# Cartilage oligomeric matrix protein (COMP) and collagen IX are sensitive markers for the differentiation state of articular primary chondrocytes

Frank ZAUCKE[1], Robert DINSER, Patrik MAURER and Mats PAULSSON

Institute for Biochemistry II, Medical Faculty, University of Cologne, Joseph-Stelzmann-Strasse 52, D-50931 Cologne, Germany

Primary chondrocytes dedifferentiate in serial monolayer with respect to their morphological and biosynthetic phenotype. They change from a round to a flattened fibroblast-like shape, and collagen I is secreted instead of the cartilage-specific collagen II. We analysed in detail the time course of dedifferentiation of mature bovine articular chondrocytes in monolayer for up to 32 weeks. Assessment of RNA expression by reverse transcription-PCR led to the identification of two novel phenotypical markers, the cartilage oligomeric matrix protein (COMP) and collagen IX, which are down-regulated faster than the widely accepted marker, collagen II. The different kinetics of COMP and collagen expression suggest differential regulation at the level of transcription. Immunostaining and metabolic labelling experiments confirmed the switch in the collagen expression pattern and the rapid down-regulation of *de novo* synthesis of COMP and collagen IX. Culture of chondrocytes in a three-dimensional matrix is known to stabilize the chondrocytic phenotype. We maintained cells for up to 28 weeks in an alginate bead system, which prevented dedifferentiation and led to a stabilization of collagen and COMP expression. Immunohistochemical analysis of the alginate beads revealed a similar distribution of matrix proteins to that found *in vivo*. Chondrocytes were transferred after a variable length of monolayer culture into the alginate matrix and the potential for redifferentiation was investigated. The re-expression of COMP and collagen IX was differentially regulated. The expression of COMP was re-induced within days after transfer into the three-dimensional matrix, while the expression of collagen IX was irreversibly down-regulated. In summary, these results demonstrate that the potential for redifferentiation decreases with increasing length of monolayer culture and show that the alginate bead system represents an attractive *in vitro* model to study the chondrocyte de- and re-differentiation processes, as well as extracellular matrix assembly.

Key words: alginate, dedifferentiation, monolayer, phenotype, redifferentiation.

## INTRODUCTION

Articular chondrocytes *in vivo* are embedded in a well organized extracellular matrix mainly consisting of collagens, proteoglycans and non-collagenous proteins. When articular chondrocytes are isolated by enzymic digestion and maintained in monolayer culture, their biosynthetic profile rapidly shifts to a fibroblast-like phenotype. Specifically, collagen I is synthesized instead of collagen II [1,2]. Freshly isolated chondrocytes are commonly cultured in three-dimensional matrices, such as agarose or alginate, to prevent the dedifferentiation induced by monolayer culture. This modification of culture conditions stabilizes the typical synthetic profile of fully differentiated chondrocytes [3–5].

Little is known about the time course of dedifferentiation in monolayer culture or about the reversibility of this process when chondrocytes are transferred into a three-dimensional matrix after prolonged periods in monolayer. The question of potential reversibility of dedifferentiation has some impact in cartilage tissue engineering and autologous chondrocyte transplantation where patient chondrocytes are amplified in monolayer culture before they are returned as a suspension [6]. The influence of a three-dimensional matrix is underscored in recent transplantation experiments in rabbits using chondrocytes embedded in different artificial polymer matrices, e.g. agarose and alginate [7,8]. To assess the differentiation status of chondrocytes used for these purposes there is still a need for the establishment of reliable and sensitive markers.

In the present study we have investigated the time course of dedifferentiation in monolayer culture and the potential for redifferentiation after transfer into an alginate matrix. This culture system has been reported to be effective in supporting the cartilaginous synthetic phenotype of bovine and human primary chondrocytes. Additionally, we searched for novel marker proteins which might complement or replace the classical chondrocyte differentiation marker, collagen II.

## EXPERIMENTAL

### Chemicals

Pronase (from *Streptomyces griseus*; Calbiochem, Bad Soden, Germany), collagenase P (from *Clostridium histolyticum*; Roche Diagnostics, Mannheim, Germany), chondroitinase ABC (Fluka, Deisenhofen, Germany) proteinase K, pepsin and testicular hyaluronidase (Sigma, Deisenhofen, Germany) were used for dissociation of cartilage slices and digestion of samples. Dulbecco's modified Eagle's medium (DMEM), methionine/cysteine-free medium, fetal calf serum (FCS) and gentamicin were purchased from Gibco (Eggenstein, Germany). Low viscosity alginate (Keltone™ LV) was kindly provided by Monsanto (Hamburg, Germany). Radiolabelling was performed using [$^{35}$S]-methionine (specific radioactivity of 43.5 TBq/mmol) and [$^{14}$C]-proline (specific radioactivity of 9.25 GBq/mmol) from NEN Life Science Products (Boston, MA, U.S.A.).

For immunostaining, antibodies directed against collagen I (Quartett GmbH, Berlin, Germany), collagen II (II-4C11; Calbiochem) and collagen IX (kindly provided by Dr P. Bruckner, Institute of Physiological Chemistry and Pathophysiology, Westfälische Wilhelms Universität Münster, Münster, Germany) were used. The polyclonal cartilage oligomeric matrix protein

Abbreviations used: AEC, 3-amino-9-ethylcarbazole; COMP, cartilage oligomeric matrix protein; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HRP, horseradish peroxidase; RT-PCR, reverse transcription-PCR; TBS, Tris-buffered saline.

[1] To whom correspondence should be addressed (e-mail frank.zaucke@uni-koeln.de).

(COMP) antiserum has been described previously [9]. Secondary horseradish peroxidase (HRP)-conjugated antibodies, and anti-mouse IgG Cy2- and anti-rabbit IgG Cy3-conjugated antibodies were purchased from Dako (Hamburg, Germany) and Jackson Immunoresearch Laboratories (distributed through Dianova, Hamburg, Germany) respectively. All other chemicals were of reagent grade and were purchased from several different companies.

### Isolation and culture of cells

Bovine humeroscapular joints of 18–24-month-old animals were obtained from the local slaugtherhouse. Articular cartilage slices were aseptically collected and chondrocytes were isolated by sequential digestion with Pronase (0.4 %; 1.5 h at 37 °C) and collagenase P (0.025 %; overnight at 37 °C) in DMEM containing 5 % (v/v) FCS and 50 $\mu$g/ml gentamicin. The isolated cells were then cultured either in monolayer cultures or in a three-dimensional alginate matrix.

For monolayer culture, chondrocytes were plated at a density of $3 \times 10^4$ cells/cm$^2$. After reaching confluency for the first time (within 6–7 days), cells were subcultured twice a week and grown in DMEM containing 100 $\mu$M ascorbate and 10 % (v/v) FCS.

Chondrocytes were encapsulated in alginate beads according to the method of Häuselmann et al. [4] immediately after isolation or after a variable length of monolayer culture. Briefly, cells were suspended in 1.2 % (w/v) sodium alginate in 0.15 M NaCl at a density of $4 \times 10^6$ cells/ml. The cell suspension was passed dropwise through a 22-gauge needle into a 102 mM $CaCl_2$ solution. Following 10 min of polymerization, beads were washed three times with 0.15 M NaCl, once with DMEM and were finally cultured in the same medium as for the monolayer culture. For analysis of different matrix compartments alginate beads were dissolved by adding solubilization buffer (55 mM sodium citrate and 0.15 M NaCl, pH 6.05) at 37 °C for 10 min. The cells were recovered by centrifugation and the supernatants (refered to as the citrate fraction) were collected.

### Reverse transcription-PCR (RT-PCR)

Monolayer cells were trypsinized and alginate beads were lysed in the solubilization buffer described above before centrifugation. Total RNA was obtained from the cell pellets by acid guanidinium thiocyanate/phenol/chloroform extraction [10]. RNA was quantified and analysed for impurities by UV spectroscopy. Reverse transcription was performed with 2 $\mu$g of RNA and 100 units of Superscript II with an oligo-(dT) primer according to the manufacturer's recommendations. The resulting cDNA (1 $\mu$l) was amplified by PCR using 2 units of Taq polymerase over 45 cycles. Conditions followed the recommendations of the manufacturer, except that 5 % (v/v) DMSO was added to the reaction. The annealing temperature was standardized to 48 °C. A RNA preparation without reverse transcription was always used as a negative control. In order to document the semi-quantitative nature of the RT-PCR, a dilution series of template cDNA was assayed and showed a dose–response relationship. Primer pairs for COMP and aggrecan were designed from the bovine sequences. The sequences of the COMP primers were 5′-AAG GAC ACA GAT AAG GAC GG-3′ and 5′-CAC TGT TGG GCA CTG TAG G-3′, and those of the aggrecan primers were 5′-ATC CCA AAA CGC CAC TCT GG-3′ and 5′-CTT GTG TCA CCA TCC ACT CC-3′. The primer sequences for the $\alpha$2 chain of collagen I and for collagen II have been published previously [11]. For collagen IX, a primer pair designed from the human $\alpha$1 chain sequence was used to obtain a short bovine sequence, from which the bovine primer pair 5′-ATG GCT GCG AGT CTG AAG C-3′ and 5′-TTT GGG ACC TCT TCA TGG G-3′ was derived. As a standard, tubulin primers with sequences 5′-GGA ACA TAG CCG TAA ACT GC-3′ and 5′-TCA CTG TGC CTG AAC TTA CC-3′ were used. In all cases, the identity of the resulting PCR bands was confirmed by sequencing.

### Metabolic labelling and immunoprecipitation of COMP

For metabolic labelling and subsequent immunoprecipitation, monolayer or alginate bead cultures were washed three times with DMEM to remove serum and equilibrated in methionine- and cysteine-free medium. Cells were labelled for 24 h with 50 $\mu$Ci/ml [$^{35}$S]methionine. After labelling, the medium was harvested, the beads were dissolved with citrate buffer as described above and the cells were extracted with lysis buffer [50 mM Tris/HCl (pH 8.0), 1 % (v/v) Nonidet P40 and 1 mM PMSF]. The different fractions were precleared by adding 25 $\mu$l of Protein A–agarose (Roche Diagnostics) for 2 h at 4 °C. Following centrifugation, pellets were collected and stored at −80 °C and the supernatants were incubated with 5 $\mu$l of COMP-specific polyclonal antiserum coupled to Protein A–agarose overnight at 4 °C. During the present study, we optimized the immunoprecipitation procedure by adding 10 % (w/v) BSA prior to incubation with the sample, thereby blocking the non-specific binding of extracellular proteins to Protein A (see Figure 3C). Following centrifugation the resulting pellets were washed three times with Tris-buffered saline (TBS) containing 0.5 % Triton X-100. Finally, reducing sample buffer was added to the pellets and the samples were boiled for 5 min. Following centrifugation, the supernatants were separated by SDS/PAGE [7.5 % (w/v) polyacrylamide] using recombinantly expressed rat COMP as a standard [9]. The gel was dried and the labelled proteins were visualized either by autoradiography or by using a PhosphorImager system (Molecular Dynamics, Krefeld, Germany).

### Metabolic labelling and determination of collagen I, II and IX proteins

For assessement of collagens I and II in monolayer culture, $0.5 \times 10^6$ cells were plated in a 6-well plate. The number of beads corresponding to $1 \times 10^6$ cells was determined for each alginate culture by use of DNA quantification [12] and were subsequently transferred to a 6-well plate. Cells were incubated with 20 $\mu$Ci of [$^{14}$C]proline/ml in fresh medium for 24 h at 37 °C, and medium was harvested. The remaining cells in monolayer culture were rinsed twice with PBS and subjected to digestion with pepsin (200 $\mu$g/ml in 0.5 M acetic acid) for 3 days at 4 °C. Alginate beads were washed with PBS and lysed in solubilization buffer as described above. The supernatant of the lysate was harvested and the pellet was digested with pepsin.

Collagens in the medium and in the citrate fraction were precipitated with 30 %-satd ammonium sulphate. The precipitate was resuspended in pepsin solution (200 $\mu$g/ml in 0.5 M acetic acid/0.2 M NaCl) and digested for 3 days at 4 °C, as above. Samples were dialysed against 0.1 M ammonium acetate and were cleaved using CNBr as described previously [13]. Formic acid and CNBr were added to the pepsin-digested samples at final concentrations of 70 % (v/v) formic acid and 50 mg/ml CNBr. The solution was deaerated by bubbling with nitrogen and was agitated at room temperature (23 ± 1 °C) for 16 h before freeze-drying. Freeze-dried samples were dissolved in reducing sample buffer and CNBr fragments were separated by SDS/PAGE on a 10–18 % (w/v) polyacrylamide gel. Fragment identity was determined by comparison with non-labelled standard collagens submitted to CNBr cleavage.

For analysis of collagen IX synthesis, $5 \times 10^6$ cells were plated in a 145 mm plate and cultured for the indicated times. Culture supernatants (10 ml) were precipitated with ammonium sulphate 3 days after cell passaging and were pepsin-digested as described above before separation by SDS/PAGE on a 10–18 % (w/v) polyacrylamide gel. The gel was then subjected to Western-blot analysis. A monoclonal anti-(collagen IX) antibody (B3-1; Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa City, IA, U.S.A.) was used at a 1:10 dilution. Recombinant human collagen IX, used as a standard, was a gift from Dr T. Pihlajamaa (Collagen Research Unit, Biocenter and Department of Medical Biochemistry, University of Oulu, Oulu, Finland).

### Immunofluorescence staining of monolayer chondrocytes

Cells were plated in chamber slides (Nunc, Wiesbaden, Germany) and grown for 3 further days under serum-free conditions. After fixation with 2 % (w/v) paraformaldehyde and permeabilization using 0.2 % Triton X-100 in PBS and three washes with PBS, the cultures were treated with 10 % (v/v) normal goat serum in PBS as a blocking reagent. The cells were incubated with primary antibodies at a dilution of 1:100 for 60 min, followed by detection with secondary Cy2-conjugated anti-mouse or Cy3-conjugated anti-rabbit IgG antibodies. The samples were finally mounted in DAKO® fluorescent mounting medium and examined under an Axiophot fluorescence microscope (Zeiss, Oberkochen, Germany).

### Immunohistochemistry of alginate bead cultures

Owing to autofluorescence, alginate beads cannot be stained using indirect immunofluorescence. Therefore collagens I, II and IX, and COMP were localized in alginate beads by HRP staining using the 3-amino-9-ethylcarbazole (AEC; Sigma) technique. The beads were embedded in paraffin after fixation with paraformaldehyde. All solutions used after removal of paraffin were supplemented with 5 mM $CaCl_2$. Slides with 10 $\mu$m sections were deparaffinized and treated with proteinase K (20 $\mu$g/ml in TBS) for collagen II staining, or pepsin [1 % (w/v) in 0.5 M acetic acid] for collagen I and collagen IX staining. After the digestion step, slides were fixed again with paraformaldehyde. Endogenous peroxidase activity was blocked with 1 % (v/v) $H_2O_2$ in methanol prior to incubation with 1 % (w/v) BSA in TBS. The antibodies directed against collagen II and collagen I were diluted 1:50 in TBS containing 1 % (w/v) BSA. The polyclonal antibody against collagen IX was used at a 1:100 dilution. No enzymic digestion was necessary before incubation with the COMP antiserum, which was diluted 1:100. Slides were treated with the primary antibody for 4–5 h. HRP-conjugated secondary antibodies (diluted 1:100) were added for a 1 h incubation period, and the sections were finally stained using the AEC protocol. In addition, proteoglycan synthesis was assessed by staining with Toluidine Blue.

## RESULTS

### Transcription of cartilage matrix protein genes during de- and redifferentiation of chondrocytes

The earliest change in gene expression during dedifferentiation in monolayer culture was the up-regulation of collagen I (Figure 1A). The appearance of the collagen I mRNA coincides with the start of proliferation of freshly isolated chondrocytes (results not shown). Cartilage-specific genes are down-regulated during the dedifferentiation process: the mRNAs for COMP and collagen IX were lost after 5–7 weeks of culture (Figure 1B). Collagen II mRNA could be detected for up to 9 weeks, but after 16 weeks this signal disappeared. A down-regulation of aggrecan transcription could not be observed (Figure 1B).

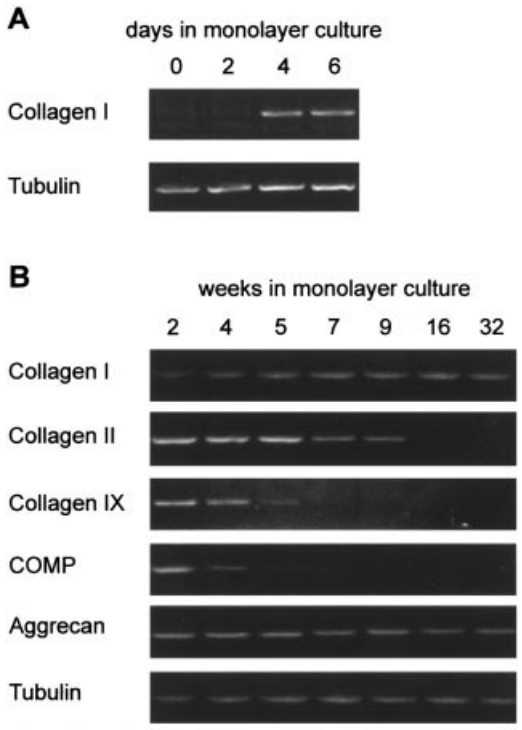


**Figure 1 Alterations in chondrocyte gene expression during monolayer culture for up to 32 weeks analysed by RT-PCR**

The time course of collagen I expression is shown in short-term (**A**) and long-term (**B**) monolayer culture. The expression pattern of the other investigated markers is presented only in long-term culture, since no changes were observed within days.

When chondrocytes were embedded in alginate directly after isolation, there was not any loss of expression of the cartilage-specific genes studied over 32 weeks of observation. However, *de novo* synthesis of collagen I mRNA could be detected (results not shown).

A delayed transfer of chondrocytes into alginate after 16 weeks of monolayer culture, when collagen II, collagen IX and COMP mRNA signals were not detectable any more (see Figure 1B), could partially reverse this dedifferentiation (Figure 2A). Within days, the mRNA for COMP was up-regulated. After 5 weeks, a weak signal for the collagen II mRNA reappeared. However, the redifferentiation was incomplete, in that the collagen I gene remained stably transcribed and the collagen IX gene transcription remained below the detection limit (Figure 2A). Transfer into alginate after only 9 weeks of monolayer culture, when the collagen II mRNA signal was still present, led to a conservation of collagen II transcription and the COMP mRNA signal reappeared as after 16 weeks of monolayer culture. The expression of collagen IX mRNA could be maintained by alginate culture only if the time in monolayer was less than 3 weeks (Figure 2B).

### Biosynthesis of COMP

To analyse the *de novo* synthesis of COMP, during the de- and redifferentiation processes, we used metabolic labelling of chondrocytes in both culture systems and subsequent immunoprecipi-

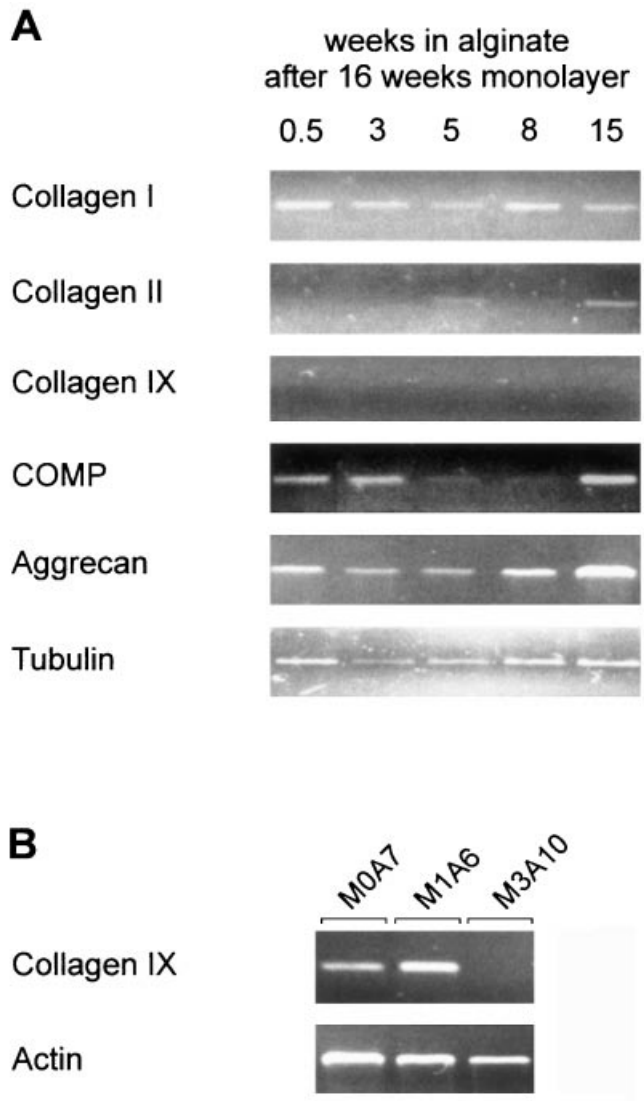


**Figure 2 Re-induction of chondrocyte-specific gene expression after transfer of monolayer chondrocytes into alginate cultures**

Gene expression was again investigated by RT-PCR. (**A**) Chondrocytes were maintained in monolayer for 16 weeks, then transferred into alginate beads and further cultured for the indicated times. (**B**) The time point of irreversible dedifferentiation with respect to collagen IX expression was investigated by embedding chondrocytes into alginate directly after isolation (M0A7), after 1 week of monolayer culture (M1A6) and after 3 weeks of monolayer culture (M3A10).

tation of cell culture supernatants and citrate fractions, followed by SDS/PAGE and autoradiography (Figure 3).

During dedifferentiation of chondrocytes, the *de novo* synthesis of COMP was abruptly down-regulated (Figure 3A). In cells grown exclusively in monolayer culture, COMP was found only during the first 2 weeks. COMP was detected not only in the cell culture supernatants but also in the corresponding cell extracts (results not shown). The specific COMP signal disappeared completely during the third week of culture (Figure 3A). The remaining bands represent non-specific binding of extracellular proteins to Protein A–agarose, which was used for immunoprecipitation.

In contrast, the expression of COMP was stabilized when freshly isolated chondrocytes were immediately embedded in alginate beads (Figures 3B and 3C). A stable synthesis of COMP was observed after 7 weeks in alginate culture and even in a long-term culture for up to 28 weeks. The analysis of different compartments revealed again that COMP could be detected in all fractions (Figure 3B), although the highest amount was clearly found in the cell culture supernatants.

In addition, the transfer of cells from monolayer to alginate culture resulted in a complete restoration of COMP expression (Figures 3B and 3C). In several independent experiments, COMP was never detected after 3 weeks of monolayer culture. However, transfer of chondrocytes cultured in monolayer for 3 or 4 weeks into the alginate bead system led to re-induction and stabilization of COMP expression within 10 and 24 weeks respectively (Figures 3B and 3C). The level of expression was then comparable with that observed in chondrocytes exclusively cultured in alginate beads.

### Biosynthesis of collagens

CNBr analysis of the collagens synthesized after 1 and 3 weeks of monolayer culture confirmed, at the protein level, the major shift in biosynthetic profile expected from the results obtained by RT-PCR (Figures 4A and 4B). While collagen II was almost exclusively present after 1 week as demonstrated by the presence of the collagen II-specific CB10 (II) and CB11 (II) cleavage products, it was completely replaced by collagen I after 3 weeks of monolayer culture (Figure 4A). At this time point, only the CNBr fragments CB7 (I) and CB8 (I), but not CB10 (II), were detected.

Alginate culture could stabilize the chondrocytic phenotype. When chondrocytes were embedded in alginate immediately after isolation, a strong signal for the CB10 (II) cleavage product was detected even after 15 weeks. However, at the position of the CB7 (I), CB8 (I) and CB11 (II) fragments, a broad band pattern was observed, suggesting that collagen I was also synthesized (Figure 4A). Embedding of chondrocytes into alginate after 1–10 weeks of monolayer culture resulted in only a partial restoration of the collagen synthesis pattern. While bands corresponding to the CB10 (II) fragment were detected after long-term culture in alginate irrespective of the length of the preceding monolayer culture, the major collagen synthesized was clearly collagen I, as demonstrated by the strong collagen I CB7 (I) and CB8 (I) signals (Figure 4A). The Western-blot analysis for collagen IX supports a down-regulation of collagen IX during prolonged monolayer culture (Figure 4B).

### Distribution of matrix proteins in monolayer cultures

Chondrocytes were processed for indirect immunofluorescence staining after 1 (Figures 5A–5C) and 4 (Figures 5D–5F) weeks of monolayer culture. After 1 week, the cells stained positively for collagen II. In some areas, even the formation of extracellular fibrillar structures was observed (Figure 5A). In contrast, collagen II was not detected after 4 weeks in monolayer culture (Figure 5D). Collagen I showed a complementary expression. After 1 week, antibodies against collagen I gave only a very weak background staining (Figure 5B), whereas after 4 weeks, nearly all cells were positively stained. The most intense signal was found intracellularly in a structure surrounding the nucleus (Figure 5E). A similar staining pattern was observed after 1 week in chondrocytes stained with an antibody directed against COMP. The strongest signal was again found close to the nucleus, but, in addition, a positive reaction was seen throughout the remaining cell body (Figure 5C). During serial monolayer culture the signal gradually disappeared until after 4 weeks only

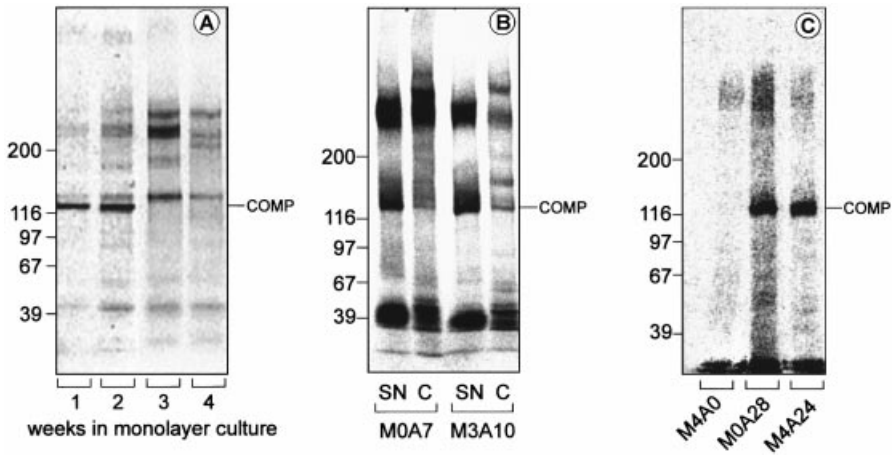


**Figure 3 Immunoprecipitation of COMP from [$^{35}$S]methionine-labelled chondrocytes grown in different culture systems followed by SDS/PAGE under reducing conditions**

(**A**) Chondrocytes were grown exclusively in monolayer for up to 4 weeks. Labelled cell culture supernatants were collected and precipitated as described in the Experimental section. (**B**) Chondrocytes were grown for up to 7 weeks exclusively in alginate cultures (M0A7) or were transferred after 3 weeks of monolayer culture into alginate for a further 10 weeks (M3A10). Cell culture supernatants (SN) and citrate fractions (C) after dissolution of the alginate cultures were analysed separately. (**C**) Chondrocytes were grown for 4 weeks in monolayer culture (M4A0) and then transferred into a long-term alginate culture for up to 24 weeks (M4A24) or cultured exclusively for 28 weeks in alginate (M0A28). Molecular-mass markers are indicated in kDa on the left-hand side of each panel.

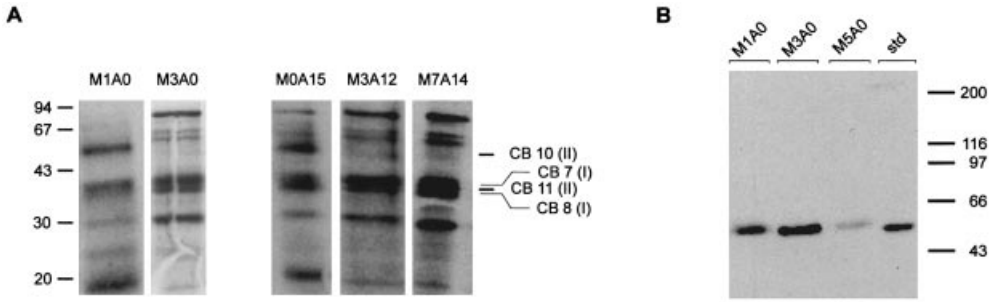


**Figure 4 SDS/PAGE analysis of [$^{14}$C]proline-labelled collagens in cell culture supernatants of chondrocytes cultured in monolayer and alginate culture**

(**A**) Cells were maintained in monolayer for 1 (M1A0) and 3 (M3A0) weeks or exclusively in the alginate system for up to 15 weeks (M0A15). In addition, monolayer cells were transferred after 3 (M3A12) and 7 (M7A14) weeks into alginate beads, in order to reveal a possible restoration of the collagen expression on protein level. Supernatants were precipitated by ammonium sulphate, pepsin-digested and cleaved by CNBr as described in the Experimental section. The samples were separated by SDS/PAGE [8–18% (w/v) polyacrylamide] under reducing conditions. Molecular-mass markers are indicated in kDa on the left-hand side. The positions of the unlabelled CB10 and CB11 fragments of collagen II, and the CB7 and CB8 fragments of collagen I are indicated on the right-hand side. (**B**) Expression of collagen IX detected by immunoblotting. Cells were cultured in monolayer culture for 1 (M1A0), 3 (M3A0) and 5 (M5A0) weeks. Cell culture supernatants were precipitated with ammonium sulphate and pepsin-digested before separation on a 6–16% (w/v) polyacrylamide gel under non-reducing conditions. Recombinant human collagen IX was used as a standard (std). Molecular-mass markers are indicated in kDa on the right-hand side.

occasional cells ($< 5\%$) were stained (Figure 5F). For collagen I and COMP no staining in the extracellular compartment could be observed at the investigated time points.

### Distribution of matrix proteins in alginate bead cultures

The collagen I signal obtained in immunolocalization studies was strongest in the chondrocytes embedded in alginate (Figure 6). There was no clear signal in the pericellular and interterritorial matrix. We could not detect a difference in collagen I distribution in cultures with a long monolayer period before transfer into alginate compared with cultures where cells were directly embedded into alginate (Figures 6E–6H). Occasionally, cells formed a monolayer around the alginate bead [4], which showed an intensive continuous staining for collagen I in contrast with the patchy staining within the alginate (results not shown).

The collagen II signal differed with the length of the monolayer period that preceded the alginate culture. When chondrocytes were directly embedded in alginate or transferred after 1 week, almost every cell appeared strongly positive and most cells were surrounded by a collagen II-containing matrix. However, when cells were cultured in monolayer for 3 weeks or more before transfer into alginate, the number of collagen II-positive cells decreased and the pericellular collagen II signal disappeared (Figures 6A–6D).

Distribution of collagen IX also depends on the length of the preceding monolayer culture. After short monolayer culture, the staining closely resembled the pattern observed for collagen II. When cells were embedded into alginate after 3 weeks of monolayer culture or later, only a few cells stained for collagen IX and there was no accumulation of collagen IX within the matrix. (Figures 6I–6L).

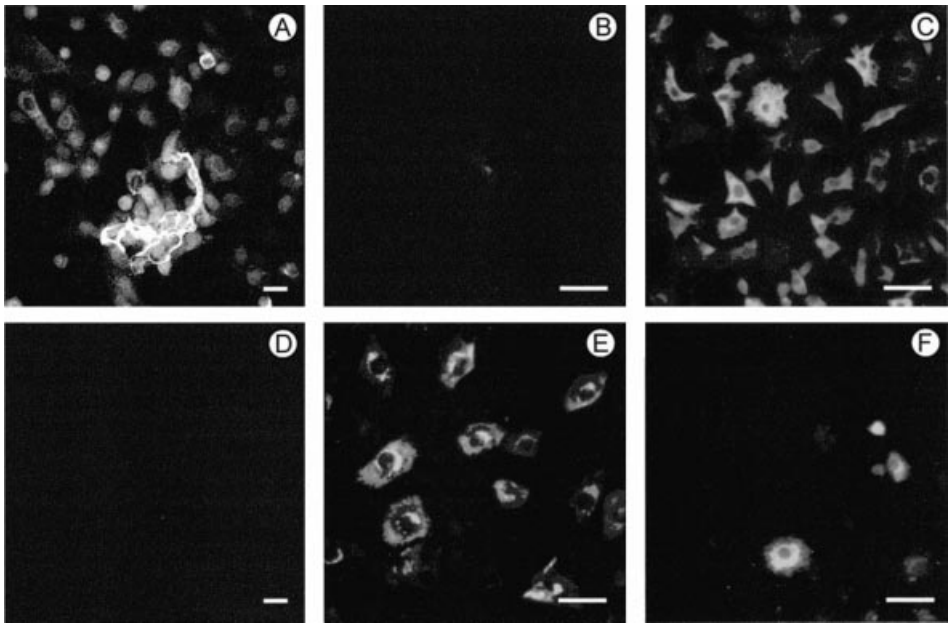


**Figure 5 Immunofluorescence staining of chondrocytes cultured in monolayer**

The cells were fixed and stained after 1 (**A**–**C**) and 4 (**D**–**F**) weeks, with specific antibodies directed against collagen II (**A** and **D**), collagen I (**B** and **E**) and COMP (**C** and **F**). In all of the panels the bar corresponds to 20 $\mu$m.

In contrast, the distribution of COMP was not affected by the length of time in monolayer culture preceding introduction into alginate. The staining was localized in the pericellular matrix and over the cell clusters with almost every cell in alginate being positive (Figures 6M–6P). Assessment of proteoglycan accumulation with Toluidine Blue yielded similar results, with strong pericellular staining around most cells irrespective of the duration of the monolayer phase (Figures 6Q–6T). Results of long-term culture over 5 or more months were qualitatively similar. The collagen II staining of chondrocytes directly embedded in alginate shifted more towards the interterritorial matrix, but was still most clearly seen in the cell clusters (results not shown).

## DISCUSSION

In the present paper we describe a detailed analysis of the time course of dedifferentiation of articular primary chondrocytes in monolayer culture using established and novel matrix proteins as indicators. Part of the changes induced by monolayer culture seem to be reversible after transfer of articular chondrocytes into a three-dimensional matrix.

Within days in monolayer culture, the collagen I mRNA becomes detectable, concordant to the cell flattening and start of proliferation of newly isolated chondrocytes. However, it has been demonstrated previously that there is no relationship between cell shape and expression pattern of collagens [14]. The gene transcription for the other classical marker of differentiation, collagen II, remains detectable for several months using RT-PCR. Two other major cartilage proteins, COMP and collagen IX are more sensitive to dedifferentiation than collagen II and disappear within 6 weeks at the RNA level. This suggests differential regulation at the level of transcription, which has also been described for the expression of the two closely related proteoglycans decorin and biglycan [15].

Analysis of protein secretion by metabolic labelling extends the results of the mRNA analysis. One week after the initiation of monolayer culture, the secretory phenotype corresponds completely to mature articular cartilage. Two weeks later, neither COMP nor collagen II is detectable any more. These results are corroborated by immunofluorescence microscopy, suggesting that the level of synthesis of the cartilage matrix proteins that we investigated is indeed very low after 3 weeks of culture.

COMP is one of the major non-collagenous proteins in cartilage [16], and the importance of COMP in cartilage development is underscored by the fact that mutations in COMP lead to skeletal dysplasias [17,18]. The down-regulation of collagens II and IX, but also COMP, might have severe consequences for the assembly, integrity and stability of the extracellular matrix synthesized. It has been shown previously that native COMP interacts with collagen I/II, procollagen I/II and collagen IX, and may play a role in fibril formation and maintenance of the extracellular collagen network [9,19].

For therapeutic applications, such as the engineering of cartilage transplants, articular chondrocytes have to be amplified in monolayer culture before they can be transferred into a three-dimensional matrix to generate a potentially cartilage-like tissue. The mostly disappointing results of autologous chondrocyte transplantation with the frequent formation of fibrocartilage instead of hyaline cartilage [20,21] suggest that the dedifferentiation due to preceding monolayer culture may not be reversible in the patient. Studies assessing the reversibility of the dedifferentiation induced by monolayer culture, whether *in vivo* or *in vitro*, must take into account that the complexity of the biosynthetic repertoire of chondrocytes is underestimated by sole measurement of collagen II.

To gain some insights into the significance of the change of gene expression that we observed, we attempted to redifferentiate chondrocytes by embedding them into alginate after different times of monolayer culture. Alginate has been shown to promote

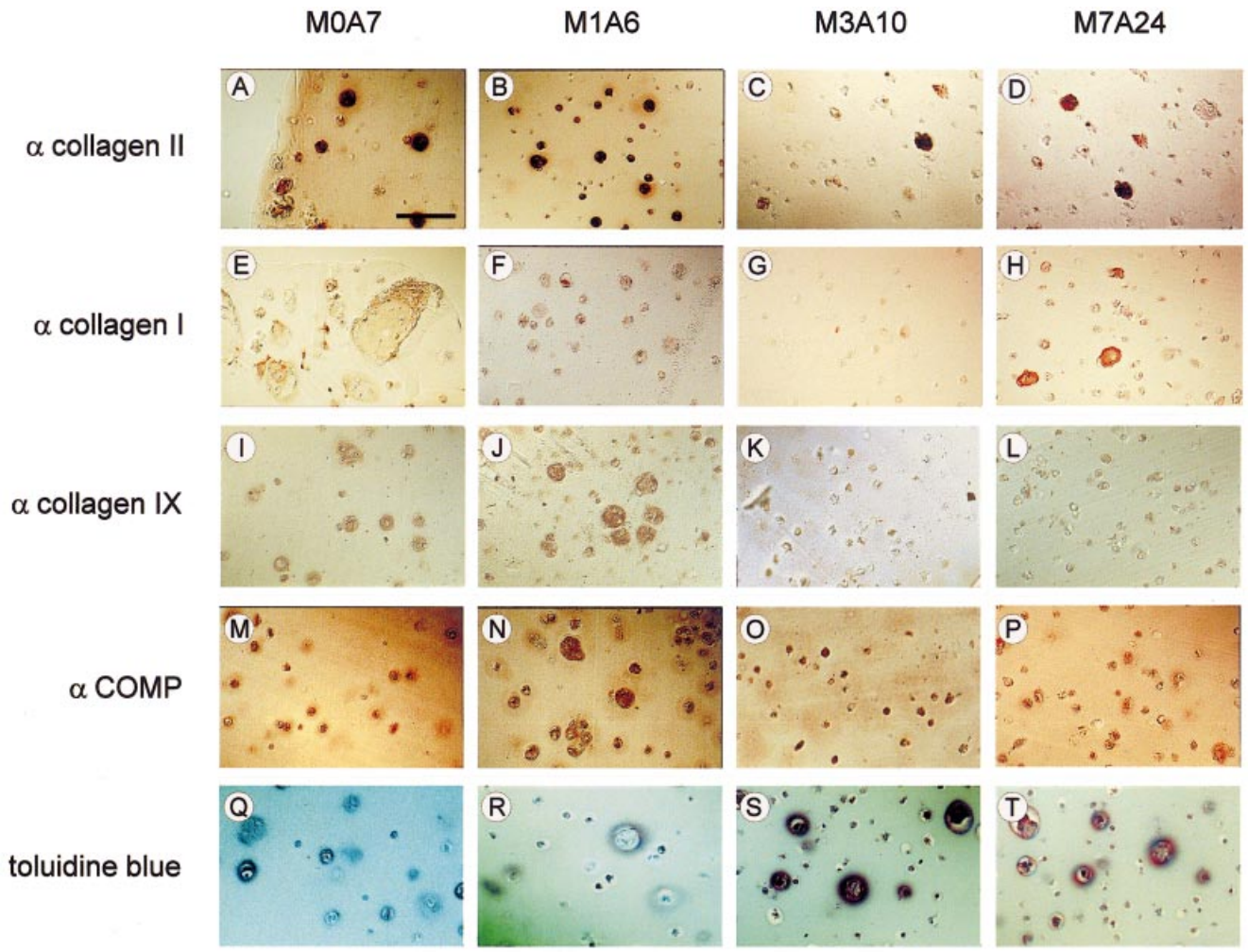


**Figure 6 Histological analysis of the synthetic repertoire of chondrocytes in alginate**

Articular chondrocytes were cultured exclusively in alginate, or in alginate after a preceding monolayer phase. Immunohistochemical analysis of cultures kept for 7 weeks in alginate (M0A7), 1 week in monolayer followed by 6 weeks in alginate (M1A6), 3 weeks in monolayer followed by 10 weeks in alginate (M3A10), and 7 weeks in monolayer followed by 24 weeks in alginate (M7A24) are shown. The sections were stained with antibodies directed against collagen II (**A**–**D**), collagen I (**E**–**H**), collagen IX (**I**–**L**), COMP (**M**–**P**), and with Toluidine Blue for proteoglycans (**Q**–**T**). Bar in (**A**) corresponds to 100 $\mu$m; all other images are of identical magnification.

the cartilaginous phenotype of articular chondrocytes from several species regarding collagen and proteoglycan expression and there is currently no superior three-dimensional culture system available [3–5].

We can confirm earlier reports that long-term culture in alginate stabilizes the differentiated phenotype of articular chondrocytes with respect to collagen II and collagen IX expression. Likewise, COMP expression is also preserved if cells are directly embedded into alginate after isolation. Even after extensive dedifferentiation in monolayer culture, the pattern of gene expression reverts to the differentiated state with respect to collagen II and COMP after transfer of chondrocytes into alginate. Again, these results are corroborated by metabolic labelling and immunohistochemistry.

While our data suggest that the potential for redifferentiation is not completely lost, even during prolonged monolayer culture, the degree of redifferentiation appears to be related to the length of the dedifferentiation period. The quantity of collagen II synthesized is irreversibly down-regulated if the monolayer period is extended beyond 1 week. Likewise, the immunohistochemical data suggest that the amount of collagen II and collagen IX incorporated into the pericellular matrix decreases with prolonged monolayer culture. This is in agreement with the finding that the number of collagen II-positive cells in alginate cultures decreases steadily with increasing length of the monolayer phase [22]. The critical influence of subculturing on the potential for redifferentiation has also been demonstrated for decorin and biglycan expression, which steadily declines with increasing passage number [15].

Both the changes in collagen I and collagen IX expression induced by monolayer culture seem to be irreversible. For collagen I it has been observed by others using both alginate and agarose as a supporting matrix that the exact quantity depends on the cell origin and culture conditions [4,23,24]. For all time periods investigated in the present study, collagen I was actively secreted by the chondrocytes in alginate, even though the immunohistochemical data suggest that it is poorly retained in the matrix. Small amounts of collagen I were also detected in freshly isolated chondrocytes that were cultured for 2 weeks in alginate in previous reports [4,24]. It was speculated that collagen

I is mainly produced by flattened cells and cell clusters at the surface of the beads, which we have also observed in our study. Once up-regulated in monolayer culture, at least 2 weeks in alginate culture was necessary for the complete suppression of collagen I synthesis in rabbit articular chondrocytes [25].

In the present study, collagens, proteoglycans and COMP are found in highest concentration in the pericellular compartment. The molecular assembly of the collagenous chondrocyte matrix is discussed controversially in the literature. The formation of collagen fibrils in the alginate system was analysed in several studies by electron microscopy and mostly a collagen meshwork surrounding the chondrocytes was demonstrated. In addition, a co-distribution of relevant chondrocyte collagens and collagen-specific cross-links surrounding chondrocytes very similar to the situation *in vivo* was found in alginate cultures [4,24,26,27]. In contrast, in alginate cultures containing chick embryo chondrocytes an abnormal collagen assembly was observed and the typical band pattern was missing, but, interestingly, this had no effects on the differentiation state, suggesting that the collagenous matrix is not essential for the phenotype [28].

At the mRNA level, our data imply that collagen IX synthesis once down-regulated is not up-regulated by changing culture conditions. It even appears that embedding chondrocytes into alginate does not halt the process of down-regulation of collagen IX. Possibly, the stimulating effect of the FCS that we used overrides the redifferentiation stimulus of the three-dimensional matrix. This may also explain the emergence or persistence of collagen I synthesis, even if collagen II synthesis could be preserved by rapid transfer of cells into alginate. As already mentioned, the embedding of chondrocytes in a three-dimensional matrix was sufficient to re-induce COMP synthesis immediately, independent of the length of the preceding monolayer phase, again suggesting a different regulatory mechanism. The potential for redifferentiation in general and especially the long-term stabilization of COMP expression within the alginate system provides an attractive *in vitro* model for the investigation of mutations involved in chondrodysplasias.

This work was supported by grants from the European Commission (BMH4-CT97-2437) and from the Köln Fortune programme of the Medical Faculty of the University of Cologne.

## REFERENCES


1 Abbott, J. and Holtzer, H. (1966) The loss of phenotypic traits by differentiated cells. III. The reversible behavior of chondrocytes in primary cultures. J. Cell Biol. **28**, 473–487

2 Benya, P. D., Padilla, S. R. and Nimni, M. E. (1978) Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. Cell (Cambridge, Mass.) **15**, 1313–1321

3 Bonaventure, J., Kadhom, N., Cohen-Solal, L., Ng, K. H., Lasselin, C. and Freisinger, P. (1994) Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. Exp. Cell Res. **212**, 97–104

4 Häuselmann, H. J., Fernandes, R. J., Mok, S. S., Schmid, T. M., Block, J. A., Aydelotte, M. B., Kuettner, K. E. and Thonar, E. J. (1994) Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. J. Cell Sci. **107**, 17–27

5 Liu, H., Lee, Y.-W. and Dean, M. F. (1998) Re-expression of differentiated proteoglycan phenotype by dedifferentiated human chondrocytes during culture in alginate beads. Biochim. Biophys. Acta **1425**, 505–515

6 Brittberg, M., Lindahl, A., Nilsson, A., Ohlsson, C., Isaksson, O. and Peterson, L. (1994) Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N. Engl. J. Med. **331**, 889–895

7 Rahfoth, B., Weisser, J., Sternkopf, F., Aigner, T., von der Mark, K. and Brauer, R. (1998) Transplantation of allograft chondrocytes embedded in agarose gel into cartilage defects of rabbits. Osteoarthritis Cartilage **6**, 50–65

8 Fragonas, E., Valente, M., Pozzi-Mucelli, M., Toffanin, R., Rizzo, R., Silvestri, F. and Vittur, F. (2000) Articular cartilage repair in rabbits by using suspensions of allogenic chondrocytes in alginate. Biomaterials **21**, 795–801

9 Thur, J., Rosenberg, K., Nitsche, D. P., Pihlajamaa, T., Ala-Kokko, L., Heinegård, D., Paulsson, M. and Maurer, P. (2000) Mutations in cartilage oligomeric matrix protein (COMP) causing pseudoachondroplasia and multiple epiphyseal dysplasia affect binding of calcium and collagen I, II and IX. J. Biol. Chem. **276**, 6083–6092

10 Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**, 156–159

11 Xu, C., Oyajobi, B. O., Frazer, A., Kozaci, L. D., Russell, G. G. and Hollander, A. P. (1996) Effects of growth factors and interleukin-1$\alpha$ on proteoglycan and type II collagen turnover in bovine nasal and articular chondrocyte pellet cultures. Endocrinology **137**, 3557–3565

12 Kim, Y. J., Sah, R. L., Doong, J. H. and Grodzinsky, A. J. (1988) Fluorimetric assay of DNA in cartilage explants using Hoechst 33258. Anal. Biochem. **174**, 168–176

13 O'Driscoll, S. W., Salter, R. B. and Keeley, F. W. (1985) A method for quantitative analysis of ratios of types I and II collagen in small samples of articular cartilage. Anal. Biochem. **145**, 277–285

14 von der Mark, K., Gauss, V., von der Mark, H. and Müller, P. (1977) Relationship between cell shape and type of collagen synthesized as chondrocytes lose their cartilage phenotype in culture. Nature (London) **267**, 531–532

15 Demoor-Fossard, M., Redini, F., Boittin, M. and Pujol, J. P. (1998) Expression of decorin and biglycan by rabbit articular chondrocytes. Effects of cytokines and phenotypic modulation. Biochim. Biophys. Acta **1398**, 179–191

16 Hedbom, E., Antonsson, P., Hjerpe, A., Aeschlimann, D., Paulsson, M., Rosa-Pimentel, E., Sommarin, Y., Wendel, M., Oldberg, Å. and Heinegård, D. (1992) Cartilage matrix proteins. An acidic oligomeric protein (COMP) detected only in cartilage. J. Biol. Chem. **267**, 6132–6136

17 Briggs, M. D., Hoffman, S. M., King, L. M., Olsen, A. S., Mohrenweiser, H., Leroy, J. G., Mortier, G. R., Rimoin, D. L., Lachman, R. S., Gaines, E. S. et al. (1995). Pseudoachondroplasia and multiple epiphyseal dysplasia due to mutations in the cartilage oligomeric matrix protein gene. Nat. Genet. **10**, 330–336

18 Hecht, J. T., Nelson, L. D., Crowder, E., Wang, Y., Elder, F. F., Harrison, W. R., Francomano, C. A., Prange, C. K., Lennon, G. G. M., Deere, M. and Lawler, J. (1995) Mutations in exon 17B of cartilage oligomeric matrix protein (COMP) cause pseudoachondroplasia. Nat. Genet. **10**, 325–329

19 Rosenberg, K., Olsson, H., Mörgelin, M. and Heinegård, D. (1998) Cartilage oligomeric matrix protein shows high affinity zinc-dependent interaction with triple helical collagen. J. Biol. Chem. **273**, 20397–20403

20 Nehrer, S., Spector, M. and Minas, T. (1999) Histologic analysis of tissue after failed cartilage repair procedures. Clin. Orthop. **365**, 149–162

21 Peterson, L., Minas, T., Brittberg, M., Nilsson, A., Sjogren-Jansson, E. and Lindahl, A. (2000) Two- to 9-year outcome after autologous chondrocyte transplantation of the knee. Clin. Orthop. **374**, 212–234

22 Marijnissen, W. J., van Osch, G. J., Aigner, J., Verwoerd-Verhoef, H. L. and Verhaar, J. A. (2000) Tissue-engineered cartilage using serially passaged articular chondrocytes. Chondrocytes in alginate, combined *in vivo* with a synthetic (E210) or biologic biodegradable carrier (DBM). Biomaterials **21**, 571–580

23 Benya, P. D. and Shaffer, J. D. (1982) Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell (Cambridge, Mass.) **30**, 215–224

24 Petit, B., Masuda, K., D'Souza, A. L., Otten, L., Pietryla, D., Hartmann, D. J., Morris, N. P., Uebelhart, D., Schmid, T. M. and Thonar, E. J. (1996) Characterization of crosslinked collagens synthesized by mature articular chondrocytes cultured in alginate beads: comparison of two distinct matrix compartments. Exp. Cell Res. **225**, 151–161

25 Lemare, F., Steimberg, N., Le Griel, C., Demignot, S. and Adolphe, M. (1998) Dedifferentiated chondrocytes cultured in alginate beads: restoration of the differentiated phenotype and of the metabolic response to interleukin-1$\beta$. J. Cell. Physiol. **176**, 303–313

26 Häuselmann, H. J., Masuda, K., Hunziker, E. B., Neidhart, M., Mok, S. S., Michel, B. A. and Thonar, E. J. (1996) Adult human chondrocytes cultured in alginate form a matrix similar to native human articular cartilage. Am. J. Physiol. **271**, C742–C752

27 Loty, S., Sautier, J. M., Loty, C., Boulekbache, H., Kokubo, T. and Forest, N. (1998) Cartilage formation by fetal rat chondrocytes cultured in alginate beads: a proposed model for investigating tissue-biomaterial interactions. J. Biomed. Mater. Res. **42**, 213–222

28 Gregory, K. E., Marsden, M. E., Anderson-MacKenzie, J., Bard, J. B., Bruckner, P., Farjanel, J., Robins, S. P. and Hulmes, D. J. (1999) Abnormal collagen assembly, though normal phenotype, in alginate bead cultures of chick embryo chondrocytes. Exp. Cell Res. **246**, 98–107


Received 3 April 2001/25 May 2001; accepted 19 June 2001