


Elevated Levels of Cartilage Oligomeric Matrix Protein during *In Vitro* Cartilage Matrix Generation Decrease Collagen Fibril Diameter

Y.M. Bastiaansen-Jenniskens,^{1,2} A.C.W. de Bart,¹
W. Koevoet,³ K.M.B. Jansen,⁴ J.A.N. Verhaar,²
G.J.V.M. van Osch,^{2,3} and J. DeGroot¹

Cartilage
1(3) 200–210
© The Author(s) 2010
Reprints and permission:
sagepub.com/journalsPermissions.nav
DOI: 10.1177/1947603510361238
http://cart.sagepub.com


Abstract

Cartilage oligomeric matrix protein (COMP) is a protein present in the cartilage matrix and is expressed more abundantly in osteoarthritis cartilage than in healthy cartilage. The present study was designed to investigate the effect of transforming growth factor β (TGF β) on COMP deposition and the influence of COMP on collagen biochemistry in a long-term 3-dimensional culture. Bovine chondrocytes in alginate beads were cultured with or without 25 ng/mL TGF β 2 for 21 or 35 days. COMP was overexpressed in bovine chondrocytes using lentiviral transfection. COMP gene expression, COMP protein production, collagen and proteoglycan deposition, and collagen fibril thickness were determined. Addition of TGF β 2 resulted in more COMP mRNA and protein than the control condition without growth factors. Lentiviral transduction with COMP resulted in elevated gene expression of COMP and increased COMP levels in the alginate bead and culture medium compared to untransfected cells. Overexpression of COMP did not affect the deposition of collagen, collagen cross-linking, proteoglycan deposition, or the mechanical properties. Stimulating COMP production by either TGF β 2 or lentivirus resulted in collagen fibrils with a smaller diameter. Taken together, COMP deposition can be modulated in cartilage matrix production by the addition of growth factors or by overexpression of COMP. Inducing COMP protein expression resulted in collagen fibrils with a smaller diameter. Because it has been demonstrated that the collagen fibril diameter is associated with mechanical functioning of the matrix, modulating COMP levels should be taken into account in cartilage regeneration strategies.

Keywords

cartilage oligomeric matrix protein, cartilage matrix, chondrocyte, lentivirus, transforming growth factor β

Introduction

Cartilage oligomeric matrix protein (COMP) is a homopentameric protein of 520 to 550 kDa with subunits of 100 to 120 kDa.¹ COMP was originally found in cartilage² but is also present in a large variety of other tissues such as tendon,³ synovium, and skin.⁴ The function of COMP in the formation of a cartilage matrix is not completely understood. It is known that COMP interacts with several components of the extracellular matrix such as collagen type I, II,⁵ and IX⁶ and the noncollagenous proteins matrilin (MATN) 1, 3, and 4 and aggrecan.^{7,8}

A large number of COMP mutations has been identified, some causing phenotypes ranging from severe pseudoachondroplasia (PSACH) to mild multiple epiphyseal dysplasia (MED)⁹ depending on the location of the mutation. Because COMP interacts with other matrix proteins, the

mutations in the *comp* gene resulting in retention in the endoplasmatic reticulum of the cell often also lead to altered secretion of collagen type IX and matrilin 3. Secretion of collagen type II is less affected by COMP mutations.^{6,10,11}

¹Business Unit BioSciences, TNO Quality of Life, Leiden, the Netherlands

²Department of Orthopaedics, University Medical Center Rotterdam, Erasmus Medical Center, Rotterdam, the Netherlands

³Department of Otorhinolaryngology, University Medical Center Rotterdam, Erasmus Medical Center, Rotterdam, the Netherlands

⁴Department of Precision and Microsystems Engineering, Faculty of Mechanical, Maritime and Materials Engineering, Delft University of Technology, Delft, the Netherlands

Corresponding Author:

Yvonne Bastiaansen-Jenniskens, Department of Orthopaedics, Erasmus MC University Hospital, Dr. Molewaterplein 50, 3015 GE Rotterdam, the Netherlands
Email: y.bastiaansen@erasmusmc.nl

In addition, COMP mutations result in alterations in cartilage matrix assembly and reduced interaction between chondrocytes and COMP, which could also contribute to the PSACH or MED phenotype.¹² Surprisingly, complete absence of COMP *in vivo* does not result in morphological or anatomical changes and does not lead to any signs of PSACH or MED.¹³ Recently, COMP was found to influence the fibril formation of collagen type I and II, leading to quicker fibrillogenesis and better organized collagen fibrils *in vitro* depending on the ratio between COMP and collagen. However, COMP was not associated with mature collagen fibrils, suggesting a role as catalyst in fibrillogenesis for COMP.¹⁴ Without the presence of COMP, collagen fibrils were formed slower and with a larger diameter.¹⁴

The distribution of COMP within healthy cartilage changes in time. In young cartilage, COMP is uniformly present in the superficial layers of the cartilage, whereas in the middle layer, the location is more territorial (around the chondrocytes), shifting with age toward a more interterritorial distribution.¹⁵ In degenerating cartilage, as seen in osteoarthritis (OA), COMP is mainly present in the pericellular matrix of cell clusters as a result of reactivation of COMP synthesis.^{16,17} Here, COMP is mainly located on the collagen fibers, whereas in healthy cartilage, almost no COMP is associated with collagen fibers.¹⁶ Besides the shift in matrix distribution, gene expression and protein expression are also increased in OA when compared to healthy cartilage.^{16,18} The interactions between COMP and other matrix components and the shift of COMP distribution in OA cartilage to a more immature distribution pattern, in a possible attempt to repair the cartilage, suggest that COMP may have a role in the development of articular cartilage.

The production of COMP can be regulated by growth factors. Recklies et al. found that synthesis of COMP by articular chondrocytes in a short monolayer culture was induced very strongly by transforming growth factor (TGF) β .¹⁹ TGF β is often mentioned to play a role in OA²⁰ and is often used to induce a chondrogenic cell phenotype or enhance extracellular matrix production. Because of the possible role of COMP in the cartilage matrix production, assembly, and regeneration, the goal of the present study was to examine the role of COMP during cartilage matrix generation *in vitro* in a long-term 3-dimensional culture. Specifically, we hypothesize that TGF β will increase COMP gene expression and protein production by isolated chondrocytes and consequently have an effect on extracellular matrix assembly, and functional properties of the generated matrix in a long-term 3-dimensional culture were examined. From our earlier studies, we know that TGF β results in less proteoglycan deposition, less collagen deposition, and fewer cross-links per collagen molecule,^{21,22} making it difficult to draw hard conclusions on the possible effects on COMP on these parameters. Therefore, we also

overexpressed COMP in primary chondrocytes using lentivirus for stable integration.

Methods

Cell Culture

Articular cartilage was harvested from metacarpophalangeal joints of calves aged 6 to 12 months. Full-thickness slices of noncalcified articular cartilage were subjected to pronase (2 mg/mL) (Sigma, St. Louis, MO) digestion for 2 hours followed by overnight collagenase B (1.5 mg/mL) (Roche Diagnostics, Basel, Switzerland) digestion. Chondrocytes were resuspended in 1.2% (w/v) low viscosity alginate (Keltone, NutraSweet, Surrey, UK) in 0.9% NaCl (Sigma) at a concentration of 4×10^6 cells/mL, and beads were made as described previously.²³ Briefly, the cell-alginate suspension was pressed through a 22-gauge needle in 105 mM CaCl₂. Beads were washed with 0.9% NaCl and Dulbecco's Modified Eagle Medium (DMEM)/F12 (GibcoBRL, Gaithersburg, MD) and inspected visually. Beads that appeared smaller or larger were not included in the experiment. The average volume of the alginate beads was 15 μ L. After transfer to a 6-well plate (BD Falcon, Bedford, MA), they were cultured in 75 μ L culture medium per bead. DMEM/F12 was used as culture medium supplemented with 10% fetal bovine serum (GibcoBRL), 50 μ g/mL L-ascorbic acid 2-phosphate (Sigma), 50 μ g/mL gentamicin (GibcoBRL), and 1.5 μ g/mL fungizone (GibcoBRL). Chondrocytes were cultured with and without 25 ng/mL TGF β 2 (recombinant human) (R&D Systems, Abington, UK) and harvested after 11 and 21 days of culture. TGF β 2 and its concentration was chosen based on our own and others' previous results.^{21,22,24,25} In previous studies, the 3 isoforms of TGF β were compared *in vitro*, and significant differences between the isoforms were never observed.²⁶

For experiments with lentivirus, chondrocytes were incubated with or without virus for 4 hours prior to suspension in alginate and cultured for 11, 21, or 35 days. Culture medium was changed 3 times per week.

COMP and GFP Lentivirus Production

A 4-plasmid expression system was used to generate lentiviral vectors by transient transfection. The 4 plasmids were the following: 1) the expression plasmid (pLV), in which human COMP or green fluorescent protein (GFP) cDNA was fused to a CMV promoter. For this, COMP in the pCMV6-XL5 vector based on NM_000095.2 (Origene, Rockville, MD) was cut, and CMV and COMP were ligated into the pLV expression plasmid. 2) The packaging plasmid pLP1 encoding Gag and Pol, 3) the packaging plasmid pLP2 encoding Rev, and 4) the envelope plasmid pLP/

VSVG for surface proteins. Lentiviral vectors were produced by transfection of plasmid DNA into 293FT cells using the transfection agent Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transfections were done in 75 cm² flasks using 9 µg expression plasmid, 6.4 µg of pLP1, 4.8 µg of pLP2, and 3.4 µg of pLP/VSVG. Medium was changed after 24 hours and collected after an additional 48 hours. The medium was filtered and concentrated by ultracentrifugation. The p24 ELISA (Gentaur, Brussels, Belgium) was used according to the manual to determine the number of infectious units. Cell transduction was performed by incubating bovine chondrocytes with COMP or GFP lentivirus at an MOI of 0.75 for 4 hours prior to suspending the chondrocytes in alginate and making beads.

Determination of Transfection Efficiency

Chondrocytes infected with GFP were recovered from alginate beads using 75 µL per bead of 55 mM sodium citrate and 20 mM ethylene diaminetetraacetate (EDTA) in 150 mM NaCl for 20 minutes at room temperature. After washing twice in PBS, chondrocytes were resuspended in PBS with 0.1% FCS. Analysis of transfection efficiency was performed using a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). A minimum of 10,000 cells per sample was collected. Data acquisition and analysis were performed using CellQuest 3.3 (Becton Dickinson, Franklin Lakes, NJ).

RNA Isolation and Quantitative RT-PCR

For total RNA isolation, alginate beads were dissolved in 150 µL/bead sodium citrate buffer. After centrifugation, cell pellets were suspended in 1000 µL RNA-Bee™ (TEL-TEST Inc., Friendswood, TX) and subsequently precipitated with 2-propanol and purified with lithium chloride. Total RNA was quantified using Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and 500 ng total RNA of each sample was reverse transcribed into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). Quantitative real-time PCR was performed using the ABI Prism 7000 Sequence Detection system (Applied Biosystems, Foster City, CA) for quantification using TaqMan (Applied Biosystems) and SYBR Green (Eurogentec, Maastricht, The Netherlands) and melting curve analysis. Primer sequences for the genes were as follows: for glyceraldehyde-3-phosphate dehydrogenase (GAPDH reference gene) forward: GTCAACGGATT TGGTCGTATTGGG, reverse: TGCCATGG GTGGAATCATATTGG, and probe: Fam-TGGCGCCCCAACCCAGCC-Tamra. For the COMP assay specific for bovine, reverse: TCTGATCTGAGTTGG GCACCTT, forward: CCAGAAGAACGACGACCAGAA. For the COMP assay specific for humans, a TaqMan gene expression assay was used (Applied Biosystems). PCR

conditions were as follows: 2 minutes at 50 °C and 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C, with data collection during the last step. For GAPDH and human COMP, TaqMan 2x Universal PCR Mastermix (Roche Diagnostics) was used in the reaction. For bovine COMP, qPCR Mastermix Plus SYBR Green I (Eurogentec, Seraing, Belgium) was used in the reaction. All PCRs were performed in a total volume of 25 µL. Relative quantification of PCR signals was performed by comparing the threshold cycle value (Ct), in duplicate, for the gene of interest in each sample with the Ct value for the reference gene GAPDH. Quantitative PCR analysis of each sample was performed in duplicate.

Isolation of Cell-Associated Matrix

Alginate beads were dissolved by adding 75 µL per bead of sodium citrate buffer for 20 minutes at room temperature. The suspension was centrifuged for 10 minutes at 1000 rpm (Eppendorf, Hamburg, Germany) to separate cells surrounded by its cell-associated matrix (CM, the pellet) from components originating predominantly from the “interterritorial” or further removed matrix (FRM, the supernatant) from the cells as described previously.^{23,27}

Biochemical Assays

Alginate beads or separated CM and FRM were digested overnight at 56 °C in papain buffer (250 µg/mL papain in 50 mM EDTA and 5 mM L-cysteine). Glycosaminoglycan (GAG) amount in the digest was quantified using dimethylmethylene blue (DMB) assay at a pH of 1.75. By lowering the pH of the DMB solution from 3.0 to 1.75, carboxyl groups of the alginate are protonated while sulfated GAGs can still form complexes with DMB.²⁸ The metachromatic reaction of GAG with DMB was monitored with a spectrophotometer, and the ratio $A_{530}:A_{590}$ was used to determine the amount of GAG present, using chondroitin sulfate C (Sigma) as a standard. High-performance liquid chromatography (HPLC) of amino acids (hydroxyproline, Hyp) and collagen cross-links (hydroxylysylpyridinoline, HP) was performed by the methods of Bank.²⁹ The quantities of cross-links were expressed as the number of residues per collagen molecule, assuming 300 Hyp residues per collagen triple helix.

COMP Detection with ELISA

To analyze the produced extracellular matrix and the culture medium for COMP, alginate beads were dissolved in the sodium citrate buffer. To block proteolysis, the broad-spectrum protease inhibitor Complete (Roche Diagnostics) dissolved in PBS was added to the alginate and culture medium samples. A COMP ELISA (Anamar, Göteborg,

Sweden) was used to quantify the COMP content according to the manual.

Mechanical Testing

For mechanical characterization, we used 4×10^6 cells/mL in 1.2% (w/v) alginate constructs that were 3 mm thick and 6 mm in diameter. Constructs were prepared as previously described.³⁰ After 35 days of culture, constructs were mounted on the Dynamic Mechanical Analyzer DMA Q800 (TA Instruments, New Castle, DE) and tested in a radially unconfined stress relaxation test as described previously.³¹ The secant modulus is related to the interaction between the solid and the liquid phase and is therefore an indication for the ability to hold water. The modulus measured at equilibrium depends strongly on the compressive stiffness of the (cartilagenous) solid matrix.³²

Electron Microscopy and Fibril Measurements

After 21 days of culture, beads were rinsed 3 times in PBS, fixed for 2 hours at room temperature in 0.1 M sodium cacodylate-buffered 1.5% glutaraldehyde (EM grade) (Sigma) and 1% paraformaldehyde, pH 6.7, and then rinsed 3 times in 0.15 M sodium cacodylate. After postfixation for 2 hours in 0.1 M sodium cacodylate-buffered 1% osmium tetroxide (OsO₄), pH 6.7, beads were dehydrated in a series of graded acetone and embedded in LX 112 (Epon, Ladd Research, Williston, Vermont, USA). Ultrathin sections (LKB Ultratome IV, LKB, Stockholm, Sweden) were mounted on copper grids (300 mesh) and contrasted with 2% uranyl acetate (10 minutes at 45 °C) and lead citrate. They were examined with a Zeiss 902 electron microscope (Oberkochen, Germany). Pictures were taken in the pericellular region at 2,800 \times , 18,000 \times , and 89,000 \times magnification. Four pictures per sample (89,000 \times magnification) were used to measure collagen fibril thickness with ImageJ 1.40g (National Institutes of Health, Bethesda, Maryland, USA). In each picture, all collagen fibrils (excluding noncollagen fibers and histology artifacts) were measured.

Statistical Analysis

The experiments were repeated 3 times. Each experiment consisted of 3 times 7 beads per experimental condition for biochemical analyses and 2 samples of 10 beads per experimental condition for gene expression analysis. Experiments for mechanical testing were repeated 2 times with a total of 6 samples per group. Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA) software. All data are presented as mean \pm standard deviation. Control groups without TGF β or lentivirus and groups supplemented with TGF β or transfected with COMP lentivirus were compared with a Mann-Whitney test.

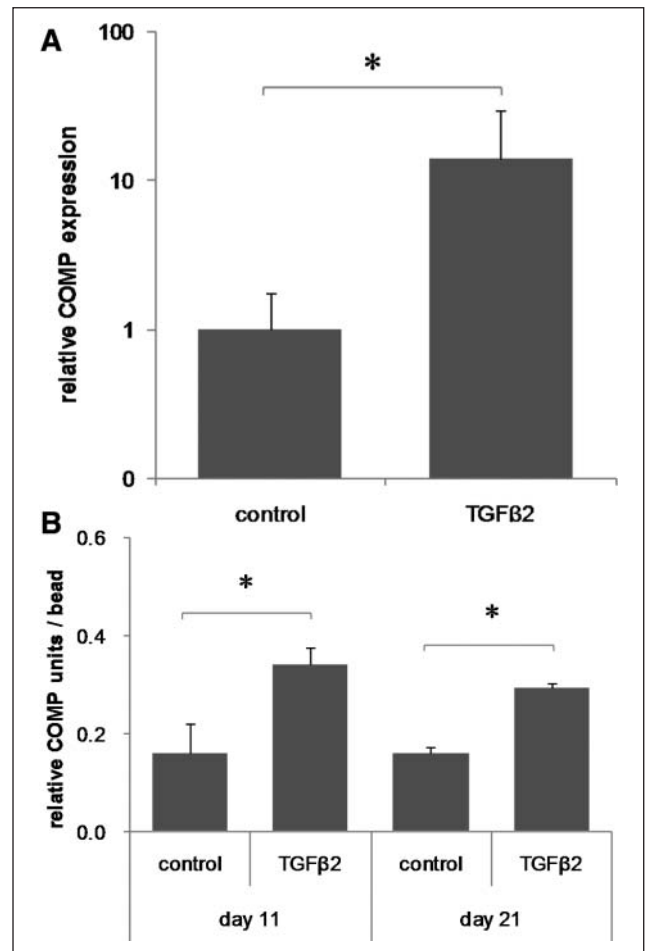


Figure 1. The effect of 25 ng/mL TGF β 2 on COMP gene and protein expression. Gene expression of bovine COMP is shown (A) by expression after culturing for 11 days relative to the control without growth factors, which is set at 1 ($n = 6$). (B) Protein deposition of COMP in the alginate beads is shown in relative COMP units as determined with ELISA ($n = 9$). *Significant difference ($P < 0.05$) between growth factor and control without growth factor.

Results

COMP Expression in Presence of TGF β

The presence of TGF β 2 increased the expression 14-fold after 11 days of culture (Fig. 1A). COMP protein deposition in the alginate bead, quantified with ELISA, increased approximately 2-fold after the addition of TGF β 2 (Fig. 1B).

Distribution of Matrix Components after Addition of TGF β 2

The distribution of COMP, collagen, and proteoglycans within the alginate bead after 21 days of culture was determined by separation of the CM and FRM. In both the

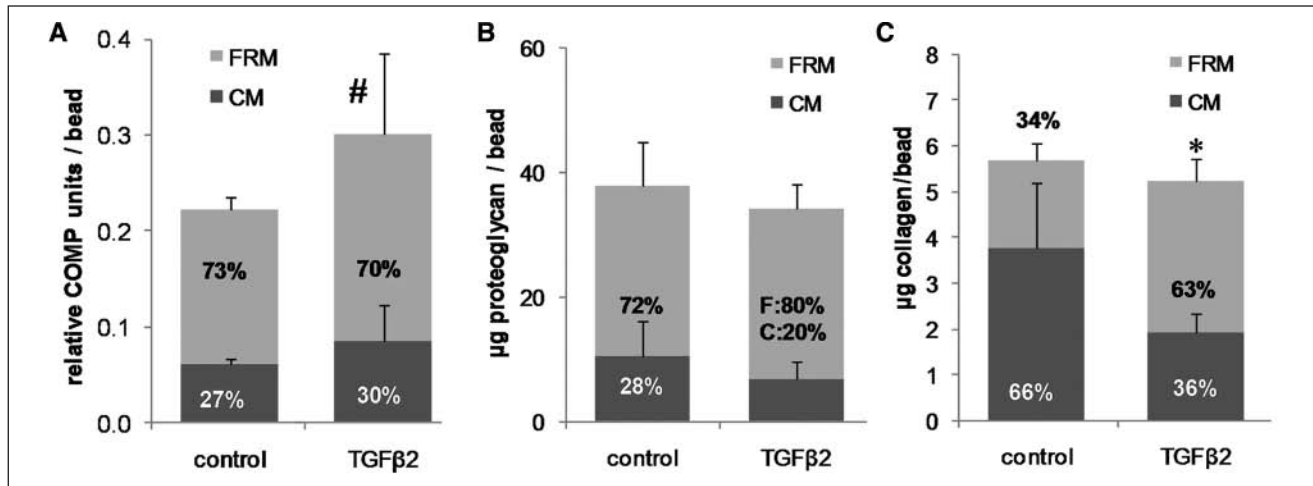


Figure 2. The effect of 25 ng/mL TGFβ2 on absolute matrix distribution with relative percentages after 21 days of culture as shown by the distribution of (A) COMP, (B) proteoglycans, and (C) collagen between the cell-associated matrix (CM, dark bars) and the further removed matrix (FRM, light bars). $N = 9$. *Significant difference ($P < 0.05$) in relative distribution between TGFβ2 and control without TGFβ2. #Significant difference ($P < 0.05$) in absolute values between the TGFβ2 condition and control condition.

control and TGFβ2 condition, approximately 30% of the COMP was located in the CM and approximately 70% of the COMP in the FRM (Fig. 2A). The same distribution for the proteoglycans was seen in the control condition. Addition of TGFβ2 resulted in a slight shift toward the FRM, although this is not significantly different from control (Fig. 2B). In the case of collagen, 66% ± 8% was cell associated, and 34% ± 4% was further removed from the cell in the control condition. This distribution changed to less collagen in the CM in the presence of TGFβ2 (Fig. 2C).

Collagen Fibril Diameter in Response to TGFβ

To further investigate the effect of increasing COMP production on the newly formed matrix, we measured collagen fibril diameter on an electron microscopic level. The matrix of chondrocytes cultured in the presence of TGFβ2 appeared more organized than the matrix produced in the corresponding control condition (Fig. 3A) because collagen fibrils were aligned more parallel to the chondrocyte surface in the presence of TGFβ2. Collagen fibril diameter also decreased from 23.0 nm to 20.0 nm when TGFβ2 was present (Fig. 3B).

COMP Gene Expression after Lentiviral Transfection

To evaluate whether the effect on matrix seen in cultures with TGFβ was due to increased COMP expression, a lentiviral vector was used to more selectively increase COMP gene expression. A GFP lentivirus was used in parallel to determine transfection efficiency. In time, the percentage of GFP-positive cells did not decrease, indicating that a stable

transfection was accomplished (Figs. 4A and B). The human COMP gene expression increased in time when bovine chondrocytes were transfected with the COMP lentivirus. In the control conditions without virus or an empty virus, no human COMP gene expression was seen (Fig. 5A). Bovine COMP gene expression was not influenced by transfection with lentivirus (Fig. 5B). As quantified by ELISA, COMP protein deposition was increased 1.5-fold after lentiviral transfection at day 21. COMP secretion into the culture medium was also increased (Fig. 5C).

Matrix Deposition and Functionality in Response to COMP Overexpression

The amount and distribution of collagen deposited were not influenced after COMP transfection (Fig. 6A). The number of HP cross-links per collagen triple helix was 0.57 ± 0.02 in the control condition and not significantly changed after COMP transfection (Fig. 6B). The proteoglycan production and distribution (Fig. 6C) were also not influenced by COMP overexpression. The ability to hold water (defined by the secant modulus) and the stiffness of the matrix (defined by the equilibrium modulus) were also not significantly different between the control and COMP transfected condition (Figs. 6D and E).

Collagen Fibril Diameter in Response to Increased COMP Production

The induction of COMP production by chondrocytes using a lentivirus also resulted in a more organized matrix compared to the corresponding control condition (Fig. 7A). As seen

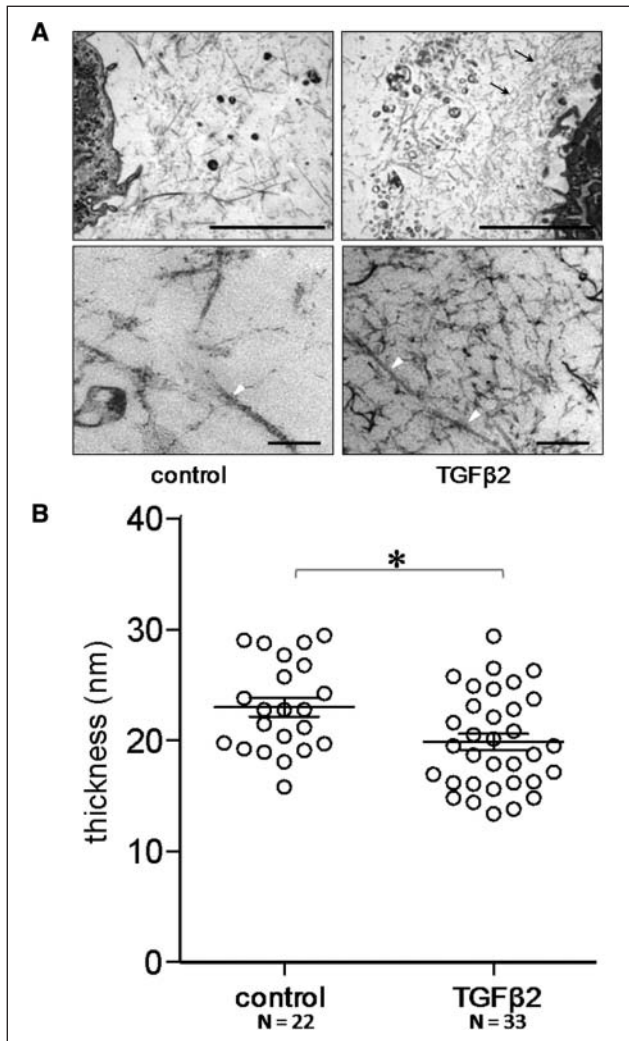


Figure 3. Representative examples of electron microscopic analysis of alginate beads cultured for 3 weeks in the presence of TGF β 2 and its corresponding control condition. **(A)** Newly formed matrix in the presence of TGF β 2 and its control condition. Arrows point out collagen fibrils parallel to the cell surface; arrowheads indicate actual collagen fibrils with their typical banded pattern. Magnifications are 18,000 \times (upper row), with the bar indicating 2 μ m, and 89,000 \times (lower row), with the bar indicating 200 nm. **(B)** Diameter of collagen fibrils with or without TGF β showed as the diameter of individual fibrils, with mean diameter \pm SD. *Significant difference, $P < 0.05$.

after the addition of TGF β to the chondrocytes leading to increased COMP production, overexpressing COMP also resulted in smaller diameters of the collagen fibrils (**Fig. 7B**).

Discussion

Osteoarthritis is characterized by cartilage damage, and once damaged, adult articular cartilage has a poor repair

capacity, which is probably due to the ineffective repair of the collagen network because proteoglycan depletion is often reversible.³³ COMP interacts with several other cartilage matrix molecules and is involved in matrix-chondrocyte interaction.³⁴ It is also up-regulated during the progression of OA¹⁸ and therefore used as a marker for OA.³⁵⁻³⁸ The present study shows that COMP gene expression and protein deposition by chondrocytes in alginate beads were up-regulated in the presence of TGF β 2. We previously examined collagen and glycosaminoglycan deposition as well as mechanical properties of these samples.²² There, we found that TGF β 2 slightly influenced collagen deposition by bovine chondrocytes in alginate and severely reduced the number of collagen cross-links without affecting functionality of the newly formed matrix. It is unclear why TGF β addition resulted in less collagen deposition in the alginate bead. This effect confirmed our observations in 2 previous studies,^{21,22} in which we found that TGF β 2 was able to induce collagen production when chondrocytes were cultured on plastic instead of in alginate beads, suggesting the culture environment plays an important role in the effect of TGF β on collagen production.²¹ Because of the pleiotropic effects of TGF β , it is not possible to determine whether the increase of COMP deposition in this condition leads to a better integrity of the newly formed matrix and whether COMP is involved in cartilage development as seen previously.¹⁶ With lentiviral overexpression, we increased COMP gene expression and protein production. This appeared to have no effect on absolute matrix deposition and functional properties. However, increasing COMP production resulted in a more organized collagen matrix and collagen fibrils with a smaller diameter, independent of the approach used to induce COMP production (i.e., lentiviral overexpression of COMP or addition of TGF β to the culture medium).

COMP interacts with collagen type 2 (COL2),⁵ and COMP could function as a catalyst in collagen fibrillogenesis. Previous experiments have shown that *in vitro* fibrillogenesis of collagen type I or II can be modulated (i.e., smaller diameter of the fibrils and quicker fibrillogenesis) when coincubated with COMP without clear attachment of COMP to the formed collagen fibrils.¹⁴ This might be an indication that COMP acts as a catalyst for collagen fibrillogenesis. A possible catalyst role of COMP in collagen fibrillogenesis might explain the results we see: more COMP production as a result of lentiviral transfection of the COMP gene, and as a result, collagen fibrils with a smaller diameter but not much extra COMP in the alginate bead and thus incorporated in the matrix. Possibly, the extra COMP is not necessary because collagen deposition is unchanged, and therefore COMP is diffused directly into the culture medium. Future experiments might include induction of collagen deposition as shown previously^{21,22} in combination with COMP overexpression to examine COMP and collagen interaction more closely.

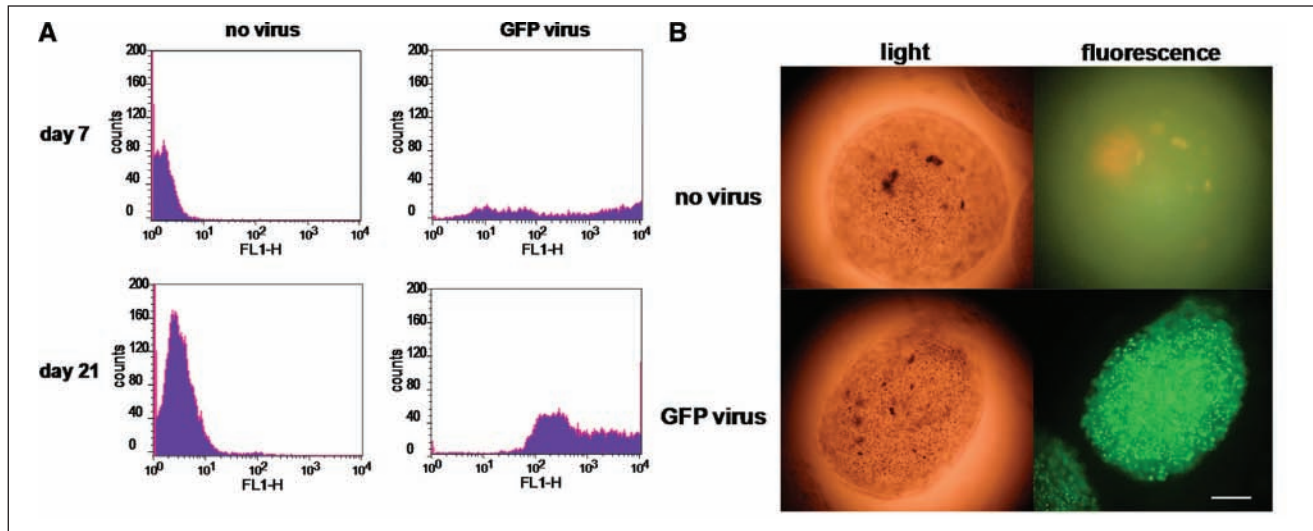


Figure 4. The effect of lentiviral transfection of GFP as a control for transfection efficiency. **(A)** The percentage of positive cells in the GFP transfected condition increased from 92% at day 7 to 99% at day 21. No GFP-positive cells were present in the untransfected control condition. $N = 2$. **(B)** Cells with or without GFP lentivirus in alginate analyzed with a fluorescence microscope. No green fluorescent cells were present in the control condition after 21 weeks of culture; green cells were visible in the GFP lentivirus condition. The bar represents 0.5 mm.

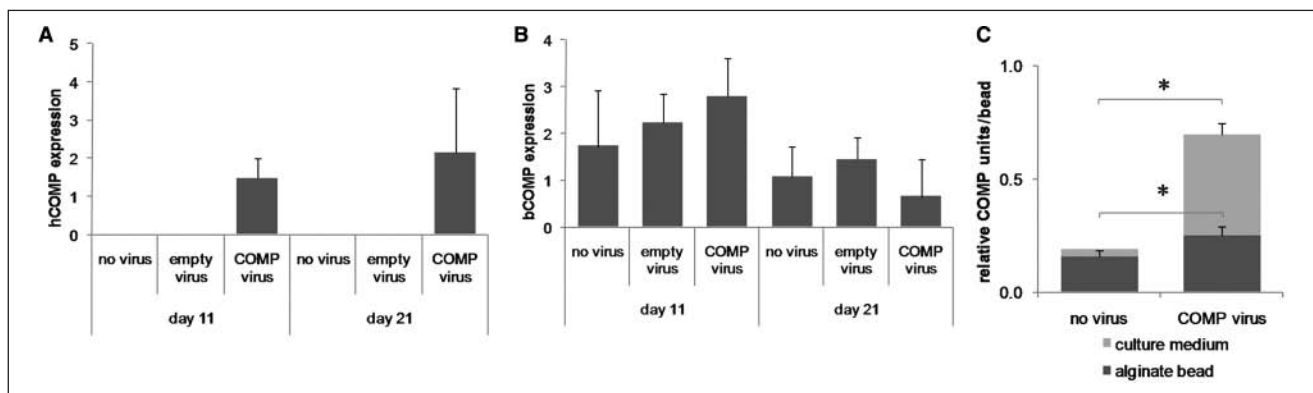


Figure 5. COMP gene expression in bovine chondrocytes, transfected with or without lentivirus containing the human COMP gene or an empty virus. **(A)** Relative human COMP gene expression corrected for GAPDH after lentiviral transduction of bovine chondrocytes. No human COMP gene expression was detected in the bovine chondrocytes in the control conditions. **(B)** Relative endogenous bovine COMP gene expression after transduction with human COMP. **(C)** The effect of lentiviral COMP overexpression on COMP production. After transfection with or without lentivirus containing the human COMP gene, alginate beads (dark bars) and the culture medium (light bars) were analyzed after 21 days of culture for the presence of COMP with ELISA. $N = 6$.

Interestingly, inducing COMP production by either TGF β stimulation or lentiviral transduction resulted in more organized collagen matrix and a decrease of collagen fibril diameter. To our knowledge, 3 other studies describe the relation between COMP amount and collagen fibril diameter. In a recent study with COMP and collagen type IX single and double knock-out chondrocytes, we found indications that COMP and collagen type IX regulate collagen fibril diameter.¹¹ In another study performed on equine tendons, a positive correlation between

high COMP levels and the percentage of small collagen fibrils was present,³⁹ which corresponds to our findings. Halász et al. concluded that COMP is involved in collagen fibrillogenesis *in vitro*, affecting intermediate forms of collagen fibrils and increasing the rate of fibril formation when COMP and collagen were coinubated in a test tube. Without the presence of COMP, collagen fibrils with a larger diameter were found.¹⁴ Next to an altered fibril diameter, the latter study also reports on altered collagen organization when COMP is absent. This confirms

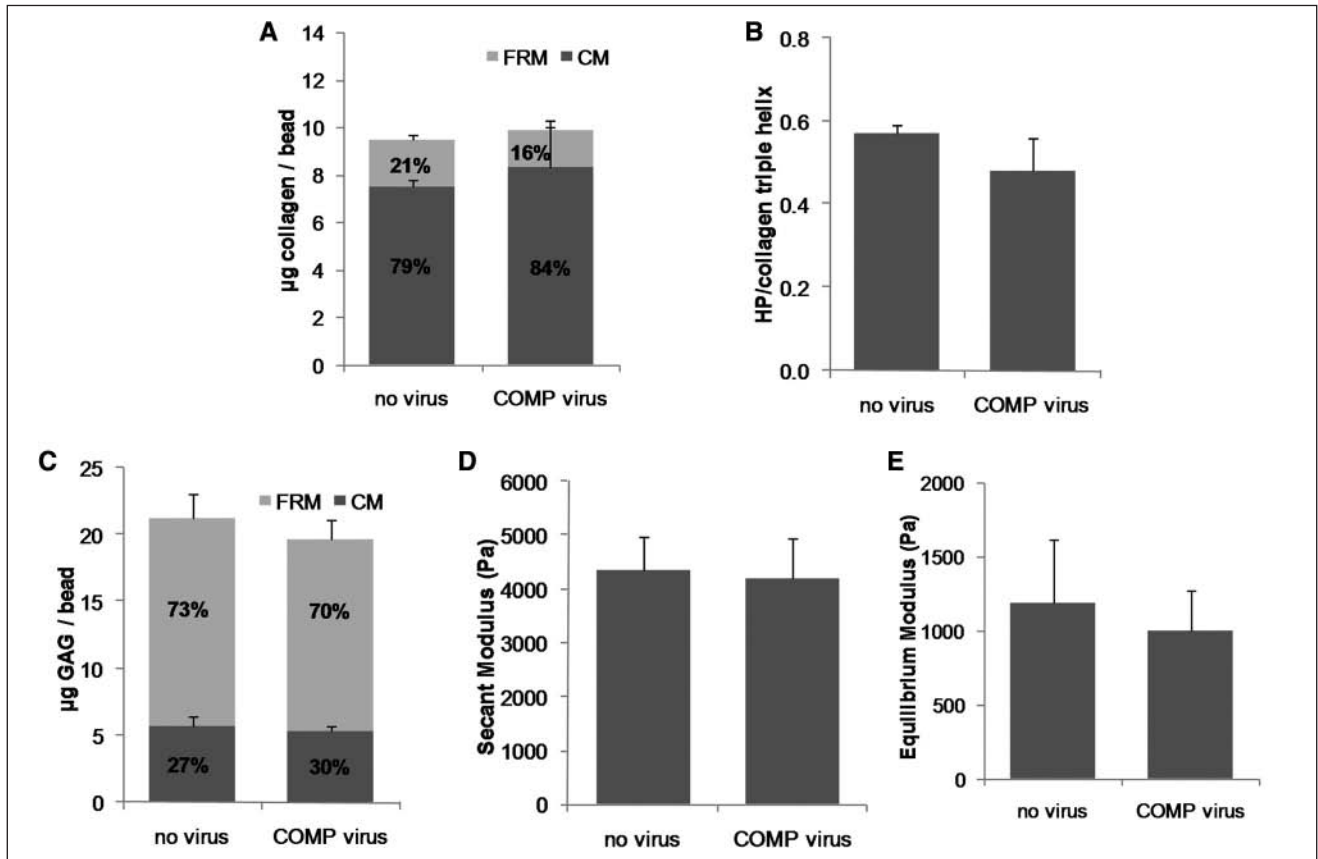


Figure 6. Collagen biochemistry and matrix functionality in lentiviral transfected chondrocytes. (A) Collagen deposition and distribution between cell-associated matrix (CM, gray) and further removed matrix (FRM, white). (B) Collagen cross-linking. (C) Proteoglycan deposition and distribution between CM (gray) and FRM (white) (all $N = 9$). (D) Secant modulus and equilibrium modulus of newly formed matrix after 35 days of culture, $N = 12$. The previously determined equilibrium aggregate modulus and the secant modulus of 1-day-old constructs were 0.36 kPa and 2.1 kPa, respectively.^{22,42}

the results of our present study, in which we also found an effect of COMP on collagen fibril organization.

The diameter of the collagen fibrils is known to play a significant role in determining the mechanical properties of the tissue. Deformation of tissues is related to the proportion of small diameter fibrils, and as the diameter increases, the flexibility of the tissue decreases.^{40,41} In previous studies, we have demonstrated that mechanical properties of alginate scaffolds with cells that are cultured for several weeks are higher than properties of alginate scaffolds without cells.^{22,42} In addition, we were able to modulate the mechanical properties of the newly formed cartilage matrix by stimulating collagen and proteoglycan production²² or inhibiting proteoglycan formation.⁴³ In the present study, we did not observe altered mechanical properties when COMP production was stimulated, despite the small diameter of the collagen fibrils. Immaturity of the newly formed matrix might explain this. This was also seen for collagen cross-links that became important when the matrix was more

mature.³¹ This suggests that cartilage matrix in which high levels of COMP had been present during formation can be of less quality in a more mature phase because of the small diameter collagen fibrils. To confirm this would require very long-term culture experiments.

Regulation of COMP gene expression and protein production by chondrocytes with growth factors has been shown before.¹⁹ In that study by Recklies et al., TGF β addition also resulted in increased synthesis of COMP and therefore confirms our results. However, monolayer cultures of chondrocytes were used, and expression was analyzed after a short-term culture. In our study, chondrocytes were cultured in a 3-dimensional environment in order to maintain their phenotype, resulting in proper matrix production. COMP distribution over the matrix is not affected by TGF β , in contrast to collagen distribution. This suggests that COMP localization is not dependent on collagen. Overexpression of COMP did not influence collagen or proteoglycan production and distribution. This confirms earlier results for COMP and collagen.¹⁶

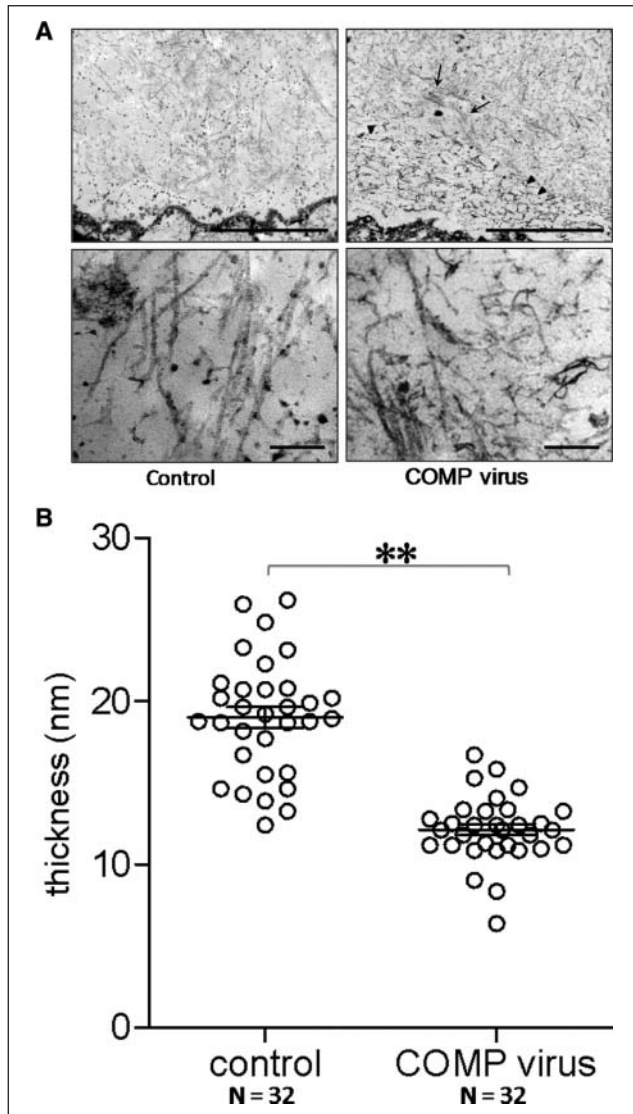


Figure 7. Representative examples of electron microscopic analysis of alginate beads cultured for 3 weeks after COMP lentiviral transfection and its corresponding control condition. **(A)** Newly formed matrix after lentiviral transfection and its control without virus. Arrows point out organized collagen fibrils; arrowheads indicate processing artefacts. Magnifications are 18,000 \times (upper row), with the bar indicating 2 μ m, and 89,000 \times (lower row), with the bar indicating 200 nm. **(B)** Diameter of collagen fibrils with or without lentiviral overexpression of COMP showed as the diameter of individual fibrils, with mean diameter \pm SD. **Significant difference, $P < 0.001$.

Taken together, COMP production and deposition were increased by TGF β and lentiviral overexpression, both leading to smaller diameters of collagen fibrils and changed organization of collagen. From the overexpression experiments, we conclude that induction of COMP production has no effect on other biochemical parameters. Increased

production of COMP is one of the features of OA,^{16,18} next to increased synthesis of other cartilage matrix molecules,^{33,44} although repair is ineffective. Increased COMP production in OA could lead to decreased stiffness of the cartilage via decreasing collagen fibril diameter, therefore negatively affecting the quality of the matrix. Lowering COMP levels could therefore contribute to successful cartilage regeneration strategies.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

Funding

Supported by a grant of The Dutch Arthritis Association (Reumafonds), NR 02-2-40.

References

1. Oldberg A, Antonsson P, Lindblom K, Heinegard D. COMP (cartilage oligomeric matrix protein) is structurally related to the thrombospondins. *J Biol Chem.* 1992;267(31):22346-50.
2. Hedbom E, Antonsson P, Hjerpe A, Aeschlimann D, Paulsson M, Rosa-Pimentel E, et al. Cartilage matrix proteins: an acidic oligomeric protein (COMP) detected only in cartilage. *J Biol Chem.* 1992;267(9):6132-6.
3. Smith RK, Zunino L, Webbon PM, Heinegard D. The distribution of cartilage oligomeric matrix protein (COMP) in tendon and its variation with tendon site, age and load. *Matrix Biol.* 1997;16(5):255-71.
4. Dodge GR, Hawkins D, Boesler E, Sakai L, Jimenez SA. Production of cartilage oligomeric matrix protein (COMP) by cultured human dermal and synovial fibroblasts. *Osteoarthritis Cartilage.* 1998;6(6):435-40.
5. Rosenberg K, Olsson H, Morgelin M, Heinegard D. Cartilage oligomeric matrix protein shows high affinity zinc-dependent interaction with triple helical collagen. *J Biol Chem.* 1998;273(32):20397-403.
6. Thur J, Rosenberg K, Nitsche DP, Pihlajamaa T, Ala-Kokko L, Heinegard D, et al. Mutations in cartilage oligomeric matrix protein causing pseudoachondroplasia and multiple epiphyseal dysplasia affect binding of calcium and collagen I, II, and IX. *J Biol Chem.* 2001;276(9):6083-92.
7. Mann HH, Ozbek S, Engel J, Paulsson M, Wagener R. Interactions between the cartilage oligomeric matrix protein and matrilins: implications for matrix assembly and the pathogenesis of chondrodysplasias. *J Biol Chem.* 2004;279(24):25294-8.
8. Chen FH, Herndon ME, Patel N, Hecht JT, Tuan RS, Lawler J. Interaction of cartilage oligomeric matrix protein/thrombospondin 5 with aggrecan. *J Biol Chem.* 2007;282(34):24591-8.
9. Briggs MD, Hoffman SM, King LM, Olsen AS, Mohrenweiser H, Leroy JG, et al. Pseudoachondroplasia and multiple epiphyseal dysplasia due to mutations in the cartilage oligomeric matrix protein gene. *Nat Genet.* 1995;10(3):330-6.

10. Hecht JT, Hayes E, Haynes R, Cole WG. COMP mutations, chondrocyte function and cartilage matrix. *Matrix Biol.* 2005; 23(8):525-33.
11. Budde B, Blumbach K, Ylostalo J, Zaucke F, Ehlen HW, Wagener R, et al. Altered integration of matrilin-3 into cartilage extracellular matrix in the absence of collagen IX. *Mol Cell Biol.* 2005;25(23):10465-78.
12. Schmitz M, Becker A, Schmitz A, Weirich C, Paulsson M, Zaucke F, et al. Disruption of extracellular matrix structure may cause pseudoachondroplasia phenotypes in the absence of impaired cartilage oligomeric matrix protein secretion. *J Biol Chem.* 2006;281(43):32587-95.
13. Svensson L, Aszodi A, Heinegard D, Hunziker EB, Reinholt FP, Fassler R, et al. Cartilage oligomeric matrix protein-deficient mice have normal skeletal development. *Mol Cell Biol.* 2002; 22(12):4366-71.
14. Halasz K, Kassner A, Morgelin M, Heinegard D. COMP acts as a catalyst in collagen fibrillogenesis. *J Biol Chem.* 2007;282(43):31166-73.
15. Shen Z, Heinegard D, Sommarin Y. Distribution and expression of cartilage oligomeric matrix protein and bone sialoprotein show marked changes during rat femoral head development. *Matrix Biol.* 1995;14(9):773-81.
16. Koelling S, Clauditz TS, Kaste M, Miosge N. Cartilage oligomeric matrix protein is involved in human limb development and in the pathogenesis of osteoarthritis. *Arthritis Res Ther.* 2006;8(3):R56.
17. Salminen H, Perala M, Lorenzo P, Saxne T, Heinegard D, Saamanen AM, et al. Up-regulation of cartilage oligomeric matrix protein at the onset of articular cartilage degeneration in a transgenic mouse model of osteoarthritis. *Arthritis Rheum.* 2000;43(8):1742-8.
18. Lorenzo P, Bayliss MT, Heinegard D. Altered patterns and synthesis of extracellular matrix macromolecules in early osteoarthritis. *Matrix Biol.* 2004;23(6):381-91.
19. Recklies AD, Baillargeon L, White C. Regulation of cartilage oligomeric matrix protein synthesis in human synovial cells and articular chondrocytes. *Arthritis Rheum.* 1998;41(6):997-1006.
20. Blaney Davidson EN, van der Kraan PM, van den Berg WB. TGF-beta and osteoarthritis. *Osteoarthritis Cartilage.* 2007; 15(6):597-604.
21. Bastiaansen-Jenniskens YM, Koevoet W, De Bart AC, Zuurmond AM, Bank RA, Verhaar JA, et al. TGFbeta affects collagen cross-linking independent of chondrocyte phenotype but strongly depending on physical environment. *Tissue Eng Part A.* 2008;14(6):1059-66.
22. Jenniskens YM, Koevoet W, de Bart AC, Weinans H, Jahr H, Verhaar JA, et al. Biochemical and functional modulation of the cartilage collagen network by IGF1, TGFbeta2 and FGF2. *Osteoarthritis Cartilage.* 2006;14(11):1136-46.
23. Hauselmann HJ, Fernandes RJ, Mok SS, Schmid TM, Block JA, Aydelotte MB, et al. Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. *J Cell Sci.* 1994;107(Pt 1):17-27.
24. Tchétina EV, Antoniou J, Tanzer M, Zukor DJ, Poole AR. Transforming growth factor-beta2 suppresses collagen cleavage in cultured human osteoarthritic cartilage, reduces expression of genes associated with chondrocyte hypertrophy and degradation, and increases prostaglandin E(2) production. *Am J Pathol.* 2006;168(1):131-40.
25. van Susante JL, Buma P, van Beuningen HM, van den Berg WB, Veth RP. Responsiveness of bovine chondrocytes to growth factors in medium with different serum concentrations. *J Orthop Res.* 2000;18(1):68-77.
26. van der Slot AJ, van Dura EA, de Wit EC, De Groot J, Huizinga TW, Bank RA, et al. Elevated formation of pyridinoline cross-links by profibrotic cytokines is associated with enhanced lysyl hydroxylase 2b levels. *Biochim Biophys Acta.* 2005;1741(1-2):95-102.
27. van Osch GJ, van den Berg WB, Hunziker EB, Hauselmann HJ. Differential effects of IGF-1 and TGF beta-2 on the assembly of proteoglycans in pericellular and territorial matrix by cultured bovine articular chondrocytes. *Osteoarthritis Cartilage.* 1998;6(3):187-95.
28. Enobakhare BO, Bader DL, Lee DA. Quantification of sulfated glycosaminoglycans in chondrocyte/alginate cultures, by use of 1,9-dimethylmethylene blue. *Anal Biochem.* 1996;243(1):189-91.
29. Bank RA, Jansen EJ, Beekman B, te Koppele JM. Amino acid analysis by reverse-phase high-performance liquid chromatography: improved derivatization and detection conditions with 9-fluorenylmethyl chloroformate. *Anal Biochem.* 1996;240(2):167-76.
30. Wong M, Siegrist M, Wang X, Hunziker E. Development of mechanically stable alginate/chondrocyte constructs: effects of guluronic acid content and matrix synthesis. *J Orthop Res.* 2001;19(3):493-9.
31. Bastiaansen-Jenniskens YM, Koevoet W, de Bart AC, van der Linden JC, Zuurmond AM, Weinans H, et al. Contribution of collagen network features to functional properties of engineered cartilage. *Osteoarthritis Cartilage.* 2008;16(3):359-66.
32. Mow VC, Holmes MH, Lai WM. Fluid transport and mechanical properties of articular cartilage: a review. *J Biomech.* 1984;17(5):377-94.
33. Lafeber FP, van Roy H, Wilbrink B, Huber-Bruning O, Bijlsma JW. Human osteoarthritic cartilage is synthetically more active but in culture less vital than normal cartilage. *J Rheumatol.* 1992;19(1):123-9.
34. Chen FH, Thomas AO, Hecht JT, Goldring MB, Lawler J. Cartilage oligomeric matrix protein/thrombospondin 5 supports chondrocyte attachment through interaction with integrins. *J Biol Chem.* 2005;280(38):32655-61.
35. Sharif M, Kirwan JR, Elson CJ, Granell R, Clarke S. Suggestion of nonlinear or phasic progression of knee osteoarthritis based on measurements of serum cartilage oligomeric matrix protein levels over five years. *Arthritis Rheum.* 2004;50(8):2479-88.
36. Wislowska M, Jablonska B. Serum cartilage oligomeric matrix protein (COMP) in rheumatoid arthritis and knee osteoarthritis. *Clin Rheumatol.* 2005;24(3):278-84.

37. Vilim V, Olejarova M, Machacek S, Gatterova J, Kraus VB, Pavelka K. Serum levels of cartilage oligomeric matrix protein (COMP) correlate with radiographic progression of knee osteoarthritis. *Osteoarthritis Cartilage*. 2002;10(9):707-13.
38. Di Cesare PE, Carlson CS, Stolerman ES, Hauser N, Tulli H, Paulsson M. Increased degradation and altered tissue distribution of cartilage oligomeric matrix protein in human rheumatoid and osteoarthritic cartilage. *J Orthop Res*. 1996;14(6):946-55.
39. Sodersten F, Ekman S, Eloranta ML, Heinegard D, Dudhia J, Hultenby K. Ultrastructural immunolocalization of cartilage oligomeric matrix protein (COMP) in relation to collagen fibrils in the equine tendon. *Matrix Biol*. 2005;24(5):376-85.
40. Bailey AJ, Paul RG, Knott L. Mechanisms of maturation and ageing of collagen. *Mech Ageing Dev*. 1998;106(1-2):1-56.
41. Parry DA. The molecular and fibrillar structure of collagen and its relationship to the mechanical properties of connective tissue. *Biophys Chem*. 1988;29(1-2):195-209.
42. Knight MM, van de Breevaart Bravenboer J, Lee DA, van Osch GJ, Weinans H, Bader DL. Cell and nucleus deformation in compressed chondrocyte-alginate constructs: temporal changes and calculation of cell modulus. *Biochim Biophys Acta*. 2002;1570(1):1-8.
43. Bastiaansen-Jenniskens YM, Koevoet W, Jansen KM, Verhaar JA, DeGroot J, VanOsch GJ. Inhibition of glycosaminoglycan incorporation influences collagen network formation during cartilage matrix production. *Biochem Biophys Res Commun*. 2009;379(2):222-6.
44. Nelson F, Dahlberg L, Laverty S, Reiner A, Pidoux I, Ionescu M, et al. Evidence for altered synthesis of type II collagen in patients with osteoarthritis. *J Clin Invest*. 1998;102(12):2115-25.