0.1	2.1*	2.1*
SH3GLB2	BC014635	SH3-domain GRB2-like endophilin B2	0.1	2.5*	2
PROS1	BC015801	Protein S (alpha)	0.2	2	2.0*
SAS	BC010377	Sarcoma amplified sequence	0.3	2.1*	2.0*
LOC51123	BC015925	HSPC038 protein	−0.3	2.3	2.3*
YAP	BC003500	YY1 associated protein	1.1	2.1*	1.9
TMSB10	BC016025	Thymosin, beta 10	0.1	2.4*	2.2*
KRT18	BC000698	Keratin 18	0.7	1.7	2.0*
UPK1B	BC012851	Uroplakin 1B	0.9	3.0**	2.8*
MGC10814	BC004943	Hypothetical protein MGC10814	−0.1	2.5*	2.3
MRPL30	NM_145212	Mitochondrial ribosomal protein L30	0.1	2.2*	2
FLJ10719	BC004277	Hypothetical protein FLJ10719	0.6	2.6*	2
FLJ11305	BC016614	Hypothetical protein FLJ11305	0	1.9	2.0*
LOC51759	BC007664	Hepatocellular carcinoma-associated antigen 59	−0.2	2.1*	1.8
CRYZL1	BC013155	Crystallin, zeta (quinone reductase)-like 1	−0.5	2.2*	2.1
UBL5	BC007053	Ubiquitin-like 5	0.2	1.9	2.1*

D1, day 1; D3, day 3; D5, day 5. (*$P < 0.05$; **$P < 0.001$). Z_{diff} computed by the formula: $Z_{diff} = Z_{chondrons} - Z_{chondrocytes}$. P value computed by Z test (See Materials and methods for details).

By design, this study directly compared chondrons and chondrocytes at the expression of each individual gene. As a result, genes expressed in both chondrons and chondrocytes but at a similar level were not shown. It is the case for most of the common cartilage specific genes, such as types II, IX, X collagen and aggrecan. The main reason could be that the phenotypes of isolated chondrocytes and chondrocytes in chondrons are generally stable in pellet culture[19]. Larson *et al.* found that it is a better retention, rather than increasing synthesis, that makes more proteoglycan in the cultured chondron pellets than in the chondrocyte pellets[9].

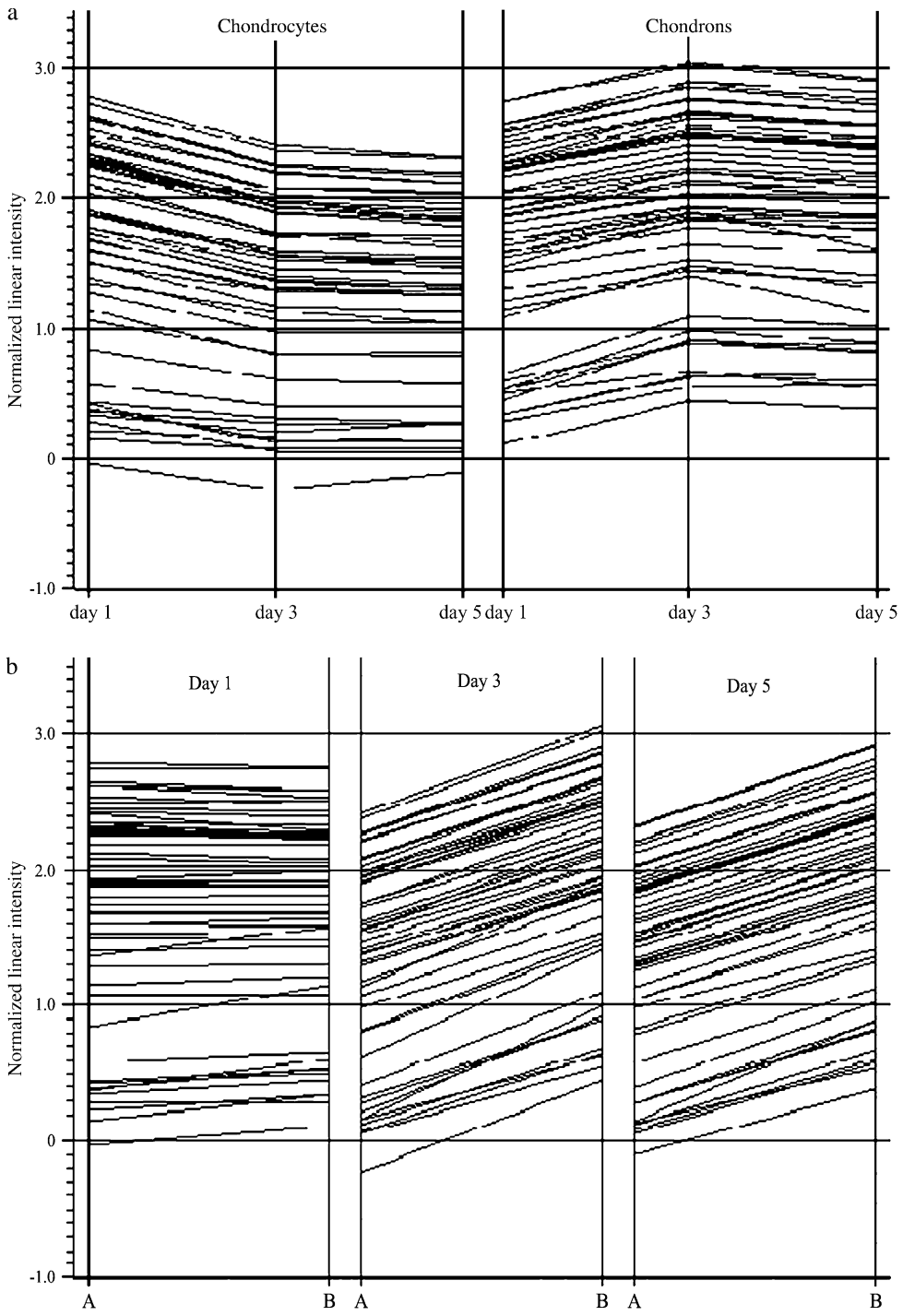

Fig. 1. Cluster A includes 42 genes. a: This cluster of genes was downregulated in chondrocytes during culture, and upregulated in chondrons after day 3 in culture. b: Direct comparison between chondrocytes (axis *A*) and chondrons (axis *B*); this cluster of genes was expressed at the same level in both chondrocytes and chondrons at day 1. The expression of this gene cluster was increased in chondrons at days 3 and 5 as compared to chondrocytes. The normalized linear intensity (*y* axis) is the result of multiple-step normalizations, involving a mean of whole array, control values and input data.

A notable group of upregulated genes in chondrons is heat shock protein genes. Heat shock proteins are molecular chaperones that participate in protein assembly, folding, transport, and promotion of cell survival during stress challenges[20]. For example, chondrocytes (HCS-2/8) respond to physiological pressure by upregulating HSP70[21,22]. The upregulation of HSPA1A, HSPA2, and HSPA8 in chondrons, which are members of the HSP70 family, may contribute to the mechanical robustness of chondrons[4].

HSP and its cofactors promote cell survival by inhibiting both the mitochondrial (intrinsic) and the death receptor

Table IV
Genes in cluster B

Symbol	GenBank	Name	Z_{diff}		
			D1	D3	D5
FLJ20813	BC017219	Hypothetical protein FLJ20813	1.3	2.4*	2.2
MGC3169	BC001195	Hypothetical protein MGC3169	0.5	2.4*	1.8
DKFZP434A0131	BC002581	DKFZp434A0131 protein	0.1	2.0*	1.8
C20orf106	BC014951		0.9	2.9*	2.5
FLJ20139	BC009620	Hypothetical protein FLJ20139	0.7	2.3*	2.4
ZNF215	BC014538	Zinc finger protein 215	1.5	2.7*	2.3
SMA3	BC002622	SMA3	−0.3	2.0*	1.7
TUBD1	BC000258	Likely ortholog of mouse tubulin, delta 1	0.4	2.5*	2
HSPC052	BC016914	HSPC052 protein	0.6	1.8	2.1*
FLJ20320	BC015324	Hypothetical protein FLJ20320	0.3	1.9	2.0*
CCBP2	BC020558	Chemokine binding protein 2	0	2.1*	1.9
NM23-H6	BC012828	Nonmetastatic cells 6	1.2	2.3*	2.3
DFFA	BC007721	DNA fragmentation factor, 45 kDa, alpha polypeptide	0.7	2.2*	1.9
MGC5457	BC000988	Hypothetical protein MGC5457	1	2.7*	2.4
SLC12A8	BC020506	Solute carrier family 12, member 8	1.1	2.3*	2.2
MGC4248	BC005871	Hypothetical protein MGC4248	0.7	2.0*	1.8
FLJ22548	BC015548		1.1	3.5*	3
CASP2	BC002427	Caspase 2	0.7	2.1*	1.7
LOC197336	BC013032	Similar to Hypothetical 43	0.4	2.6*	1.1
CHEK2	BC000004	CHK2 checkpoint homolog (*S. pombe*)	0.4	2.1*	2
LOC201853	BC009926	Similar to hypothetical protein (L1H 3 region) − human	1.2	3.4*	3.2
AP4S1	BC001259	Adaptor-related protein complex 4, sigma 1 subunit	1	2.4*	2.3
ICAM4	BC000046	Intercellular adhesion molecule 4	0.7	2.4*	1.9
RFC2	BC002813	Replication factor C (activator 1) 2, 40 kDa	0.9	2.4*	2.5*
PTD015	BC016854	PTD015 protein	1.1	2.6*	2.4
MGC21675	BC013775	Hypothetical protein MGC21675	0.7	2.4*	2.4
MGC14254	NM173562	Hypothetical protein MGC14254	1.2	2.2*	2.1
HZF12	BC011502	Zinc finger protein 12	1.2	2.6*	2.4
FUSIP1	BC005039	FUS interacting protein (serine−arginine rich) 1	0.3	2.1*	2
PPM1F	BC013798	Protein phosphatase 1F (PP2C domain containing)	0.4	2.0*	1.9
AP1S3	BC021898	Adaptor-related protein complex 1, sigma 3 subunit	0.9	2.1*	1.6
UEV3	BC011011	Ubiquitin-conjugating enzyme E2-like	1	2.4*	2.1
LOC115761	BC013150	Hypothetical protein MGC17429	0.5	2.4*	2.5
RBM3	BC006825	RNA binding motif protein 3	0.8	2.2**	1.9
KDELR2	BC008081	KDEL endoplasmic reticulum protein retention receptor 2	0.4	2.2*	1.8
PTAFR	BC013816	Platelet-activating factor receptor	0.7	2.7*	2.3
RAD1	BC006837	RAD1 homolog (*S. pombe*)	0.9	2.1*	1.9
MGC3113	BC000203	Hypothetical protein MGC3113	1	2.5*	2.2
LOC57146	BC015812	Hypothetical protein from clone 24796	0.5	2.0*	1.6
KIAA0101	BC005832	KIAA0101 gene product	1.1	2.8*	2.3
DBR1	BC009472	Debranching enzyme homolog 1 (*S. cerevisiae*)	0.9	2.2*	2.2
FLJ13614	BC016905	Hypothetical protein FLJ13614	0.3	2.9*	2.1
LOC90141	BC001787	Chromosome 14 open reading frame 143	0.5	2.1*	2
PECR	BC002529	Peroxisomal trans 2-enoyl CoA reductase	1.8	2.4*	2
MGC16037	BC007651	Hypothetical protein MGC16037	0.5	2.4*	2
MGC29762	BC021264	Hypothetical protein MGC29762	0.6	2.2*	1.9

D1, day 1; D3, day 3; D5, day 5. (*$P < 0.05$; **$P < 0.001$). Z_{diff} computed by the formula: $Z_{diff} = Z_{chondrons} - Z_{chondrocytes}$. P value computed by Z test (See Materials and methods for details).

(extrinsic) cell-death pathways[23,24]. The upregulation of HSP genes (HSPA1A, HSPA2 and HSPA8) and BAG3, which is a cochaperone partner of HSP[29], can be one of the mechanisms that protect chondrons from stresses. On the other hand, the decreased expression of SSP1, expressed by hypertrophic chondrocytes[25], in chondrons may suggest a reduction of terminal differentiation and apoptosis in the chondron population. A healthy population

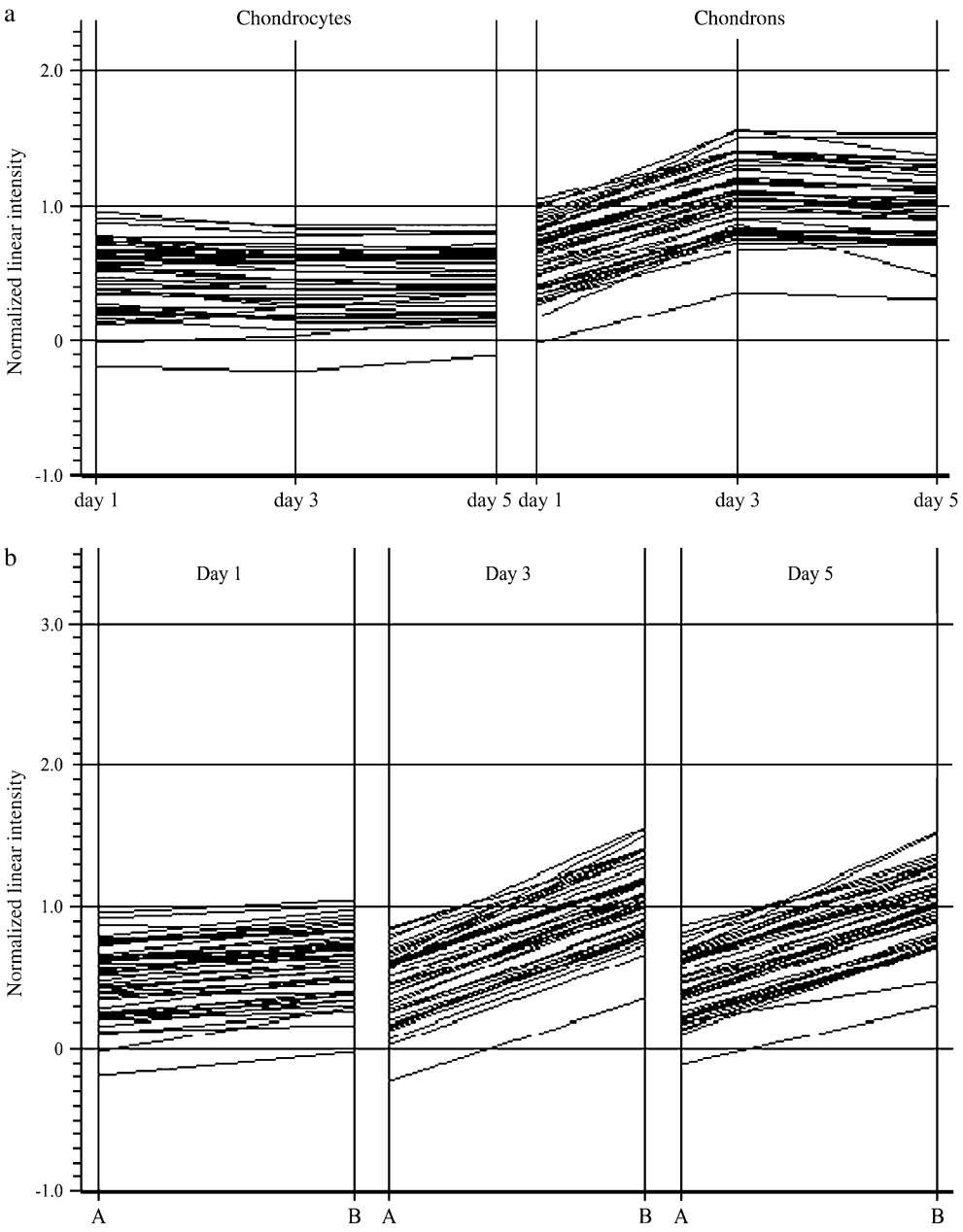

Fig. 2. Cluster B includes 46 genes. a: The genes of cluster B were expressed at a steady level in chondrocytes during culture. In chondrons, this group of genes was upregulated after day 3 in culture. b: Comparison of cluster B gene expressions between chondrocytes (axis *A*) and chondrons (axis *B*) showed no difference between chondrocytes and chondrons at day 1. However, the expression of this cluster of genes increased in chondrons as compared to chondrocytes at days 3 and 5. The normalized linear intensity (*y* axis) is the result of multiple-step normalizations, involving a mean of whole array, control values and input data.

of chondrons could benefit the rebuilding of ECM in tissue culture for cartilage engineering[8,9].

Chondrocytes display various phenotypes according to physiological stages or pathological conditions[26]. Classical chondrocyte dedifferentiation includes a fibroblast-like morphology and expression of type I collagen instead of type II collagen[27]. It is now agreed that the dedifferentiation process is much more complex and involves multiple genes[28]. DCN is a small leucine-rich proteoglycan in articular cartilage matrix[29]. On both mRNA and protein level, DCN content increases in osteoarthritic cartilage[30], due to increased expression by dedifferentiated chondrocytes[31]. In cultured chondrons, DCN expression was decreased compared to chondrocytes. This might suggest that the chondrocyte phenotype is further stabilized by the presence of a PCM in chondrons, even though the pellet culture technique utilized for the current study retains the chondrocyte phenotype[19].

The robust response to physical stimulation and efficacy of matrix production by chondrons gives great potential for tissue engineering[32]. It is encouraging that the volume and content of PCM in chondrons can be manipulated

Table V
Genes in cluster C

Symbol	GenBank	Name	Z_{diff}		
			D1	D3	D5
NEIL1	BC010876	Nei endonuclease VIII-like 1 (*E. coli*)	0.4	−2.2*	−1
GNG11	BC009709	Guanine nucleotide binding protein (G protein), gamma 11	0.7	−2.5**	−1.1
3-Apr	BC011006	Apoptosis related protein APR-3	−0.6	−2.0*	−1.3
SMAP	BC007103	Small acidic protein	2.6**	−1.1	−0.7
KIAA0092	BC001233	KIAA0092 gene product	1.9	−2.4*	−0.9
LOC116123	BC014341	Hypothetical protein BC014341	−1.2	−2.0*	−1.7
CTSL	BC012612	Cathepsin L	−1.2	−2.3*	−1.9
GLRX	BC005304	Glutaredoxin (thioltransferase)	0.9	−2.5*	−1.5
RNF40	BC018647	Ring finger protein 40	0.1	−2.4*	−0.5
HSPC194	BC002496	Hypothetical protein HSPC194	0.4	−2.2*	−1
ALDH1A1	BC001505	Aldehyde dehydrogenase 1 family, member A1	−2.1	−1.1	−2.0*
RPS27L	BC003667	Ribosomal protein S27-like	0.5	−2.1**	−1.6
MGC1223	BC001033	Hypothetical protein MGC1223	0.5	−2.0*	−0.9

D1, day 1; D3, day 3; D5, day 5. (*$P < 0.05$; **$P < 0.001$). Z_{diff} computed by the formula: $Z_{diff} = Z_{chondrons} - Z_{chondrocytes}$. P value computed by Z test (See Materials and methods for details).

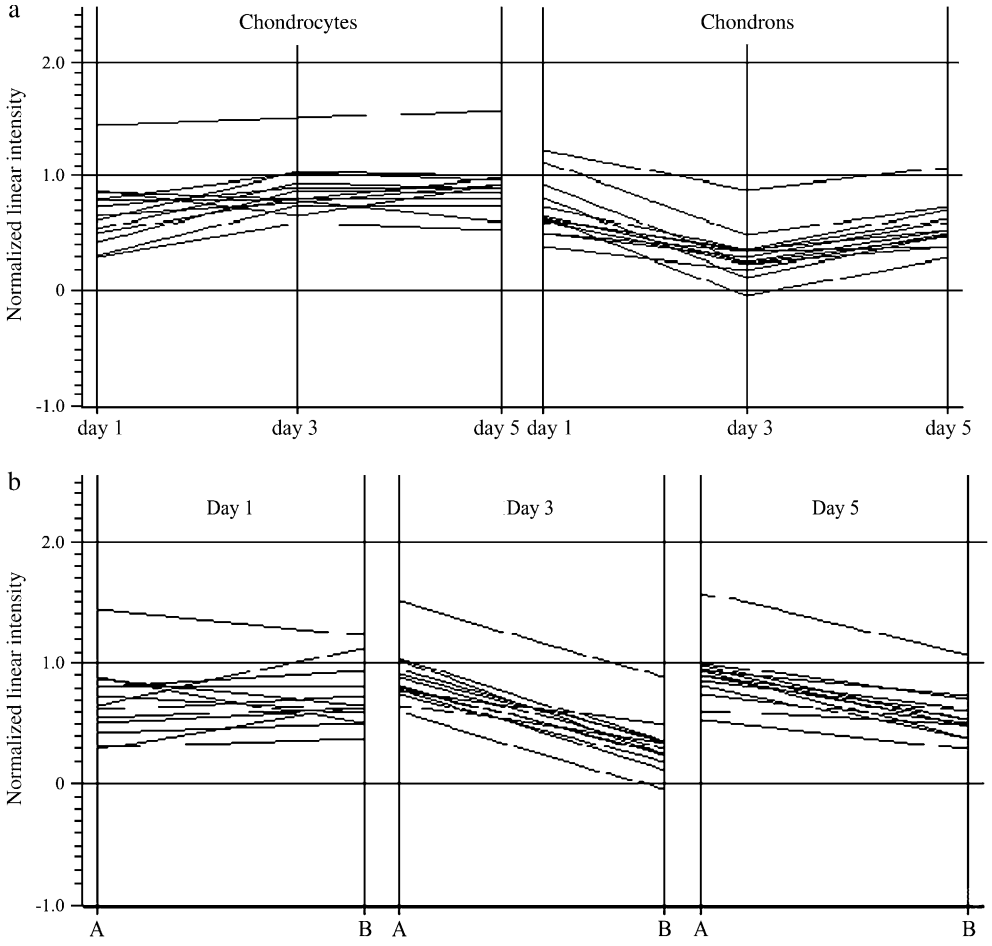

Fig. 3. Cluster C includes 13 genes. a: Generally, expression of genes in cluster C was level in chondrocytes during culture. However, this cluster of genes was downregulated in chondrons at day 3, and slightly upregulated by day 5. b: At day 1, the expression of cluster C varied in a small range between chondrocytes (axis *A*) and chondrons (axis *B*). On days 3 and 5, the expression of this cluster of genes in chondrons was downregulated when compared to chondrocytes. The normalized linear intensity (*y* axis) is the result of multiple-step normalizations, involving a mean of whole array, control values and input data.

Table VI
Selected genes of chondrons in comparison with chondrocytes in microarray and RT-PCR (in bracket)

Gene name	Symbol	Z_{differ}		
		D1	D3	D5
Heat shock 70 kDa protein 1A	HSPA1A		4.0 (5.8)	2.3 (5.2)
Heat shock 70 kDa protein 8	HSPA8	2.5 (2.1)	2.3 (4.4)	
Heat shock 70 kDa protein 2	HSPA2		3.6 (1.1)	
BCL2-associated athanogene 3	BAG3	3.1 (7.1)	2.2 (2.4)	
Secreted phosphoprotein 1 (osteopontin)	SPP1	−3.7 (7%)	−4.8 (3%)	−4.9 (1%)
Decorin	DCN		−2.6 (17%)	−3.4 (1%)

in vitro[33]. The current study adds favorable information to this approach. By comparison of the gene expression profiles of chondrons and chondrocytes, the current study reveals that the PCM in chondrons regulates the expression of a large number of genes. Secondly, the upregulation of HSP70 may contribute to the better survival of chondrons from cell stress.

Acknowledgments

This study was supported by Intramural Research Program, National Institute on Aging, National Institutes of Health and an NIH grant (AR43883) to GML. The authors thank J. Tran for his critical review and Inna Tchivilev for technical assistance.

References

1. Poole CA, Flint MH, Beaumont BW. Chondrons in cartilage: ultrastructural analysis of the pericellular microenvironment in adult human articular cartilages. J Orthop Res 1987;5:509−22.
2. Poole CA. Articular cartilage chondron: form, function and failure. J Anat 1997;191:1−13.
3. Poole CA, Ayad S, Schofield JR. Chondrons from articular cartilage: I. Immunolocalization of type VI collagen in the pericellular capsule of isolated canine tibial chondrons. J Cell Sci 1988;90:635−43.
4. Knight MM, Ross JM, Sherwin AF, Lee DA, Bader DL, Poole CA. Chondrocyte deformation within mechanically and enzymatically extracted chondrons compressed in agarose. Biochim Biophys Acta 2001; 1526:141−6.
5. Guilak F, Jones WR, Ting-Beall HP, Lee GM. The deformation behavior and mechanical properties of chondrocytes in articular cartilage. Osteoarthritis Cartilage 1999;7:59−70.
6. Hing WA, Sherwin AF, Ross JM, Poole CA. The influence of the pericellular microenvironment on the chondrocyte response to osmotic challenge. Osteoarthritis Cartilage 2002;10:297−307.
7. Lee GM, Poole CA, Kelley SS, Chang J, Caterson B. Isolated chondron: a viable alternative for studies of chondrocyte metabolism *in vitro*. Osteoarthritis Cartilage 1997;5:261−74.
8. Graff RD, Kelley SS, Lee GM. Role of pericellular matrix in development of a mechanically functional neocartilage. Biotechnol Bioeng 2003;82:457−64.
9. Larson CM, Kelley SS, Blackwood AD, Banes AJ, Lee GM. Retention of the native chondrocyte pericellular matrix results in significantly improved matrix production. Matrix Biol 2001;21:349−59.
10. Kawai T, Fan J, Mazan-Mamczarz K, Gorospe M. Global mRNA stabilization preferentially linked to translational repression during the endoplasmic reticulum stress response. Mol Cell Biol 2004;24:6773−87.
11. Cheadle C, Vawter MP, Freed WJ, Becker KG. Analysis of microarray data using *Z* score transformation. J Mol Diagn 2003;5:73−81.
12. Becker KG, Hosack DA, Dennis Jr G, Lempicki RA, Bright TJ, Cheadle C, *et al*. PubMatrix: a tool for multiplex literature mining. BMC Bioinformatics 2003;4:61.
13. Behonick DJ, Werb Z. A bit of give and take: the relationship between the extracellular matrix and the developing chondrocyte. Mech Dev 2003;120:1327−36.
14. Malda J, Martens DE, Tramper J, van Blitterswijk CA, Riesle J. Cartilage tissue engineering: controversy in the effect of oxygen. Crit Rev Biotechnol 2003;23: 175−94.
15. Urban JP, Hall AC, Gehl KA. Regulation of matrix synthesis rates by the ionic and osmotic environment of articular chondrocytes. J Cell Physiol 1993;154:262−70.
16. Benz K, Breit S, Lukoschek M, Mau H, Richter W. Molecular analysis of expansion, differentiation, and growth factor treatment of human chondrocytes identifies differentiation markers and growth-related genes. Biochem Biophys Res Commun 2002;293: 284−92.
17. Chang J, Poole CA. Confocal analysis of the molecular heterogeneity in the pericellular microenvironment produced by adult canine chondrocytes cultured in agarose gel. Histochem J 1997;29:515−28.
18. Knight MM, Lee DA, Bader DL. The influence of elaborated pericellular matrix on the deformation of isolated articular chondrocytes cultured in agarose. Biochim Biophys Acta 1998;1405:67−77.
19. Zhang Z, McCafery JM, Spencer RGS, Francomano CA. Hyaline cartilage engineered by pellet culture: histologic, immunohistochemical and ultrastructural analysis. J Anat 2004;205:229−37.
20. Kregel KC. Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. J Appl Physiol 2002;92:2177−86.
21. Takahashi K, Kubo T, Kobayashi K, Imanishi J, Takigawa M, Arai Y, *et al*. Hydrostatic pressure influences mRNA expression of transforming growth factor-β1 and heat shock protein 70 in chondrocyte-like cell line. J Orthop Res 1997;15:150−8.
22. Sironen RK, Karjalainen HM, Elo MA, Kaarniranta K, Torronen K, Takigawa M, *et al*. cDNA array reveals mechanosensitive genes in chondrocytic cells under hydrostatic pressure. Biochim Biophys Acta 2002; 1591:45−54.
23. Garrido C, Gurbuxani S, Ravagnan L, Kroemer G. Heat shock proteins: endogenous modulators of apoptotic cell death. Biochem Biophys Res Commun 2001; 286:433−42.
24. Takayama S, Reed JC, Homma S. Heat-shock proteins as regulators of apoptosis. Oncogene 2003;22:9041−7.
25. Shibata S, Fukada K, Suzuki S, Ogawa T, Yamashita Y. *In situ* hybridization and immunohistochemistry of bone sialoprotein and secreted phosphoprotein 1 (osteopontin) in the developing mouse mandibular condylar cartilage compared with limb bud cartilage. J Anat 2002;200:309−20.

26. Sandell LJ, Aigner T. Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. Arthritis Res 2001;3:107–13.
27. Schnabel M, Marlovits S, Eckhoff G, Fichtel I, Gotzen L, Vecsei V, *et al*. Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture. Osteoarthritis Cartilage 2002;10:62–70.
28. Stokes DG, Liu G, Coimbra IB, Piera-Velazquez S, Crowl RM, Jimenez SA. Assessment of the gene expression profile of differentiated and dedifferentiated human fetal chondrocytes by microarray analysis. Arthritis Rheum 2002;46:404–19.
29. Knudson CB, Knudson W. Cartilage proteoglycans. Semin Cell Dev Biol 2001;12:69–78.
30. Cs-Szabo G, Melching LI, Roughley PJ, Glant TT. Changes in messenger RNA and protein levels of proteoglycans and link protein in human osteoarthritic cartilage samples. Arthritis Rheum 1997;40:1037–45.
31. Bock HC, Michaeli P, Bode C, Schultz W, Kresse H, Herken R, *et al*. The small proteoglycans decorin and biglycan in human articular cartilage of late-stage osteoarthritis. Osteoarthritis Cartilage 2001;9: 654–63.
32. Kelly TA, Wang CC, Mauck RL, Ateshian GA, Hung CT. Role of cell-associated matrix in the development of free-swelling and dynamically loaded chondrocyte-seeded agarose gels. Biorheology 2004;41:223–37.
33. Horikawa O, Nakajima H, Kikuchi T, Ichimura S, Yamada H, Fujikawa K, *et al*. Distribution of type VI collagen in chondrocyte microenvironment: study of chondrons isolated from human normal and degenerative articular cartilage and cultured chondrocytes. J Orthop Sci 2004;9:29–36.