Osteoarthritis and Cartilage (2002) **10,** 62–70
© 2002 OsteoArthritis Research Society International
doi:10.1053/joca.2001.0482, available online at http://www.idealibrary.com on IDEAL®
1063–4584/02/010062+09 $35.00/0

Osteoarthritis and Cartilage
Journal of the OsteoArthritis Research Society International

Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture

M. Schnabel*, S. Marlovits†, G. Eckhoff*, I. Fichtel*, L. Gotzen*, V. Vécsei† and J. Schlegel‡
*Department of Traumatology, Philipps-University of Marburg, Germany
†Department of Traumatology, University of Vienna, Austria; Ludwig Boltzmann Institute of Biomechanics and Cell Biology, Vienna, Austria
‡Institute of Pathology, Munich Technical University, Germany

Summary

Objective: The aim of the present study was the investigation of differential gene expression in primary human articular chondrocytes (HACs) and in cultivated cells derived from HACs.

Design: Primary human articular chondrocytes (HACs) isolated from non-arthritic human articular cartilage and monolayer cultures of HACs were investigated by immunohistochemistry, Northern analysis, RT-PCR and cDNA arrays.

Results: By immunohistochemistry we detected expression of collagen II, protein S-100, chondroitin-4-sulphate and vimentin in freshly isolated HACs. Cultivated HACs, however, showed only collagen I and vimentin expression. These data were corroborated by the results of Northern analysis using specifc cDNA probes for collagens I, II and III and chondromodulin, respectively, demonstrating collagen II and chondromodulin expression in primary HACs but not in cultivated cells. Hybridization of mRNA from primary HACs and cultivated cells to cDNA arrays revealed additional transcriptional changes associated with dedifferentiation during propagation of chondrocytes *in vitro*. We found a more complex hybridization pattern for primary HACs than for cultivated cells. Of the genes expressed in primary HACs the early growth response (EGR1) transcription factor showed the strongest expression whereas D-type cyclin was expressed in proliferating cells. Other factors associated with differentiated HACs were the adhesion molecules ICAM-1 and VCAM-1, VEGF, TGFβ2, and the monocyte chemotactic protein receptor.

Conclusions: Our data support the hypothesis that HACs dedifferentiate when grown in monolayer cultures. Moreover, the expression patterns also show that proliferation and differentiation are exclusive features of human chondrocytes. © 2002 OsteoArthritis Research Society International

Key words: Chondrocytes, Cartilage, Gene expression, cDNA array, Morphology, Differentiation.

Introduction

Pathologic changes of cartilage due to osteoarthritis (OA) as well as developmental, metabolic, and inflammatory disorders, or after trauma are an unsolved contemporary medical problem. The intrinsic capacity of cartilage to repair articular chondral defects is poor[1,2]. Unfortunately there is also a lack of causative therapies. Consequently, biological approaches to restore the unique mechanical competence of normal articular cartilage are of major interest. Recently, autologous chondrocyte transplantation (ACT), first inaugurated in animal experiments by Smith and Chesterman[3], has been introduced into clinical use as a novel biological approach[4]. Human articular chondrocytes (HACs) are therefore harvested from non-affected, non-weight-bearing areas of the same joint during arthroscopy. HACs are subsequently grown in monolayer cell culture in order to achieve sufficient amounts of cells to fill the articular defect covered by a periosteal flap in a second operation[4]. It has been shown previously, however, that HACs dedifferentiate to a fibroblast-like state when cultivated in monolayers[5]. This shift in cellular differentiation has been demonstrated both by morphological changes and by alterations in collagen expression patterns[6,7]. The extent and the reversibility of this process is actually unknown. Consequently, the identification of the genes involved in chondrocyte differentiation will be of major interest in biological therapy approaches. The aim of the present study was the identification of genes which are differentially regulated between primary and dedifferentiated HACs after cultivation in monolayers by novel cDNA array technologies.

Received 26 October 2000; revision requested 11 January 2001; revision received 13 April 2001; accepted 4 June 2001.

Address correspondence to: Juergen Schlegel, M.D., Institute of Pathology, Munich Technical University, Ismaninger Straße 22, D-81675 Muenchen, Germany. Tel: +49-89-4140-6142; Fax: +49-89-4140-4915; E-mail: juergen.schlegel@lrz.tu-muenchen.de

Material and methods

CELL CULTURE

Non-arthritic human articular cartilage was isolated from femoral heads of five patients with displaced femoral neck fractures (two male, three female, patients' mean age was 84 years). The cartilage specimens were taken immediately after surgery, placed into Petri dishes and moistened with phosphate buffered saline (PBS). Two sterile scalpels were used to dice the cartilage into 1–3 mm^3 pieces. The PBS

was removed, and diced tissue was placed in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc., Rockville, MD, U.S.A.) with 200 U/ml collagenase type II from clostridium histolyticum (Life Technologies, Inc., Rockville, MD, U.S.A.). The tube was capped, covered with Parafilm, placed sideways on an orbital shaker at 25 rpm, and incubated at 37°C for 20 h. The following day the digestate was resuspended, and centrifuged at 1000 rpm for 10 min. The supernatant was removed and the pellet gently resuspended with 10 ml calcium- and magnesium-free PBS. The suspension was filtered through a 100 μm mesh into a sterile polypropylene tube. Cells were centrifuged and washed twice, then counted in a hemocytometer, resuspended in medium, and placed into T-75-flasks at a density of $1.5\times10^4/cm^2$ and grown at 37°C and 5%CO_2 as monolayer cultures in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 50 μl/ml ascorbic acid.

Total RNA was isolated from primary HACs at day 7 and at day 42 (passage 2) using the RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. Briefly, cells were directly lysed in culture flasks by addition of Qiagen Buffer RLT. Subsequently, cell lysates were collected with a rubber policeman and homogenized by passing the lysate at least 5–10 times through an 20-gauge needle. After addition of 2.0 ml of 70% ethanol the homogenized lysate was applied to an RNeasy spin column placed in a 15-ml centrifuge tube and centrifuged for 5 min at 5000×***g*** two times. Columns were washed twice with Buffer RW1 and in Buffer RPE for 2 min at 5000×***g*** and subsequently eluted in 150 μl of RNase-free water for 3 min at 5000×***g*****.**

IMMUNOHISTOCHEMISTRY

Morphological characterization was performed immunocytochemistry using mouse monoclonal antibodies directed against conformational determinants of human collagen type I (Southern Biotechnology Associates Inc., Birmingham, AL, U.S.A.), directed against the carboxyl-terminal part of the a1(II) chain of collagen type II, (clone 2B1.5, Neomakers, Union City, CA, U.S.A.), chondroitin-4-sulphate (clone BE-123, Chemicon Int. Inc., Temecula, CA), and vimentin (clone V9, Dako A/S, Glostrup, Denmark). Briefly, cells grown on sterile slides were washed in phosphate-buffered saline (PBS). Slides were incubated with the primary antibodies diluted 1:100 in PBS/2% bovine albumin (BSA, Sigma Chemical) for 1 h at room temperature in a humidified chamber. Slides were then washed three times with PBS, incubated at room temperature in a humidified chamber for 1 h with biotin-conjugated horse antimouse secondary antibody (Vector Laboratories, Inc., Burlingame, CA, U.S.A.) diluted 1:100 into PBS/2% bovine albumin and subsequently treated with avidin biotin (Vectastain ABC Kit, Vector Laboratories) for 30 min. For negative controls the first antibody was omitted. Immunoreactivity was visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB, final concentration 5 mg/ml in 0.05 M Tris buffer, pH 7.6, 15 min). Sections were counterstained with Mayer's haemalaun.

NORTHERN-ANALYSIS

Total RNA was isolated from primary HACs and after two passages of monolayer cell culture at day 42 using the RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. For Northern analysis, 7 μg of total RNA were separated by agarose gel electrophoresis and subsequently transferred to nitrocellulose membranes using standard protocols[8]. Hybridization was performed using cDNA fragments radioactively labelled with [^{32}P]dATP (specific activity: 2×10^9 cpm/μg) following standard protocols[8]. For low expression products, RT-PCR using specific primer pairs was performed as previously described[9].

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

First strand synthesis was carried out using 250 ng RNA in a final volume of 20 μl containing 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 250 μM each dNTP, 0.5 μl of hexanucleotide mix and 100 U M-MLV reverse transcriptase at 37°C for 2 h. PCR was performed using 2.5 μl of the first strand reaction in a 50 μl reaction volume containing 0.3 μM primer (Table I), 10 mM Tris-Cl, 75 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP and 1 U Taq DNA polymerase. The reaction mixture was heated at 95°C for 5 min to denature the DNA and then subjected to 28 cycles of PCR (94°C for 1 min, specific annealing temperature for 1 min, 72°C for 2 min) followed by a final extension at 72°C for 5 min. The oligonucleotides used as primers (synthesized by MWG-Biotech, Ebersberg, Germany) are summarized in Table I. PCR products were analysed by non-denaturing PAGE (12.5% polyacrylamide) and visualized by silver staining as previously described[10].

ATLAS ARRAYS

Hybridization on Atlas cDNA expression arrays (Clontech, Heidelberg, Germany) was performed following the manufacturer's recommendation with minor modifications. Briefly, 5 μg total RNA were labelled by reverse transcription using MMLV reverse transcriptase (Promega, Madison) in a reaction buffer supplemented with DTT, [^{32}P]dATP (3000 Ci/mmol, 10 mCi/ml), and the Atlas CDS Primer Mix. Subsequently, free nucleotides were separated from labelled cDNA using Chroma Spin-200 DEPC-H20 columns. Atlas membranes were prehybridized in ExpressHyb hybridization solution containing denatured sheared salmon testes DNA (3%) for 30 min at 68°C. Hybridization was performed after adding the labelled cDNA probes (2×10^6 cpm) and C_0t-1 DNA to the hybridization solution for 14 h at 68°C. Filters were exposed to Kodak BioMax MR film overnight at −70°C in presence of intensifying screens. The quantitative analysis was carried out by phosphorimager using the Software Image Gauche V 3.1 (Fuji).

Results

IMMUNOHISTOCHEMISTRY

HACs showed extensive morphological changes during cultivation as monolayer cell cultures. Cytologically, freshly isolated HACs showed a round to polygonal cell morphology whereas cultivated cells exhibited an elongated fibroblast-like phenotype (Fig. 1). By IHC we found expression of collagen II, protein S-100, chondroitin-4-sulphate and vimentin in freshly isolated HACs. Cultivated HACs, however, showed only collagen I and vimentin expression. The changes in expression pattern occured around day 21

Table I
Oligonucleotide used as primers for RT-PCR

Gene	Acc no.	Temp	Size	Ori	Sequence
Collagen 1	X066269	56.8	159	Sense	CCC AAG GAC AAG AGG CAT
				Antisense	GCA GTG GTA GGT GAT GTT CTG
Collagen 2	X 16711	57.7	225	Sense	GGT CCT TCT GGA AAA GAT GG
				Antisense	AGG GAA TCC TCT CTC ACC AC
Collagen 3	X 15332	57.9	229	Sense	GGG TCC TAC TGG TCC TAT TGG
				Antisense	GCC TCC TTC ACC TTT CTC AC
Chondr 4S	NM001897	58.2	242	Sense	CCC CCA TCC TCA CTA CAA AC
				Antisense	ATC CAG GGT TCC TCT GTG TG
Chondr 6S	AB012192	58.1	216	Sense	CCC GTC TTT CTG GCT GAA
				Antisense	GTT GGG TCT GGG TGT GAT G
Chond Mod	AF050147	56.8	156	Sense	GCT GGA GGA CTA TGA AGC ACT
				Antisense	GGT GGG GTT GTC AGT TAT CTC
BMP 7	NM001719	57.4	198	Sense	ATG CCA TCT CCG TCC TCT AC
				Antisense	AGG GGA AGG TCT CAC AAA AG
BMP 8	NM001720	57.5	185	Sense	AGC CGA AGA AAA GCA ACG
				Antisense	GTC CCC CTC ACA GTA ATA GGC
CD 44	M59040	56.6	203	Sense	GGG GTC CCA TAC CAC TCA T
				Antisense	TTT TCT TCT GCC CAC ACC T
Cyclin D1	X59798	58.6	179	Sense	GCG GAG GAG AAC AAA CA
				Antisense	GTG AGG CGG TAG TAG GAC A
Cyclin D3	M92287	58.8	169	Sense	TTT TTG GCC CTC TGT GCT A
				Antisense	GCA GGC AGT CCA CTT CA
ICAM I	J03132	58.4	212	Sense	GAT CTT GAG GGC ACC TAC C
				Antisense	CCT TTT TGG GCC TGT TGT A
ICAM II	X15606	58.6	183	Sense	GTT CCG TGG CAA TGA GAC T
				Antisense	GGG GCT GAG TGT TTG TG
ICAM I R	X69711	58.6	200	Sense	AGC TCA CGA GGC AAA TAC A
				Antisense	GAC GTG AGG GGC AGA TAG
IGF I R	X 04434	58.5	168	Sense	ACG GGG CGA TCT CAA AAG T
				Antisense	CGG GCA GCA AGG TCT CTG T
IGF II R	Y 00285	58.9	283	Sense	CTG TTT CTT CCA CCA TCT T
				Antisense	TCC CTC CTC TCC TTC TT
IGF BP I	M 31145	58.6	266	Sense	TGC GTG CAG GAG TCT GAC
				Antisense	TCC TGT GCC TTG GCT AAA C
IL 2 R	X 01057	58.6	168	Sense	AAG CGA GCC TTC CAG GTC A
				Antisense	TCC CGT GGG TCA TTT TGC
EGR-1	X 52541	54.9	168	Sense	TCT TCG TCC TTT TGG TTT
				Antisense	CCC TCT TCC TTA TTT TGC T
TGF B2	M 19154	58.6	171	Sense	CCC CAG AAG ACT ATC CTG A
				Antisense	GCG GCA TGT CTA TTT TGT
TGF B3	J 03241	55.4	208	Sense	GTG AGT GGC TGT TGA GAA
				Antisense	AGA TGA GGG TTG TGG TGA
TGF B1	X 02812	58.6	189	Sense	AAT TCC TGG CGA TAC CTC A
				Antisense	GAA CCC GTT GAT GTC CAC
TNF R	M 33294	58.6	155	Sense	GGG GAG CTT GAA GGA AC
				Antisense	GCA AAG TTG GGA CAG TCA C
VCAM 1	X 53051	58.2	150	Sense	CAA TGT TGC CCC CAG AG
				Antisense	GGC TGT AGC TCC CCG TTA G
IGF BP 3	M 35878	56.4	308	Sense	TTA TGG CAC GGT TTC CTG T
				Antisense	CCC TCT CCC TCC CTT TCT C
BMP 2	M 22490	57.5	290	Sense	TGC TTT TCG TTT CCT CTT
				Antisense	CAT AGT TTG GCT GCT TCT C

(Acc No.=Gene Bank Accession Number; ori=orientation; temp=annealing temperature; size=size of PCR product.)

(range 14–28). These data were corroborated by the results of Northern analysis using specifc cDNA probes for collagens II and III and chondromodulin, respectively (Fig. 2). By Northern analysis collagen I, however, also showed expression in primary HACs (Fig. 2).

ATLAS ARRAYS

Atlas blots exhibited complex hybridization patterns when hybridized with cDNAs generated from RNAs of primary HACs and RNA of cultivated fibroblast-like chondrocytes (Fig. 3). By densitometry, highest intensities of hybridization signals were detected for the guanine nucleotide-binding protein G-s alpha subunit (GNAS), for the 60S ribosomal protein L6 (RPL6) and for the nuclease-sensitive element DNA-binding protein (NSEP) both in primary HACs and in cultivated cells (Table II). In addition, the early growth response protein 1 (EGR1), also known as KROX24 showed a very intense hybridization signal in primary HACs but not in cultivated cells (Table III).

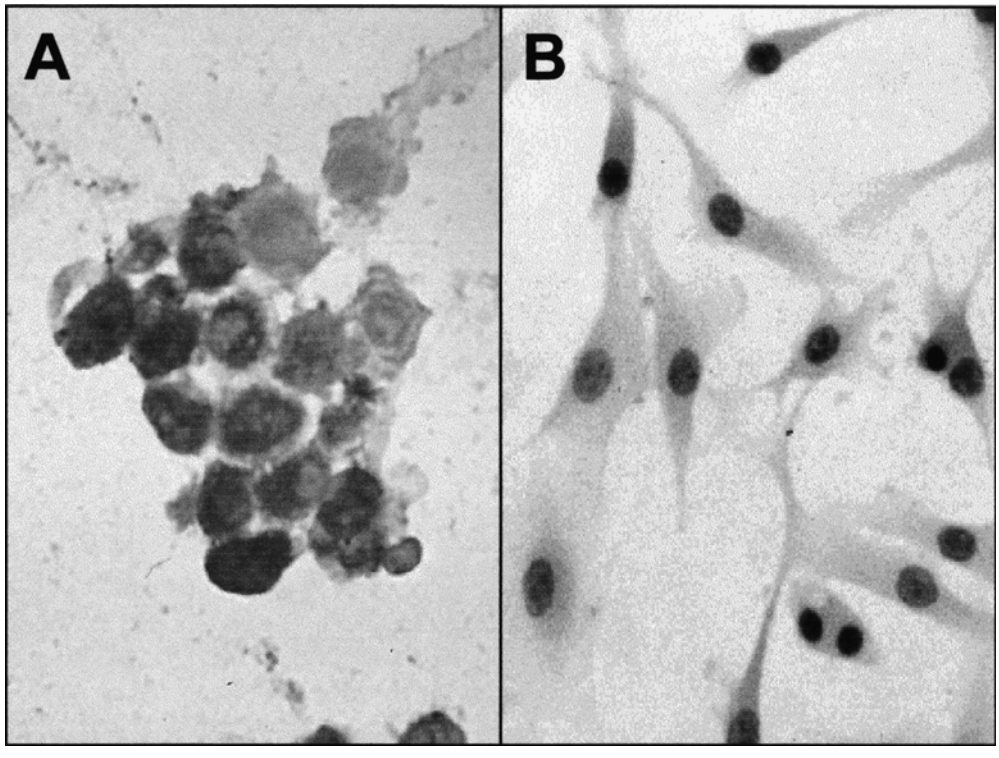

Fig. 1. Immunocytochemistry of primary (A) and cultivated (B) HACs using monoclonal antibodies raised against collagen II revealed expression of collagen II only in primary but not in cultivated HACs. Morphology of cells changed from a differentiated round to polygonal cell shape (A) to an elongated fibroblast-like phenotype (B) when grown in monolayer cultures.

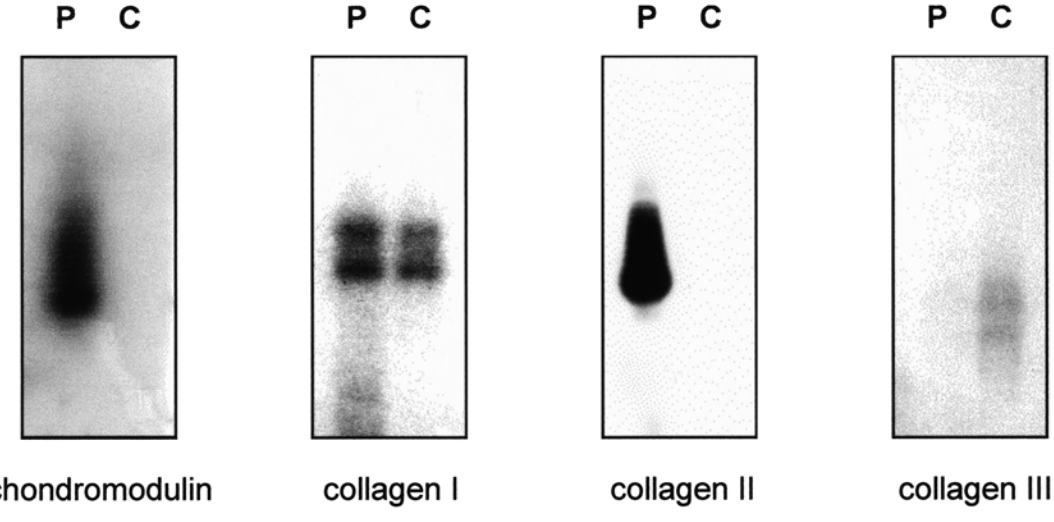

Fig. 2. Northern blots of primary (P) and cultivated (C) HACs showed expression of collagen II and chondromodulin only in primary HACs and collagen III only in cultivated cells. By Northern analysis collagen I expression could be detected in both cell types.

There were additional quantitative and qualitative differences between these two cell types. By hybridization using cDNAs generated from RNA of primary HACs to Atlas arrays, 93 spots could be visualized, however, only 64 spotted cDNA clones on Atlas arrays showed hybridization with cDNA generated from RNA of cultivated HACs. There were also multiple differences in the signal intensities of individual hybridization spots. Clones exhibiting more than 2.5-fold densitometric ratios between the two cell types were regarded as possibly differentially regulated genes (Tables III and IV). These differences were most prominent in the clones representing the genes for cyclinD1, the TNF receptor alpha, and prothymosin alpha, which were expressed exclusively in cultivated but not in primary HACs, and the IGF binding protein 3, which exhibited a 3-fold up-regulation in cultivated cells (Table III).

On the other hand, the clones representing EGR1, MCP-1RA, V-CAM1, ICAM-1, TGFβ2 and VEGF showed an exclusive hybridization in primary HACs or less than 10% of the level of primary HACs in cultivated cells (Table III). Atlas array results were confirmed by Northern analysis and RT-PCR (Fig. 3).

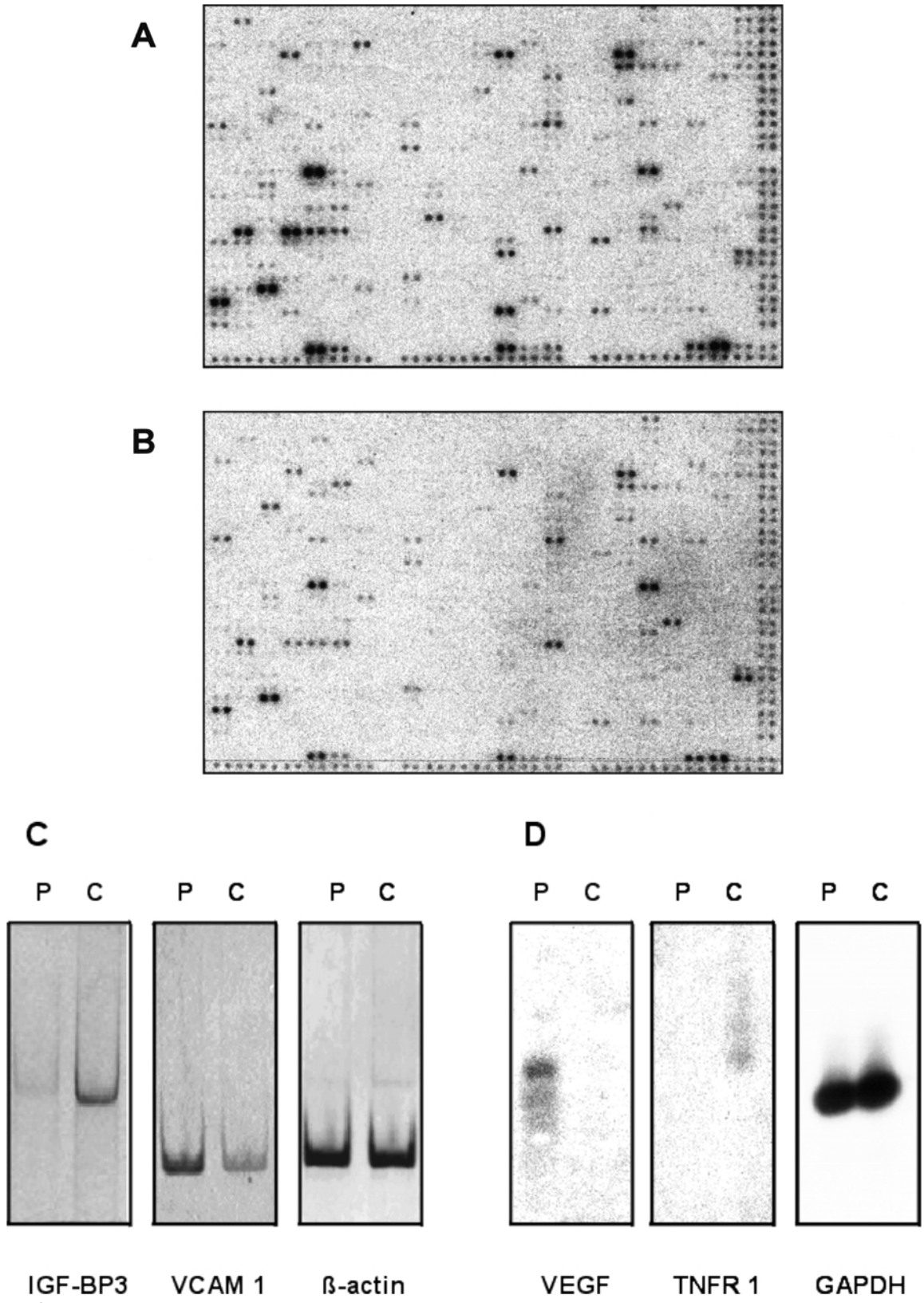

Fig. 3. Atlas™ cDNA arrays of differentiated primary HACs (A) and dedifferentiated HACs after monolayer cultivation (B) showing multiple qualitative and quantitative differences. Results were confirmed by RT-PCR (C), representative RT-PCR results of VCAM 1, IGF-BP3 and β-actin or by Northern analysis (D), representative Northern blots with probes of transcripts expressed in primary HACs (VEGF), in cultivated HACs (TNFR1) or in both cell types (GAPDH), P=primary, C=cultivated.

Table II
Genes equally (less than 2.5-fold) expressed in primary HACs and after cultivation

Name	Acc no.	Primary	Culture	X-fold
Guanine nucleotide-binding protein G-s alpha subunit (GNAS)	M14631	392.23	180.17	2.2
60S ribosomal protein L6 (RPL6)	X69391	370.97	150.12	2.5
Nuclease-sensitive element DNA-binding protein (NSEP)	M83234	333.83	171.90	1.9
Transcription factor ATF-4; cAMP-response element binding prot. (CREB2)	D90209	257.61	99.46	2.5
Defender against cell death 1 (DAD1)	D15057	218.95	88.24	2.5
Mitogen-activated protein kinase p38 (MAP kinase p38)	L35253	131.56	81.91	1.6
Nucleoside diphosphate kinase B (NDP kinase B; NDKB)	L16785	106.13	53.13	2.0
Fos-related antigen (FRA1)	X16707	82.54	31.75	2.6
Cyclin-dependent kinase inhibitor 1 (CDKN1A. CIP1. WAF1)	U09579	74.84	43.33	1.7
Acyl-CoA-binding protein (ACBP); diazepam binding inhibitor (DBI)	M14200	61.79	36.32	1.7
Glutathione S-transferase mu1 (GSTM1; GST1); HB subunit 4; GTH4	X68676	56.81	26.36	2.2
c-jun proto-oncogene; transcription factor AP-1	J04111	50.27	46.49	1.1
Macrophage-specific colony-stimulating factor (CSF-1; MCSF)	M37435	41.92	31.22	1.3
Clusterin precursor (CLU); complement-associated protein SP-40,40	M74816	41.73	23.44	1.8
Ephrin type-A receptor 1 precursor; tyrosine-protein kinase receptor eph	M18391	40.88	17.99	2.3
Glutathione S-transferase theta 1 (GSTT1)	X79389	39.54	21.88	1.8
cAMP-responsive element-binding protein (CREB1)	L05515	38.74	15.26	2.5
Natural killer cell enhancing factor (NKEFB)	L19185	37.90	27.02	1.4
NADH-ubiquinone oxidoreductase B18 subunit	M33374	37.48	17.96	2.1
CACCC-box DNA-binding protein	L04282	32.25	14.23	2.3
Platelet-derived growth factor receptor alpha subunit (PDGFRA)	M21574	31.80	26.13	1.2
Putative transcription activator DB1	D28118	31.57	13.28	2.4
Zinc finger X-chromosomal protein (ZFX)	X59738	28.58	17.96	1.6
Growth arrest & DNA-damage-inducible protein (GADD45)	M60974	28.47	22.14	1.3
Integrin beta 5 subunit precursor (ITGB5)	J05633	28.43	14.88	1.9
mutL protein homolog; DNA mismatch repair protein MLH1; COCA2	U07418	28.06	16.18	1.7
40S ribosomal protein S19 (RPS19)	M81757	24.39	19.95	1.2
Activator 1 37-kDa subunit; replication factor C 37-kDa subunit (RFC37)	M87339	24.02	15.08	1.6
Neurotrophic tyrosine kinase receptor-related 3; TKT precursor	X74764	20.14	12.83	1.6
Nuclear factor I (NFI); NFI-X	L31881	19.72	8.45	2.3
Cytoplasmic dynein light chain 1 (HDLC1)	U32944	19.05	16.02	1.2
Transforming growth factor beta receptor III (TGFR-III)	L07594	19.00	15.51	1.2
Monocyte chemotactic protein 1 precursor (MCP1)	M24545	138.09	169.92	1.2
Mitochondrial matrix protein P1 precursor; heat shock protein 60 (HSP-60)	M34664	76.37	83.85	1.1
Fibronectin receptor beta subunit (FNRB);CD29 antigen	X07979	75.36	81.18	1.1
Thymosin beta-10 (TMSB10; THYB10); PTMB10	M92381	33.62	82.18	2.4
Transforming protein rhoA H12 (RHO12; ARH12; ARHA)	L25080	33.07	51.63	1.6
Fas-activated serine/threonine (FAST) kinase	X86779	30.94	41.62	1.3
Cytosolic superoxide dismutase 1 (SOD1)	K00065	23.40	23.57	1.0
Insulin-like growth factor binding protein 1 (IGFBP1)	M31145	22.41	34.95	1.6
Replication factor C 38-kDa subunit (RFC38); activator 1 38-kDa subunit	L07541	20.29	24.55	1.2
Keratinocyte growth factor (KGF); fibroblast growth factor 7 (FGF7)	M60828	11.23	24.11	2.1

(Acc No.=Gene Bank Accession Number.)

Discussion

Pathological alterations of articular cartilage represent a major clinical problem and may occur in different circumstances including OA and after trauma. So far, no causative treatment is known and the depth of sientific evidence supporting clinically used methodes to restore articular surfaces is limited[11]. The understanding of the underlying mechanisms of proliferation and differentiation is a necessary contribution to develop new biological therapies to a better outcome of patients suffering from joint diseases. Recently, autologous chondrocyte transplantation (ACT) has been introduced as a novel biological treatment[4]. This approach, however, is hampered by its dependence of prior cultivation in order to increase the number of chondrocytes for transplantation. In the present study chondrocytes changed their morphology from a differentiated round to polygonal cell shape to an elongated fibroblast-like phenotype when grown in monolayer cultures. This phenotypic alteration was accompanied by changes in collagen expression patterns, and most prominently monolayer cells lose their collagen II and chondromodulin expression, which is highly specific in chondrocytes[5–7].

Thus, the prerequisite for a biological therapy using ACT is the understanding of the differentiation/dedifferentiation processes in chondrocytes. We therefore used a novel cDNA array technology to identify genes which are involved in the complex mechanisms of chondrocyte dedifferentiation in monolayer cultures. The use of cDNA arrays gained widespread acceptance for high-throughput analysis of gene expression. The array technology is based on cDNA fragments spotted to solid phase support, e.g. glass slides or nylon membranes. After hybridization with mRNA the intensity of the hybridization signal (radioactivity or fluorescence) depends on the quantity of mRNA molecules in the sample. Therefore, cDNA arrays allow a comprehensive analysis of gene expression by comparing the hybridization of two or more mRNA samples. The Atlas™ Human cDNA Expression Array I includes 588 genes that play key

Table III
Genes exclusively or >2.5-fold expressed in primary HACs

Name	Acc no.	Primary	Culture	X-fold
Intercellular adhesion molecule-1 precursor (ICAM1); CD54 antigen	J03132	97.26	<1	
Transforming growth factor beta2 precursor (TGF-beta2; TGFB2)	M19154	65.71	<1	
Vascular endothelial growth factor precursor (VEGF)	M32977	58.85	<1	
Cellular nucleic acid binding protein (CNBP)	M28372	36.17	<1	
CCAAT-binding transcription factor subunit B (CBF-B)	M59079	32.92	<1	
Tristetraproline (TTP; TIS11; ZFP36; NUP475)	M92843	29.55	<1	
Glutathione S-transferase pi (GSTP1; GST3)	X08058	28.72	<1	
Caspase-4 precursor (CASP4)	U28014	28.44	<1	
Alpha1 catenin (CTNNA1); cadherin-associated protein; alpha E-catenin	D13866	28.37	<1	
Transcription elongation factor SII	M81601	27.84	<1	
Heat shock 90-kDa protein A (HSP90A; HSPCA); HSP86	X07270	26.65	<1	
Transcription initiation factor TFIID 31-kDa subunit; TAFII31; TAF2G	U30504	26.20	<1	
Dioxin-inducible cytochrome P450 1B1 (CYP1B1)	U03688	23.84	<1	
Growth arrest & DNA-damage-inducible protein 153 (GADD153)	S40706	23.75	<1	
GA-binding protein (GABP-beta2); transcription factor E4TF1-60	D13318	23.00	<1	
Ini1	U04847	21.61	<1	
Extracellular signal-regulated kinase 3 (ERK3); MAP kinase 3 (MAPK3)	X80692	20.42	<1	
DNA-binding protein TAXREB302; albumin D box-binding protein (DBP)	D28468	18.08	<1	
Interleukin-1 beta precursor (IL-1; IL1B); catabolin	K02770	17.33	<1	
Platelet-derived growth factor B subunit precursor (PDGFB; PDGF2)	X02811	16.97	<1	
Erythroid differentiation protein (EDF); inhibin beta A; activin beta-A	J03634	16.84	<1	
HHR23A; UV excision repair protein protein RAD23A	D21235	16.65	<1	
Insulin-like growth factor II receptor (IGFR II); mannose-6-p receptor	Y00285	16.44	<1	
Protein C inhibitor (PROCI; PCI)	M68516	16.19	<1	
Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1 (GNB1)	M36430	16.17	<1	
Xeroderma pigmentosum group C repair complementing protein p58	D21090	15.64	<1	
Beta catenin (CTNNB)	X87838	15.13	<1	
CCAAT-binding transcription factor subunit B (CBF-B)	M59079	15.05	<1	
Glutathione S-transferase A1 (GTH1; GSTA1)	M25627	13.79	<1	
Guanine nucleotide-binding protein G-i/G-s/G-t beta subunit 2	M36429	13.74	<1	
Related to receptor tyrosine kinase (RYK)	S59184	13.68	<1	
Erythrocyte urea transporter (UTE; UT1); SLC14A1; HUT11; RACH1	U35735	13.41	<1	
Ski oncogene	X15218	12.37	<1	
Extracellular signal-regulated kinase 1 (ERK1; p44-ERK1); MAP kinase 1	X60188	10.27	<1	
Neuromedin B receptor (NMBR); NMB-preferring bombesin receptor	M73482	10.04	<1	
Early growth response protein 1 (EGR1. KROX24. zif-268)	X52541	358.30	35.74	10.03
Vascular cell adhesion protein 1 precursor (V-CAM 1); CD106 antigen	M30257	158.14	12.88	12.28
Nucleobindin precursor (NUC)	M96824	134.16	36.63	3.66
Monocyte chemoattractant protein 1 receptor (MCP-1RA)	U03882	74.76	4.40	16.99
Transducer of erbB2 (TOB)	D38305	63.39	14.94	4.24
Vascular endothelial growth factor receptor 1 (VEGFR1)	X51602	37.86	10.44	3.63
R kappa B DNA-binding protein	U08191	36.61	9.09	4.03

Table IV
Genes exclusively or >2.5-fold expressed in proliferating fibroblast-like HACs

Name	Acc no.	Primary	Culture	X-fold
Insulin-like growth factor-binding protein 3 (IGFBP3; IBP3)	M31159	44.67	125.62	0.36
G1/S-specific cyclin D1 (CCND1); cyclin PRAD1; bcl-1 oncogene	X59798	<1	41.56	
Tumor necrosis factor receptor 1 (TNFR1)	M33294	<1	21.02	
Prothymosin alpha (ProT-alpha; PTMA)	M26708	<1	16.62	
Ubiquitin-conjugating enzyme E2 17-kDa (UBE2A)	M74524	<1	12.43	
Tyrosine-protein kinase receptor UFO precursor; axl oncogene	M76125	<1	9.74	
cdc2-related protein kinase PISSLRE	L33264	<1	7.78	

roles in a variety of biological processes including differentiation, proliferation and apoptosis. The utility of Atlas arrays for accurately and reproducibly assessing gene expression is well established[12,13]. It is therefore particular suitable for the screening of differential gene expression in human chondrocytes.

Using this approach we identified multiple changes in gene expression patterns between primary HACs and cultivated cells after two passages of monolayer culture (42 days). In primary HACs 93 of 588 genes spotted on Atlas arrays could be detected by autoradiography; however, in cultivated cells only 64 genes were detected. These data indicate that the expression pattern in differentiated primary HACs was more complex than that of dedifferentiated fibroblast-like cells. Moreover, the data also indicate that several genes are down-regulated during monolayer propagation. Among the genes which are mainly expressed in primary HACs is the transcription factor EGR-1 (also

called KROX24 and zif-268) which belongs to the class of immediate early genes[14]. EGR-1 is crucial in the regulation of growth factors, hormones, cytokines, lipoproteins and adhesion molecules[15]. It has been shown that EGR-1 is highly expressed in fetal and human chondrocytes[16]. In addition, EGR-1 expression was always detectable in immortalized chondrocytes which exhibited signs of differentiation, i.e. collagen II and IX expression[17,18]. Recently, downregulation of EGR-1 in chondrocytes has been attributed to osteoarthritis[19]. Thus, EGR-1 is considered as a differentiation specific transcription factor in chondrocytes. EGR-1 has also been demonstrated as a transcriptional regulator of important differentiation factors in several tissues[15]. So far, however, its target genes in chondrocyte differentiation are not established. It seems not to be directly involved in collagen expression, since no EGR-1 binding site has been identified in the promoter regions of the collagen genes. Therefore, the identification and functional analysis of target genes regulated by EGR-1 in chondrocytes could substantially attribute to the better understanding of the molecular biology of cartilage. Other factors exclusively expresed in primary HACs include the genes for the adhesion molecules V-CAM1, the vascular endothelial growth factor and the transforming growth factor β2 which have been linked to chondrocyte differentiation in some recent reports[20–23]. The data of the present study also corroborate recent results indicating a role of chemokines in normal cartilage biology[24,25]. In addition, we found both the monocyte chemotactic protein (MCP1, SCYA2) and its receptor (MCP1-RA, CCR2) highly expressed in chondrocytes. MCP1 was expressed both in primary and fibroblast-like HACs, its receptor, however, was dramatically downregulated in proliferating cells.

Among the factors which were exclusively expressed in cultivated fibroblast-like cells cyclinD1 showed the highest expression both on Atlas arrays and by RT-PCR. D-type cyclins are essential factors in cell cycle regulation of proliferating cells including chondrocytes[26]. Its expression is important for cell proliferation and it is not surprising that it could be detected in the proliferating chondrocytic cells. It has been shown, however, that overexpression of cyclinD in different cells types inhibits terminal differentiation of these cells[27]. Thus, its expression is not only important for proliferation but it also interferes with differentiation. Another factor which is upreglated in proliferating cells is the IGF binding protein 3 (IGF-BP3). IGF-BPs are important factors in insulin-like growth factor signalling[15]. Their exact role is not completely understood. There are several reports indicating a modulating effect in IGF signalling by binding to IGF-BPs[15]. Therefore, its expression could contribute to dedifferentiation in chondrocytes since IGF seems to be involved in cartilage differentiation. However, the IGF-BP1 exhibited expression both in primary and in fibroblast-like cells.

The data presented here also support the hypothesis that proliferation and differentiation are exclusive features in human chondrocytes. Therefore, prior cultivation to obtain high cell counts for ACT must result in loss of differentiation. The understanding of the cell type specific gene expression could open new approaches to cultivate and redifferentiate chondrocytes for novel biological therapies of joint diseases.

The identification of genes known to be associated with cartilage dedifferentiation and proliferation confirms the reliability of the Atlas™ Array procedure. Results obtained by this method, however, need to be confirmed by other methods, e.g. immunohistochemistry, Northern analysis and RT-PCR, which has also been demonstrated here. While some of the genes presented here have been implicated in chondrocyte biology previously, the identification of transcripts that have not been linked to cartilage before represents one type of novel information which can only be obtained using screening methods like the array technology. The wealth of sequence information made available trough the human genome project will lead researchers towards systemic approaches that involve the simultaneous analysis of hundreds and thousands of genes. Analysing chondrocytes using current cDNA array technologies and additional arrays containing cartilage-specific genes will allow an analysis of complex gene expression patterns involved in chondrocyte differentiation and may therefore help to obtain detailed functional insights into cartilage metabolism.

Acknowledgments

This study was supported by the Lorenz Böhler Gesellschaft (Project 5/96), the Hoschuljubilaeumsstiftung of the University of Vienna (Project: H-276/98) and the Jubilaeumsfonds der Oesterreischischen Nationalbank (Project 7991).

References

1. Buckwalter JA, Mankin HJ. Articular cartilage. Part II: Degeneration and ostheoarthritis, repair, regeneration and transplantation. JBJS 1997;79:612–32.
2. Newman AP. Articular cartilage repair. Am J Sports Med 1998;26(2):309–24.
3. Chesterman PJ, Smith AU. Homotransplantation of articular cartilage and isolated chondrocytes. An experimental study in rabbits. J Bone Joint Surg 1968;50:184–97.
4. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L, *et al*. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994;331:889–95.
5. Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 1982;30:215–24.
6. Fuss M, Ehlers EM, Russlies M, Rohwedel J, Behrens P. Characteristics of human chondrocytes, osteoblasts and fibroblasts seeded onto a type I/III collagen sponge under different culture conditions. A light, scanning and transmission electron microscopy study. Anat Anz 2000;182:303–10.
7. Stewart MC, Saunders KM, Burton-Wurster N, Macleod JN. Phenotypic stability of articular chondrocytes in vitro: the effects of culture models, bone morphogenetic protein 2, and serum supplementation. J Bone Miner Res 2000;15:166–74.
8. Sambrook J, Frisch EF, Maniatis T. Molecular Cloning. A Laboratory Manual. 2nd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press 1989.
9. Kraus A, Neff F, Behn M, Schuermann M, Muenkel K, Schlegel J. Expression of alternatively spliced mdm2 transcripts correlates with stabilized wild-type p53 protein in human glioblastoma cells. Int J Cancer 1999;80:930–4.
10. Schlegel J, Vogt T, Münkel K, Rüschoff J. DNA fingerprinting of mammalian cell lines using non-radioactive arbitrarily primed PCR (AP-PCR). Biotechniques 1996;20:178–9.

11. Buckwalter J, Coutts R, Hunziker E, van Mow C. Breakout Session 3: Articular cartilage. Clin Orthop Related Res 1999;367:239–43.
12. Sehgal A, Boynton AL, Young RF, *et al*. Application of the differential hybridization of Atlas Human expression arrays technique in the identification of differentially expressed genes in human glioblastoma multiforme tumor tissue. J Surg Oncol 1998;67: 234–41.
13. Wellmann A, Thieblemont C, Pittaluga S, Sakai A, Jaffe ES, Siebert P, *et al*. Detection of differentially expressed genes in lymphomas using cDNA arrays: identification of clusterin as a new diagnostic marker for anaplastic large-cell lymphomas. Blood 2000;96: 398–404.
14. Sukhatme VP, Cao XM, Chang LC, Tsai-Morris CH, Stamenkovich D, Ferreira PC, *et al*. A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. Cell 1988;53:37–43.
15. Khachigian LM, Collins T. Early growth response factor 1: a pleiotropic mediator of inducible gene expression. J Mol Med 1998;76:613–6.
16. McMahon AP, Champion JE, McMahon JA, Sukhatme VP. Developmental expression of the putative transcription factor Egr-1 suggests that Egr-1 and c-fos are coregulated in some tissues. Development 1990;108:281–7.
17. Goldring MB, Birkhead JR, Suen LF, Yamin R, Mizuno S, Golwacki J, *et al*. Interleukin-1 beta-modulated gene expression in immortalized human chondrocytes. J Clin Invest 1994;94:2307–16.
18. Haas C, Aicher WK, Dinkel A, Peter HH, Eibel H. Characterization of SV40T antigen immortalized human synovial fibroblasts: maintained expression patterns of EGR-1, HLA-DR and some surface receptors. Rheumatol Int 1997;16:241–7.
19. Wang F, Connor JR, Dodds RA, James IE, Kumar S, Zou C, *et al*. Differential expression of egr-1 in osteoarthritic compared to normal adult human articular cartilage. Osteoarthritis Cart 2000;8:161–9.
20. Kienzle G, von Kempis J. Vascular cell adhesion molecule 1 (CD106) on primary human articular chondrocytes: functional regulation of expression by cytokines and comparison with intercellular adhesion molecule 1 (CD54) and very late activation antigen 2. Arthritis Rheum 1998;41:1296–305.
21. Bujia J, Behrends U, Rotter N, Pitzke P, Wilmes E, Hammer C. Expression of ICAM-1 on intact cartilage and isolated chondrocytes. In Vitro Cell Dev Biol Anim 1996;32:116–22.
22. Carlevaro MF, Cermelli S, Cancedda R, Descalzi Cancedda F. Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. J Cell Sci 2000;113:59–69.
23. Fukumura K, Matsunaga S, Yamamoto T, Nagamine T, Ishidou Y, Sakou T. Immunolocalization of transforming growth factor-betas and type I and type II receptors in rat articular cartilage. Anticancer Res 1998;18:4189–93.
24. Borzi RM, Mazzetti I, Cattini L, Uguccioni M, Baggiolini M, Facchini A. Human chondrocytes express functional chemokine receptors and release matrix-degrading enzymes in response to C-X-C and C-C chemokines. Arthritis Rheum 2000;43:1734–41.
25. Pulsatelli L, Dolzani P, Piacentini A, Silvestri T, Ruggeri R, Gualtieri G, *et al*. Chemokine production by human chondrocytes. J Rheumatol 1999;26:1992–2001.
26. Beier F, Lee RJ, Taylor AC, Pestell RG, LuValle P. Identification of the cyclin D1 gene as a target of activating transcription factor 2 in chondrocytes. Proc Natl Acad Sci USA 1999;96:1433–8.
27. Skapek SX, Rhee J, Spicer DB, Lassar AB. Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase. Science 1995;267: 1022–4.