Regulation of type-II collagen gene expression during human chondrocyte de-differentiation and recovery of chondrocyte-specific phenotype in culture involves Sry-type high-mobility-group box (SOX) transcription factors

David G. STOKES, Gang LIU, Rita DHARMAVARAM, David HAWKINS, Sonsoles PIERA-VELAZQUEZ and Sergio A. JIMENEZ[1]

Department of Medicine, Division of Rheumatology, 233 S. 10th Street, Thomas Jefferson University, Philadelphia, PA 19107, U.S.A.

During *ex vivo* growth as monolayer cultures, chondrocytes proliferate and undergo a process of de-differentiation. This process involves a change in morphology and a change from expression of chondrocyte-specific genes to that of genes that are normally expressed in fibroblasts. Transfer of the monolayer chondrocyte culture to three-dimensional culture systems induces the cells to re-acquire a chondrocyte-specific phenotype and produce a cartilaginous-like tissue *in vitro*. We investigated mechanisms involved in the control of the de-differentiation and re-differentiation process *in vitro*. De-differentiated chondrocytes re-acquired their chondrocyte-specific phenotype when cultured on poly-(2-hydroxyethyl methacrylate) (polyHEMA) as assayed by morphology, reverse transcriptase PCR of chondrocyte-specific mRNA, Western-blot analysis and chondrocyte-specific promoter activity. Essentially, full recovery of the chondrocyte-specific phenotype was observed when cells that had been cultured for 4 weeks on plastic were transferred to culture on polyHEMA. However, after subsequent passages on plastic, the phenotype recovery was incomplete or did not occur. The activity of a gene reporter construct containing the promoter and enhancer from the human type-II collagen gene (*COL2A1*) was modulated by the culture conditions, so that its transcriptional activity was repressed in monolayer cultures and rescued to some extent when the cells were switched to polyHEMA cultures. The binding of Sry-type high-mobility-group box (SOX) transcription factors to the enhancer region was modulated by the culture conditions, as were the mRNA levels for SOX9. A transfected human type-II collagen reporter construct was activated in de-differentiated cells by ectopic expression of SOX transcription factors. These results underscore the overt change in phenotype that occurs when chondrocytes are cultured as monolayers on tissue-culture plastic substrata.

Key words: cartilage, *COL2A1* gene, enhancer.

INTRODUCTION

Cartilage is a complex tissue containing a unique set of extracellular matrix molecules that provide the tissue with its ability to withstand compression and frictional stresses associated with joint movement [1–4]. The composition of the cartilage extracellular matrix is governed by chondrocytes, which express cartilage-specific genes in response to an intricate array of regulatory signals, including those that occur during development and tissue repair [5–7]. Following the pioneering studies of Benya and colleagues [8–10] on the phenotypic changes of rabbit chondrocytes in culture, modulation of the morphological and biosynthetic phenotype of cartilage cells has been the subject of intensive investigations. Numerous studies have examined the plasticity of the chondrocyte phenotype, employing cells of human, avian, lapine and rodent origin. It has become apparent from these collective studies that culturing chondrocytes as an adherent monolayer invariably leads to a process of de-differentiation whereby the cells acquire a fibroblastic morphology and lose their chondrocyte-specific gene-expression pattern [8–21]. Specifically, when chondrocytes are cultured in monolayers there is a prompt down-regulation of the expression of cartilage-specific genes, such as those encoding type-II, -IX and -XI collagens and aggrecan, and a concomitant initiation or up-regulation of the expression of fibroblast-associated genes such as those for type-I, -III and -V collagens and versican [8–21]. The cells re-acquire their chondrocyte-specific phenotype when transferred to a non-adherent, three-dimensional culture system, such as cultures in agarose or alginate beads, or in suspension on a hydrogel substrate [12,18,21–29]. However, the molecular mechanisms governing this process are not well understood.

We have previously established a human chondrocyte culture system that allows examination of the reversible loss of the chondrocyte-specific phenotype and its subsequent recovery in a predictable manner [28,29]. Furthermore, we have shown that the morphological and biochemical changes associated with these processes are accompanied by remarkable changes in the levels of DNA-binding nuclear proteins that specifically interact with important regulatory regions in the promoter of the cartilage-specific type II collagen gene *COL2A1* [30]. In the present work we investigated further the plasticity of the chondrocyte-specific phenotype afforded by this system by testing whether the de-differentiated phenotype of chondrocytes grown as a monolayer for varying periods can be fully reversed upon return to the poly-(2-hydroxyethyl methacrylate) (polyHEMA) culture system. We

Abbreviations used: β-Gal, β-galactosidase; CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; polyHEMA, poly-(2-hydroxyethyl methacrylate); SOX, Sry-type high-mobility-group box; L-SOX5, long-form SOX5; DMEM, Dulbecco's minimal essential medium; FBS, fetal bovine serum; P, passage; RT, reverse transcriptase; DTT, dithiothreitol; SV40, simian virus 40; EMSA, electrophoretic mobility-shift assay.

[1] To whom correspondence should be addressed (e-mail Sergio.Jimenez@mail.tju.edu).

show that the recovery of the chondrocyte-specific phenotype following growth as a monolayer is dependent on the number of passages. Furthermore, we show that the phenotypic changes are accompanied by modulation of the transcriptional activity of a *COL2A1* promoter/enhancer gene construct and that differences in Sry-type high-mobility-group box (SOX) transcription factor binding to a *COL2A1* enhancer segment also occur. We also found that the expression of a human type-II collagen reporter construct was activated in de-differentiated chondrocytes by ectopic expression of long-form SOX5 (L-SOX5), SOX6 and SOX9. The SOX transcription factors SOX6, SOX9 and L-SOX5 have been shown previously to be important for activation of the *COL2A1* gene during chondrogenesis in mice and for expression of the *COL2A1* gene in both human and mouse primary chondrocytes [31–35]. Skeletal formation is dependent on *SOX9*, as mutations in the human *SOX9* gene cause campomelic dysplasia, a disease associated with abnormal skeletal structures [36,37]. *SOX9* has also been shown to be down-regulated by pro-inflammatory cytokine treatment of chondrocytes and is therefore implicated in inflammatory joint diseases [38]. Thus the SOX factors (L-SOX5, SOX6 and SOX9) are thought to be master regulatory genes that are essential for the expression of the chondrocyte-specific phenotype. Therefore the results presented in this work provide the foundation for further studies that may allow an improved preservation and recovery of the chondrocyte phenotype *in vitro*.

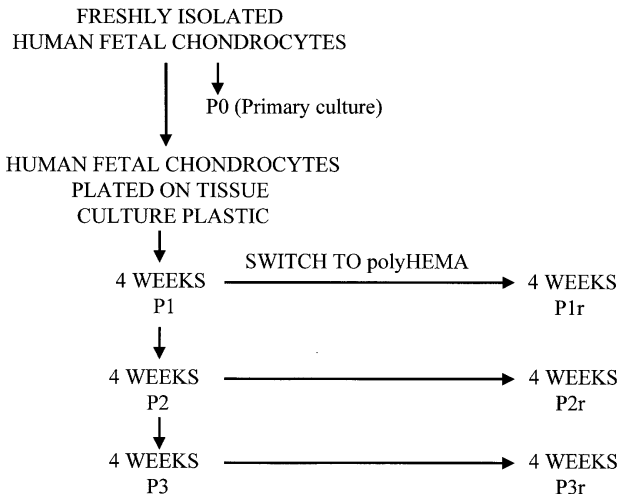
EXPERIMENTAL

Isolation of human fetal and bovine fetal chondrocytes

Human fetal chondrocytes were obtained from epiphyseal cartilage removed under sterile conditions from femoral heads, knee condyles and tibial plateaux of 20–24-week-old fetuses. The tissues were obtained from the Anatomical Gift Foundation (Laurel, MD, U.S.A.). To remove adherent fibrous tissues, the cartilage was incubated in Hank's medium containing trypsin and bacterial collagenase (2 mg/ml each) for 1 h at 37 °C. The medium was discarded and the tissue fragments minced and digested overnight at 37 °C in Dulbecco's minimal essential medium (DMEM) with 4.5 g/l glucose containing 10 % fetal bovine serum (FBS) and 0.5 mg/ml of bacterial collagenase. The released cells were filtered through a 70 μm nylon membrane into a vessel containing fresh DMEM and 10 % FBS. The cells were collected by centrifugation at 250 ***g*** for 5 min and washed four times with collagenase-free medium. The average yield of chondrocytes obtained with this procedure was $(3.0 \pm 0.4) \times 10^8$ chondrocytes/g of cartilage (wet weight). For experiments employing fetal bovine chondrocytes, articular cartilage from second trimester bovine fetuses was obtained from Animal Technologies (Tyler, TX, U.S.A.) and the chondrocytes were liberated as above. The yield of chondrocytes from the fetal bovine cartilage was 1×10^8 chondrocytes/g of wet cartilage.

Culture of chondrocytes

The isolated chondrocytes were cultured in DMEM containing 10 % FBS, 2 mM glutamine, 1 % vitamin supplements and 40 μg/ml ascorbic acid. This medium was employed because it was found in previous studies to cause a rapid and consistent loss of the cartilage-specific phenotype and a conversion of chondrocytes into cells with fibroblastic characteristics when cultured in plastic culture dishes. It also allowed for the maintenance of the chondrocyte-specific phenotype for long periods when the cells were cultured on polyHEMA-coated dishes [28,29]. To induce the loss of the chondrocyte-specific phenotype in the human fetal chondrocytes and bovine fetal chondrocytes the isolated cells were plated into plastic tissue culture dishes and were cultured until confluent [passage (P) 0]. The cells were then dissociated with trypsin and collagenase and subcultured (1:2) in new plastic dishes for 4 weeks (P1). These cells were subcultured on plastic twice more at 4 week intervals (P2 and P3). To attain recovery of the chondrocyte-specific phenotype of the de-differentiated cells, cells from the monolayer cultures on tissue-culture plastic substrata at P1, P2 and P3 were trypsinized and plated at a density of 5×10^6 cells in 60 mm plastic Petri dishes that had been coated previously with 0.9 ml of a 10 % solution of polyHEMA (Poly Sciences, Malvern, PA, U.S.A.) as described previously [28,29]. Following each sequential passage on tissue-culture plastic, the cells were cultured on the polyHEMA substrata for 4 weeks to allow the recovery of their chondrocyte-specific phenotype (P1r, P2r and P3r). A flow-chart for this experimental protocol is depicted in Scheme 1. For the transfection experiments involving bovine fetal chondrocytes the cells were initially plated at a density of 30000/cm^2 on tissue-culture plastic and cultured for 11 days, when they were transiently transfected using the Profection kit (Promega, Madison, WI, U.S.A.) as described below.



Scheme 1 Experimental protocol for investigating the de-differentiation/re-differentiation process in cultured human chondrocytes

Reverse transcriptase (RT)-PCR amplification of *COL2A1*, *COL11A2* and aggrecan from chondrocytes cultured on either polyHEMA or plastic substrates

Total RNA was isolated from chondrocytes under the various culture conditions described above using the Trizol reagent (Gibco BRL, Rockville, MD, U.S.A.). RT-PCR was carried out utilizing the GeneAmp RNA PCR kit (Perkin Elmer, Norfolk, CT, U.S.A.). The reverse transcription and PCR were carried out with the primers shown in Table 1 using the following conditions: 95 °C for 2 min followed by 95 °C for 45 s, 56 °C for 1 min and 72 °C for 1 min 5 s for 35 cycles. The PCR products were analysed by agarose-gel electrophoresis. An RNA dot-blot hybridization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a probe was carried out and quantified with a PhosphorImager and Imagequant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.) to ensure that equal amounts of RNA were used in the RT-PCR reaction.

Table 1 Primers utilized for RT-PCR amplification of *COL2A1*, *COL11A1*, *COL1A1*, *COL3A1* and aggrecan cDNA

Gene	Primer sequence (5′ → 3′)	Nucleotide location	Expected product size
COL2A1	AAGATGGTCCCAAAGGTGCTCG	2567–2588	500 nt
	AGCTTCTCCTCTGTCTCCTTGC	3066–3045	
COL11A1	ATGGAAGCCGTCTGATGC	50C–67C	410 nt
	GTAGGAGACGTCCTGCTG	459C–442C	
Aggrecan	AGAGAAGATTCTGGGTC	793–810	657 nt
	AGCATCCACCCAGGTCT	1449–1432	
COL1A1	TAAAGGGTCACCGTGGCT	3439–3456	355 nt
	CGAACCACATTGGCATCA	3793–3776	
COL3A1	CATCTTGGTCAGTCCTATGCGGAT	202–225	399 nt
	TACTGCTACTCCAGACTTGACATC	600–577	

Western-blot analysis

For Western-blot analysis, chondrocytes cultured under the various conditions described above were suspended and then centrifuged at 1500 ***g*** for 5 min and washed twice with PBS. The cells were re-suspended in 1% SDS/50 mM dithiothreitol (DTT)/1% (v/v) glycerol and heated to 100 °C for 5 min. The solubilized cell-associated proteins were separated by electrophoresis in 6% polyacrylamide gels on a mini-gel apparatus at 125 V for 90 min. The proteins were electroblotted at 40 V for 90 min from the polyacrylamide gel on to a supported nitrocellulose membrane and the transferred proteins were incubated for 30 min with a 1:500 dilution (v/v) of SJ441 antibody, a polyclonal antibody that recognizes the C-terminal telopeptide of human type-II collagen and also cross-reacts with the $\alpha 2$ chain of type-XI collagen [30]. The proteins on the filter were detected using the ECL® Western-blotting detection reagent (Amersham, Arlington Heights, IL, U.S.A.).

Transient transfection, chloramphenicol acetyltransferase (CAT) assays and Northern-blot analyses

The human fetal chondrocytes were transfected with 30 μg of the various reporter constructs by the calcium phosphate precipitation method using the Profection kit (Promega), followed 16 h later by a 15% glycerol shock for 1 min. Fresh medium was added and the cells were harvested 48 h after transfection and lysed by sonication. Total protein was measured by the Bradford assay [39]. CAT activity was determined in cell extracts by TLC [40] and quantified by PhosphorImager using Imagequant software (Molecular Dynamics). The level of CAT activity was normalized to the amount of β-galactosidase (β-Gal) activity by co-transfection with a simian virus 40 (SV40)–β-Gal construct (pSV-β-Galactosidase Control Vector; Promega) in the reversal experiments. For determination of β-Gal activity the β-Gal enzyme assay system from Promega was utilized. The COL2A1 promoter–CAT constructs used for experiments shown in Figure 4 (see below) were a gift from Mary Goldring (Harvard Medical School, Boston, MA, U.S.A.) [41]. pB/4.0 contains from −577 to +3248 bp and pB/2.9 contains from −577 to +2370 bp of the human *COL2A1* gene linked to the *CAT* gene. The region from +2497 to +3428 bp contains the chondrocyte-specific enhancer region [42]. pE/0.7 and pE/0.2 contain from −577 to +63 bp and −131 to +63 bp of the human *COL2A1* gene linked to the *CAT* gene, respectively, and also contain the SV40 enhancer region.

Bovine chondrocytes cultured in six-well plates were transiently transfected using the FuGene reagent (Roche) with 1.5 μg of the human *COL2A1*–luciferase reporter construct and either 0.5 μg of pCDNA3 vector alone or 0.5 μg of either the L-SOX5, SOX6 or SOX9 expression constructs alone or 0.25 μg each of the L-SOX5, SOX6 and SOX9 expression constructs together. All transfections included 0.5 μg of pCMV-βGAL (Clontech, Palo Alto, CA, U.S.A.) as a control for transfection efficiency. The human *COL2A1*–luciferase construct contains from −531 to +63 bp of the promoter region linked to the luciferase reporter gene followed by the region from +1286 to +4011 bp from the first intron, which contains the enhancer [42]. After addition of the FuGene/DNA mix (48 h) the cells were harvested and both β-Gal and luciferase activities were determined using assay kits from Promega. Luciferase activity was normalized to both protein amount and β-Gal activity.

Preparation of nuclear extracts and electrophoretic mobility-shift assays (EMSAs)

Nuclear extracts from differentiated and de-differentiated chondrocytes were prepared by standard methods [43] with minor modifications as described previously [29]. Briefly, cells were lysed by Dounce homogenization in hypotonic buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF and 0.5 mM DTT). Nuclei were recovered by centrifugation at 3300 ***g*** for 15 min at 4 °C and their volume estimated. The nuclei were then resuspended in a volume of low-salt buffer (20 mM Hepes, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT) equal to one-half the estimated volume of pelleted nuclei. High-salt buffer (same as low-salt buffer except that 1.2 M KCl was used) was added slowly until the final KCl concentration was 0.4–0.6 M. The nuclei were extracted for 30 min at 4 °C with gentle stirring. The extract was centrifuged for 30 min at 25000 ***g*** and then the supernatant was dialysed against 50 vol. of dialysis buffer (20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM PMSF and 0.5 mM DTT) overnight at 4 °C. Protein concentration was determined by absorbance at 280 nm.

EMSAs were carried out as follows. The 84 bp *COL2A1* enhancer probe (probe 45/46; see Figure 5A, below) was synthesized by PCR with the primers DS45 (5′-CTGGTTCCTCGCAGAGAC-3′) and DS46 (5′-AACCCACTGGACCTCGTC-3; positions 2190–2273 in *COL2A1*) and the plasmid pB/4.0 as a template [41,42,44]. These primers flank a region in the first intron of human *COL2A1* that is homologous to the mouse 48 bp enhancer sequence described by Lefebvre et al. [33]. The probe was ^{32}P-end-labelled by T4 polynucleotide kinase. Binding reactions consisted of 12.5 mM Hepes, pH 7.9, 50 mM KCl, 5% glycerol, 2 mg/ml BSA, 1–2 μg of poly(dG-dC), 0.1 mM EDTA, 0.1 mM DTT, 3–5 ng of labelled probe and 10–15 μg of nuclear protein. For competition experiments, a 100-fold excess of unlabelled 45/46 probe or unlabelled high-mobility-group (HMG) consensus probe was added. The HMG consensus probe was a double-stranded oligonucleotide prepared from the following oligonucleotides: DS33, 5-GGACACTGAGAACAAAGCGCTCTCAC-3′, and DS34, 5-GGGTGAGAGCGCTTTGTTCTCAGTGT-3′ [33]. For supershift assays, 1 μl of anti-L-SOX5, anti-SOX6 or anti-SOX9 antibodies was added to the binding reactions. The antibodies were a gift from Dr Veronique Lefebvre (University of Texas, M. D. Anderson Cancer Center, Houston, TX, U.S.A.) [34]. Binding reactions were incubated at room temperature for 30 min and then loaded on to 5% TAE (40 mM Tris/acetate/1 mM EDTA) polyacrylamide gels. Gels were electrophoresed at 140 V for 2–3 h, vacuum dried and exposed to X-ray film at −70 °C.

RESULTS

Morphological appearance of chondrocytes cultured under conditions that allow the loss of the chondrocyte-specific phenotype and its subsequent recovery

The morphological appearance of the chondrocytes is shown in Figure 1. The loss of chondrocyte morphology and the acquisition of fibroblastic shape was progressively apparent as the cells were subcultured on plastic. Recovery of the spherical shape morphology was observed when the fibroblast-like cells from P1 and P2 were transferred to polyHEMA-coated dishes (P1r, P2r; Figure 1). The P1r cells were photographed at an earlier time point during the re-differentiation process and had not yet formed the clusters observed in the P2r cells, which were photographed at a later stage. P1r cells were observed to form clusters at later time points (results not shown). However, fibroblast-like cells from P3 failed to fully re-acquire the chondrocyte shape when transferred to the suspension culture (P3r; Figure 1).

Analysis of chondrocyte-specific gene expression

In order to determine the effect of the culture conditions on the chondrocyte-specific gene expression pattern, RT-PCR analysis was performed on RNA isolated from the cells at the different passages on plastic and following their transfer to polyHEMA. Primers were used that would specifically amplify human *COL2A1*, *COL11A2* and aggrecan transcripts, three chondrocyte-specific gene products (Table 1). Two independent experiments were analysed in this manner. As shown in Figure 2, the pattern of expression of these genes was modulated by the culture conditions. In the experiment shown in Figure 2(A), the expression of COL11A1 mRNA in chondrocytes cultured on plastic diminished in P1 and P2 cells and was essentially undetectable in P3 cells. Aggrecan mRNA expression was lost by P2 on plastic. When cells from P2 on plastic were cultured on polyHEMA (P2r) the expression of COL11A1 and aggrecan mRNAs returned to the levels observed in P0 cells; however, COL2A1 mRNA expression, although higher, remained below the levels found in P0 cells (results not shown). When P3 cells were transferred to polyHEMA (P3r), the expression of both COL11A1 and aggrecan was restored, whereas COL2A1 mRNA expression was not detected in P3r cells (results not shown).

In another experiment (Figure 2B) the expression of COL2A1 and COL11A1 mRNA was analysed in P1 and P2 cells (on plastic) and following their culture on polyHEMA (P1r and P2r). In this experiment, the expression of the two genes also closely mirrored the culture conditions. P1 cells on plastic lost the expression of COL11A1, and COL2A1 expression was diminished compared with P0 cells. When P1 cells were switched to polyHEMA for 4 weeks (P1r), expression of both genes returned to the levels observed in P0 cells. COL2A1 mRNA in P2 cells on plastic was lower, as compared with P0 cells, and COL11A1 mRNA was reduced even further. When P2 cells were transferred to polyHEMA for 4 weeks (P2r cells) the expression of COL11A1 was seen to return to near the levels observed in P0

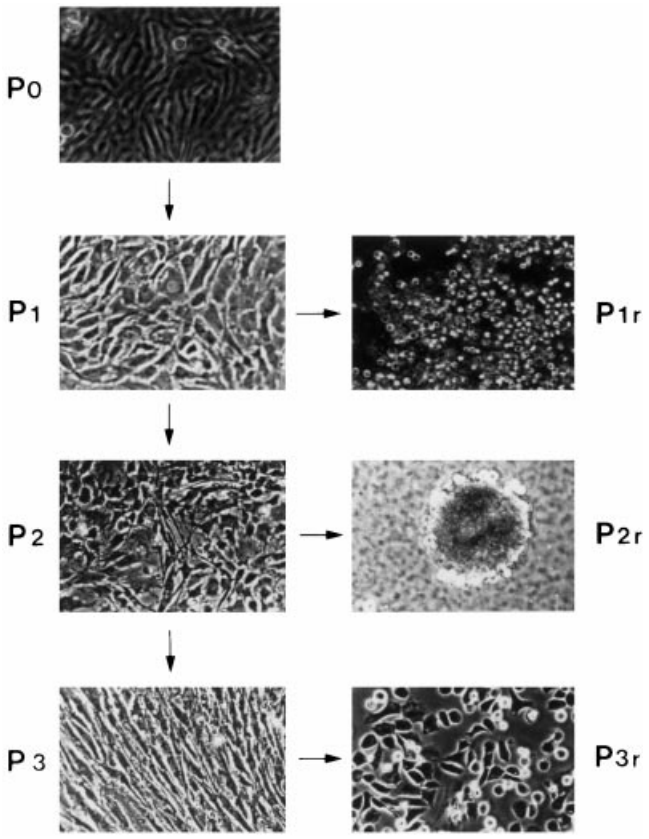


Figure 1 Morphological appearance of human chondrocytes under culture conditions for de-differentiation and re-differentiation

Morphological appearance of human fetal epiphyseal chondrocytes plated on plastic-coated dishes as primary cultures for 1 week (P0), during sequential sub-passages to plastic dishes (P1–P3), or following transfer from the plastic dishes to polyHEMA-coated dishes at each of the indicated passages (P1r–P3r).

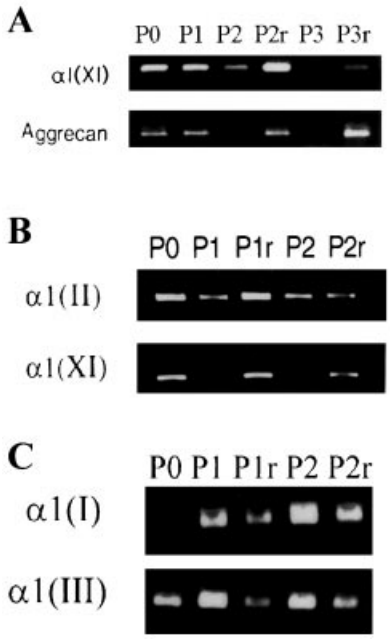


Figure 2 RT-PCR analysis of collagen and aggrecan gene expression

(**A**) RT-PCR of total RNA from chondrocytes shown in Figure 1 for the expression of the *COL11A1* [α1(X1)] and aggrecan genes. (**B**) RT-PCR of total RNA from another experiment in which the cells were taken to P2r. RNA was isolated and subjected to RT-PCR utilizing the primers described in the Experimental section for *COL2A1* [α1(II)] and *COL11A1* [α1(X1)] mRNA. (**C**) RT-PCR of total RNA from the same cells shown in (**B**) with primers designed to detect transcripts from the *COL1A1* [α1(I)] and *COL3A1* [α1(III)] genes.

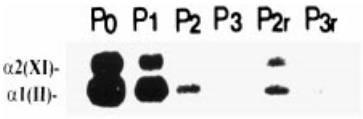


Figure 3 Western-blot analysis of collagen protein expression

Western-blot analysis of α1(II) and α2(XI) collagen chains in extracts from chondrocytes shown in Figure 1. A polyclonal antibody which recognizes the C-terminal telopeptide of α1(II) collagen and cross-reacts with the α2(XI) collagen chain [30] was employed as described in the Experimental section.

cells whereas, as was observed in the experiment in Figure 2(A), COL2A1 mRNA levels remained lower. In a third experiment, shown in Figure 2(C), the expression levels of the *COL1A1* and *COL3A1* genes, which are more highly expressed in de-differentiated cells, were investigated by employing RT-PCR. The expression patterns of those genes were again reflective of the culture conditions in that they were more highly expressed in the de-differentiated chondrocytes (P1 and P2) compared with the differentiated chondrocytes (P0, P1r and P2r).

Recovery of the cartilage-specific phenotype was also investigated by performing Western-blot analysis to ascertain the level of production of α1 type-II and α2 type-XI collagen chains. As shown in Figure 3 the expression of these chains diminished progressively with subsequent passages on plastic (P1–P3), whereas there was an increase in their expression in P2r cells (4 weeks on polyHEMA after two passages on plastic over 8 weeks) compared with both P2 and P3 cells. However, the expression of the cartilage-specific collagens did not recover to the levels seen in either P0 and P1 cells. P3r cells (4 weeks on polyHEMA after three passages over 12 weeks on plastic) exhibited detectable although diminished amounts of both collagens, which were undetectable in cells passaged three times on plastic (P3).

Effects of different culture conditions on COL2A1 promoter activity

Based on the results obtained from the RT-PCR and Western-blot experiments and our previous findings of differences in transcription-factor expression patterns between chondrocytes cultured on plastic and polyHEMA [29], we next determined whether the modulation of COL2A1 mRNA expression was controlled at the transcriptional level. For this experiment, cells cultured under conditions that either led to their de-differentiation into fibroblast-like cells or allowed them to recover their chondrocyte-specific phenotype were transiently transfected with constructs containing the COL2A1 promoter either with or without the first-intron enhancer region linked to the *CAT* reporter gene. The constructs employed for transfections were those described previously by Goldring et al. [41] and are depicted in Figure 4(A). We first performed an initial experiment in which we transfected chondrocytes that had been cultured on either polyHEMA or plastic in comparison with human skin fibroblasts. The results of this experiment are shown in Figure 4(B). We observed that only chondrocytes cultured on polyHEMA showed a high level of expression of the pB/4.0 construct, which contains both the promoter and enhancer regions of *COL2A1*. The other three constructs, pB/2.9, pE/0.7 and pE/0.2, showed equally low expression levels, although there was a small variation in expression of pB/2.9 between the three cell types. Since high-level expression was dependent on inclusion of the enhancer sequences present in the pB/4.0 construct (Figure 4A), we next investigated the differences in expression levels of the pB/4.0 and pB/2.9 constructs in chondrocytes that had been re-differentiated by transfer from monolayer culture to polyHEMA-coated dishes. We transfected P0, P1, P1r, P2 and P2r cells (see Scheme 1) with pB/4.0, pB/2.9 or pSV2-CAT. The results of this experiment are shown in Figure 4(C). There was very little difference in CAT activity between the chondrocytes cultured on plastic or polyHEMA when transfected with the pSV2-CAT control construct. However, when the construct pB/4.0 containing the COL2A1 promoter and first-intron enhancer was used, the resulting CAT activity was reflective of the differentiation/de-differentiation state of the cells (Figure 4C), with a high level of expression in cells cultured on polyHEMA in contrast to the very low levels observed in cells cultured on plastic. Interestingly, when construct pB/2.9 lacking the enhancer region was examined, no differences in CAT activity were observed between the cells cultured on either plastic or re-differentiated on polyHEMA (Figure 4C). These results indicate that the decrease in *COL2A1* gene expression observed during the de-differentiation process, which chondrocytes undergo as a result of culture in monolayers on plastic, is controlled largely at the level of transcription of the gene and that this control is dependent on the presence of the first-intron enhancer region.

Changes in nuclear-factor binding to the *COL2A1* enhancer during de-differentiation

The transfection results indicated that the enhancer region of the *COL2A1* gene was important for the regulation observed between the different culture conditions. It is known that members of the SOX family of transcription factors regulate the human *COL2A1* gene in chondrocytes through binding sites within the enhancer region [31]. A 48 bp region found in the mouse *COL2A1* enhancer region, which is conserved in the human gene, contains four SOX-protein binding sites, binds to three different SOX factors (SOX6, SOX9 and L-SOX5) and has been shown to be important for the chondrocyte-specific expression of this gene [34]. We therefore examined whether the interaction of these factors with the corresponding region from the human gene was altered in response to de-differentiation. For this purpose, nuclear extracts were prepared from human fetal chondrocytes grown on polyHEMA dishes or from chondrocytes cultured and passaged as monolayers on tissue-culture plastic at 1 week intervals for up to 4 weeks. An 84 bp probe was synthesized by PCR from the human *COL2A1* gene that contained the conserved SOX binding sites as delineated in the mouse gene and this probe was used in an electrophoretic mobility-supershift assay (Figure 5A). Figure 5(B) shows that the pattern of nuclear-factor binding to this probe changes after four sequential 1-week passages. The DNA–protein complexes from the cells cultured on plastic for either 1 or 4 weeks displayed slower electrophoretic mobility in comparison with the pattern displayed by complexes from P0 chondrocytes (Figure 5B, lanes 1–3, bracket). Supershift analysis with antibodies against L-SOX5 and SOX6 resulted in a partial shift of nuclear extracts from P0 chondrocytes and chondrocytes cultured on plastic for 1 or 4 weeks (Figure 5B, lanes 4–9, arrow). We observed the strongest shift and an apparent disruption in the DNA–protein complex in P0 extracts with the SOX9 antibodies (Figure 5B, lane 10). Supershift analysis with the anti-SOX9 antibodies employing nuclear extracts isolated from 1 and 4 week cells also showed partial shifts (Figure 5B, lanes 11 and 12, arrow), consistent with the partial activity of the *COL2A1* gene observed in chondrocytes cultured for 4 weeks in monolayers (P1, Figures 2 and 4). However, the anti-SOX9 antibodies resulted in a weaker shift of the DNA–protein complex in 1- and 4-week extracts (Figure 5B, lanes 11 and 12) compared with the

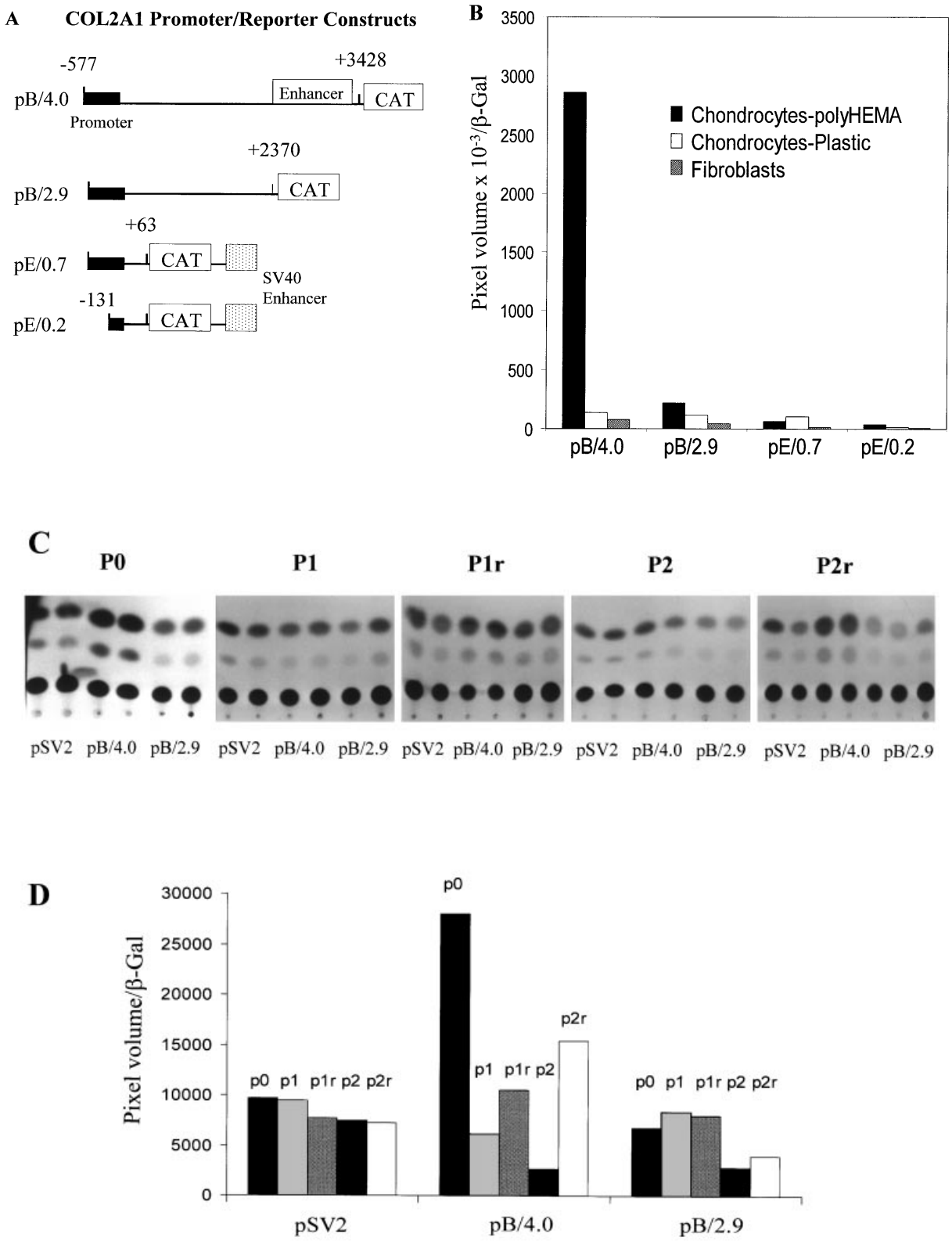


Figure 4 ***COL2A1*** **reporter constructs and transient-transfection analyses of differentiated, de-differentiated and re-differentiated human chondrocytes**

(**A**) Schematic representation of the human *COL2A1* enhancer–CAT reporter constructs that were transfected into de-differentiating and re-differentiating chondrocytes (see the Experimental section). (**B**) CAT activity of the human *COL2A1* promoter/enhancer constructs pB/4.0, pB/2.9, pE/0.7 and pE/0.2 in differentiated chondrocytes, de-differentiated chondrocytes and human dermal fibroblasts. (**C**) Representative CAT assays of the human *COL2A1* promoter/enhancer constructs pB/4.0 and pB/2.9 and the control construct pSV2-CAT (see panel **A**) in chondrocytes (P0) and chondrocytes transferred from monolayer cultures (P1, P2) to polyHEMA cultures (P1r, P2r) as outlined in Scheme 1. (**D**) Quantification of the CAT assays shown in (**C**). Results shown are from duplicate assays. Pixel volume/β-gal on the y-axis are counts collected by a PhosphorImager and corrected for β-Gal activity (co-transfection).

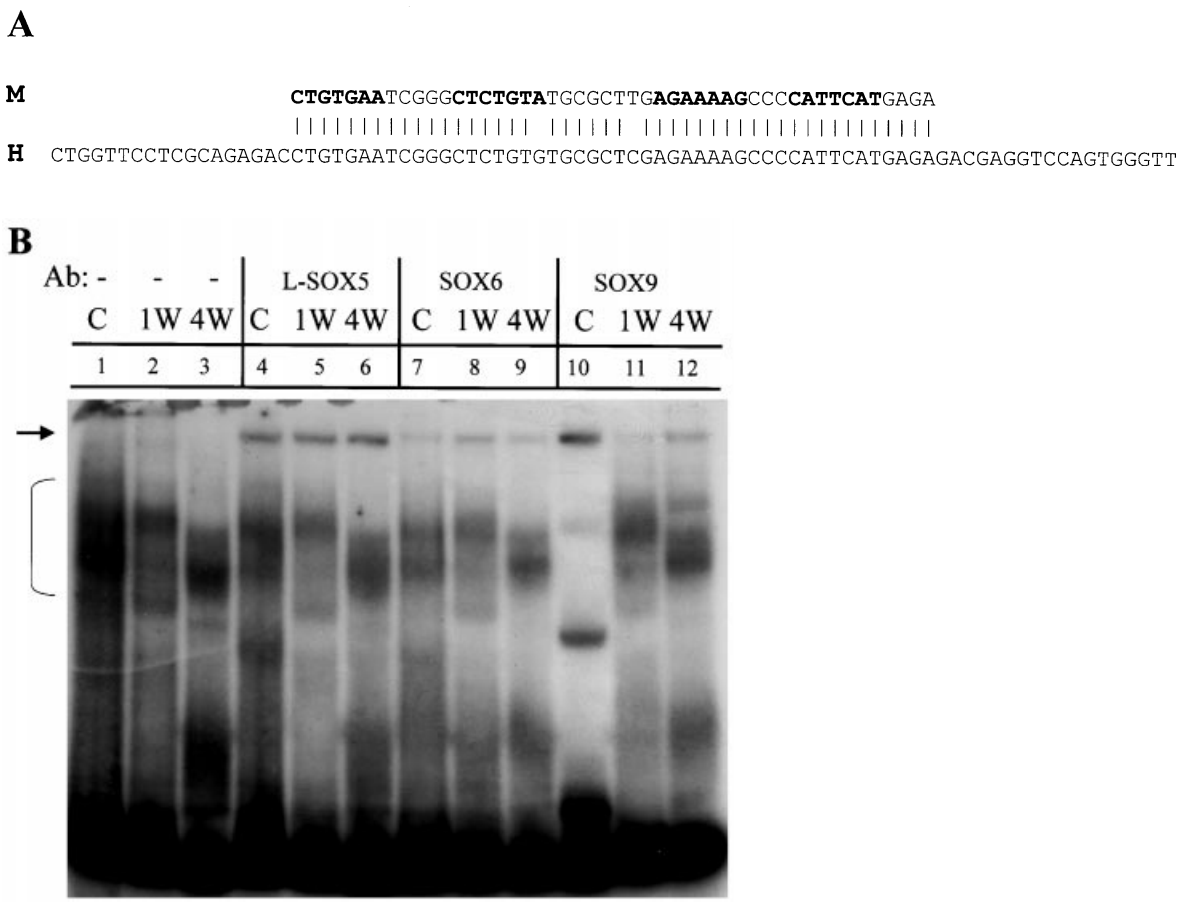


Figure 5 EMSAs and supershift assays of differentiated and de-differentiated human chondrocyte nuclear extracts with a *COL2A1* enhancer probe

(**A**) Sequence comparison of the 84 bp probe used in the EMSA shown in (**B**) from the human *COL2A1* enhancer (H) with the homologous region in the mouse *COL2A1* (M). The SOX transcription factor-binding sites are shown in bold. (**B**) Electrophoretic mobility-supershift assay with nuclear extracts from human fetal chondrocytes cultured on polyHEMA (C) or on tissue-culture plastic for either one (1W) or four 1-week passages (4W) with antibodies against L-SOX5, SOX6 and SOX9 and the probe depicted in (**A**). Nuclear protein (10 μg) from control chondrocytes cultured on polyHEMA-coated dishes or chondrocytes cultured on tissue-culture plastic for one or four 1-week passages was incubated with ^{32}P-labelled enhancer probe alone (lanes 1–3) or with anti-L-SOX5, SOX6 or SOX9 antibodies before addition of the labelled probe (lanes 4–12). Bracket, DNA–protein complexes; arrow, supershifted complexes.

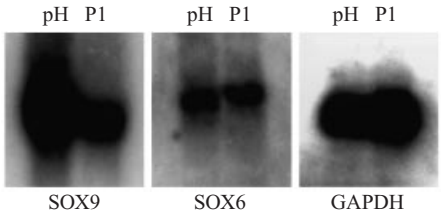


Figure 6 Northern-blot analysis of total RNA isolated from differentiated and de-differentiated human chondrocytes hybridized with cDNA probes for *SOX9* and *SOX6*

Total RNA was isolated from differentiated (pH) or de-differentiated (Pl) human fetal chondrocytes cultured on polyHEMA or tissue-culture plastic for 11 days, respectively. Each lane contained 10 μg of total RNA. The blot was also probed with a cDNA for GAPDH as a control.

P0 nuclear extract (Figure 5B, lane 10). The results indicate that the process of de-differentiation results in a changed pattern of nuclear-factor interaction with this region of the human *COL2A1* gene enhancer, involving differences in interaction with the SOX family of transcription factors. To support these results, SOX9 mRNA levels were examined by Northern-blot analysis of mRNA isolated from human fetal chondrocytes cultured as a monolayer or on polyHEMA for 11 days. The results showed a substantial decrease in the steady-state level of SOX9 transcripts in comparison with the levels found in chondrocytes cultured on polyHEMA-coated dishes for the same length of time (Figure 6).

Up-regulation of the *COL2A1* gene by expression of SOX proteins in de-differentiated chondrocytes

To determine whether the down-regulation of *COL2A1* gene expression observed in de-differentiated chondrocytes was due to the lack of availability of SOX transcription factors, de-differentiated bovine fetal chondrocytes were transiently transfected with expression constructs for L-SOX5, SOX6 and/or SOX9 along with a human *COL2A1* reporter construct containing the proximal promoter and the enhancer region (Figure 7B). As shown in Figure 7(A), fetal bovine chondrocytes exhibit the same dramatic down-regulation of type-II collagen mRNA levels as was observed in human fetal chondrocytes when they were grown as a monolayer. Fetal bovine chondrocytes were

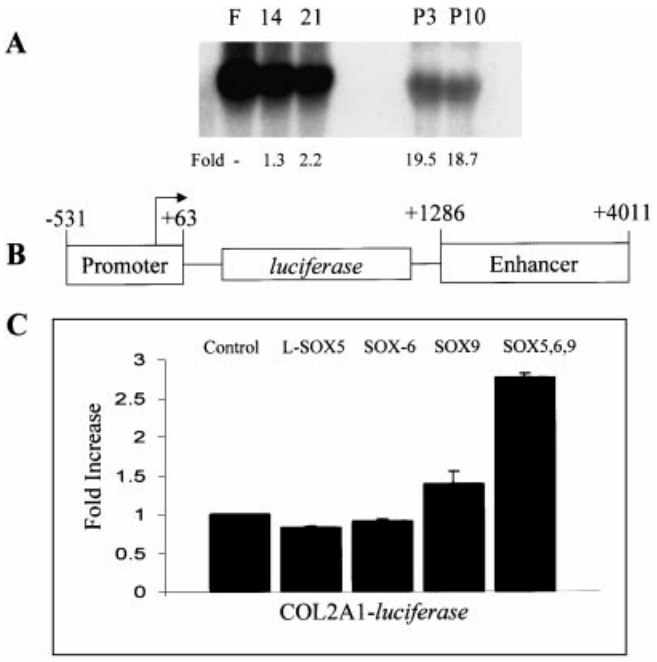


Figure 7 Effect of transient transfection of SOX expression plasmids on the expression levels of a human *COL2A1*–luciferase reporter construct in de-differentiated fetal bovine chondrocytes

(**A**) Northern-blot analysis of total RNA isolated from freshly isolated fetal bovine chondrocytes (F), cultured on polyHEMA-coated dishes for either 14 days (14) or 21 days (21), or fetal bovine chondrocytes cultured on plastic tissue-culture flasks for either three passages (P3) over 4 weeks or ten passages (P10) over 10 weeks. The blot was hybridized with cDNA probes for bovine type-II collagen and human GAPDH (results not shown). The numbers shown below the lanes represent the fold reduction in COL2A1 mRNA expression compared with freshly isolated fetal bovine chondrocytes after normalization to the GAPDH mRNA signal. (**B**) Schematic depiction of the human *COL2A1*–luciferase reporter construct. Numbers refer to nucleotide positions within the human *COL2A1* gene with reference to the transcription start site. (**C**) Quantification of luciferase activity from the transient-transfection analysis. The bar graph shows the fold difference in COL2A1–luciferase activity in the de-differentiated bovine fetal chondrocytes co-transfected with the indicated SOX expression vectors, either alone or together (SOX5,6,9).

grown as a monolayer for 8 weeks, co-transfected with the SOX expression constructs and the human *COL2A1*–luciferase reporter construct, and then 48 h later luciferase activity was determined. We observed a 2.5–3.5-fold increase in the levels of the *COL2A1*–luciferase reporter construct in the cells that were co-transfected with the L-SOX5, SOX6 and SOX9 expression constructs as compared with cells transfected with vector alone (Figure 7C).

DISCUSSION

The well-known instability of the chondrocyte phenotype remains a major problem that has hampered *in vitro* studies of chondrocyte gene expression and of their modulation by cytokines and growth factors, as well as the detailed study of pharmacological agents that may be capable of modulation of cartilage matrix production or that may potentially allow regeneration of cartilage. Indeed, a large number of studies that have examined the pharmacological modulation of chondrocyte functions or the effects of cytokines and growth factors on these cells have employed chondrocytes cultured as monolayers on plastic substrata that have presumably undergone variable (and therefore uncontrolled) levels of de-differentiation.

The results presented here document further the plasticity of the chondrocyte-specific phenotype *in vitro*. We have shown that the phenotype of human fetal epiphyseal chondrocytes can be modulated by culturing them as monolayers on plastic followed by culture on dishes coated with the hydrogel poly-HEMA. The polyHEMA culture system allows chondrocytes to maintain or re-acquire their spherical morphology and establish three-dimensional contacts with other cells, whereas when cultured on plastic, the cells attach to the substratum, spread and become fibroblastic in appearance [28,29]. The polyHEMA culture system allowed the re-acquisition of a chondrocyte-specific morphology and gene-expression pattern following one 4-week passage of culture on plastic. The ability of chondrocytes to recover their specific phenotype was diminished following two 4-week passages as a monolayer, and was almost totally lost after three 4-week passages. Other culture systems, such as alginate, agarose and certain artificial matrices, also allow re-differentiation of chondrocytes following culture in monolayer conditions on plastic [12,18,21–26]. However, few studies have systematically addressed the de-differentiation/re-differentiation process exhibited by human chondrocytes.

We have shown here that the decrease in expression of chondrocyte-specific genes observed in de-differentiating chondrocytes is largely controlled at the level of gene transcription. These findings support previous work by Maatta et al. [45], in which it was shown that de-differentiating human chondrocytes initiate expression of transcription factors that bind to an AP-1 (activator protein 1) site located in the first intron of the non-cartilage-specific *COL1A1* gene. Furthermore, they showed that a mutation in this AP-1-binding site resulted in substantially lower expression of a *COL1A1* reporter plasmid in the de-differentiating chondrocytes. These results suggest that up-regulation of genes associated with the de-differentiated phenotype such as those encoding the interstitial type-I and -III collagens may be mediated by an increase in the level of certain transcription factors as a mechanism for the phenotypic switch. In support of this notion, it has been shown previously that de-differentiating chondrocytes express greater levels of the transcription factors Sp1 and nuclear factor-κB and lower levels of C-Krox [29,46]. Interestingly, Sp1 is a positive regulator of the *COL1A1* gene, but the mechanism controlling the de-differentiation/re-differentiation process appears more complex since Sp-1 is known to also stimulate the expression of the human *COL2A1* gene [29,47,48]. Thus it is very likely that other transcription factors which control the chondrocyte-specific gene expression pattern are concomitantly down-regulated during de-differentiation. Here we have shown that the pattern of nuclear-factor binding to a region of the human *COL2A1* first-intron enhancer changes upon de-differentiation of the chondrocytes. Indeed, our studies indicate that these factors are SOX transcription factors (SOX6, SOX9 and L-SOX5), which have been implicated previously in the positive regulation of both mouse and human *COL2A1* genes through binding to the same region of the first-intron enhancer contained within the probe that we employed. These results are supported by the findings of Lefebvre and colleagues [33,34], who showed that mRNA for the SOX9 transcription factor is down-regulated during de-differentiation of mouse rib chondrocytes. We have also shown that expression of the chondrocyte-specific gene *COL2A1* can be activated in de-differentiated cells by ectopic expression of the SOX factors. Interestingly, our results showed that co-expression of three SOX transcription factors, L-SOX5, SOX6 and SOX9, was more effective than any of the transcription factors alone in activating a human *COL2A1* promoter/enhancer reporter construct in de-differentiated bovine chondrocytes. Elegant work by Lefebvre et

al. [34] has shown that L-SOX5 and SOX6 co-operate with SOX9 to activate the mouse *COL2A1* gene through the enhancer region. In agreement with our results, they showed that all three SOX factors were required for high-level activation of the *COL2A1* gene in both undifferentiated C3H101/2 mesenchymal cells and a SV40-transformed mouse embryonic limb cartilage cell line (MC615 cells) that had lost their chondrocyte phenotype through repeated passaging. These findings provide a better understanding of the regulation of the chondrocyte-specific phenotype, indicating that factors which modulate the expression of the *SOX* genes could enable a more efficient reversal to this phenotype. This is especially noteworthy since the maintenance of the chondrocyte-specific phenotype is of crucial importance to the preservation of the structure and biomechanical properties of cartilage, and alterations in the production of cartilage-specific collagens that occur when chondrocytes become de-differentiated will obviously affect severely the function of the tissue. Although there is abundant literature demonstrating a change in the pattern of extracellular matrix molecules synthesized by chondrocytes undergoing de-differentiation during culture as a monolayer on plastic, certain therapeutic approaches for treatment of articular cartilage defects incorporate an extended *ex vivo* expansion phase with multiple passages in monolayer cultures followed by re-implantation of the cells [49–52]. Furthermore, *ex vivo* expansion of chondrocytes followed by re-implantation of the cells is now being explored for treatment of degenerative joint diseases [49]. Therefore further study into the mechanisms that determine the chondrocyte-specific phenotype *in vitro* and into conditions that allow its preservation and recovery is needed to develop these therapies fully. Research aimed at elucidating these mechanisms and their control will undoubtedly lead to better ways to modulate the chondrocyte phenotype *in vitro* and thereby improve the ability to implement gene- and cell-therapy approaches for various heritable and acquired cartilage diseases.

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