Osteoarthritis and Cartilage 20 (2012) 162–171

Osteoarthritis and Cartilage

# Molecular differentiation between osteophytic and articular cartilage – clues for a transient and permanent chondrocyte phenotype

K. Gelse †*, A.B. Ekici ‡, F. Cipa †§, B. Swoboda §, H.D. Carl §, A. Olk †, F.F. Hennig †, P. Klinger †§

† Department of Orthopaedic Trauma Surgery, University Hospital Erlangen, Germany
‡ Institute of Human Genetics, University of Erlangen-Nürnberg, Erlangen, Germany
§ Department of Orthopaedic Rheumatology, University of Erlangen-Nürnberg, Germany

ARTICLE INFO

*Article history:*
Received 24 July 2011
Accepted 1 December 2011

*Keywords:*
Osteophyte
Chondrogenesis
Hypertrophic chondrocyte
Endochondral ossification
Gremlin-1
Microarray

SUMMARY

*Objective:* To identify the molecular differences between the transient and permanent chondrocyte phenotype in osteophytic and articular cartilage.
*Methods:* Total RNA was isolated from the cartilaginous layer of osteophytes and from intact articular cartilage from knee joints of 15 adult human donors and subjected to cDNA microarray analysis. The differential expression of relevant genes between these two cartilaginous tissues was additionally validated by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and by immunohistochemistry.
*Results:* Among 47,000 screened transcripts, 600 transcripts were differentially expressed between osteophytic and articular chondrocytes. Osteophytic chondrocytes were characterized by increased expression of genes involved in the endochondral ossification process [bone gamma-carboxyglutamate protein/osteocalcin (BGLAP), bone morphogenetic protein-8B (BMP8B), collagen type I, alpha 2 (COL1A2), sclerostin (SOST), growth arrest and DNA damage-induced gene 45ß (GADD45ß), runt-related transcription factor 2 (RUNX2)], and genes encoding tissue remodeling enzymes [matrix metallopeptidase (MMP)9, 13, hyaluronan synthase 1 (HAS1)]. Articular chondrocytes expressed increased transcript levels of antagonists and inhibitors of the BMP- and Wnt-signaling pathways [Gremlin-1 (GREM1), frizzled-related protein (FRZB), WNT1 inducible signaling pathway protein-3 (WISP3)], as well as factors that inhibit terminal chondrocyte differentiation and endochondral bone formation [parathyroid hormone-like hormone (PTHLH), sex-determining region Y-box 9 (SOX9), stanniocalcin-2 (STC2), S100 calcium binding protein A1 (S100A1), S100 calcium binding protein B (S100B)].
Immunohistochemistry of tissue sections for GREM1 and BGLAP, the two most prominent differentially expressed genes, confirmed selective detection of GREM1 in articular chondrocytes and that of BGLAP in osteophytic chondrocytes and bone.
*Conclusions:* Osteophytic and articular chondrocytes significantly differ in their gene expression pattern. In articular cartilage, a prominent expression of antagonists inhibiting the BMP- and Wnt-pathway may serve to lock and stabilize the permanent chondrocyte phenotype and thus prevent their terminal differentiation. In contrast, osteophytic chondrocytes express genes with roles in the endochondral ossification process, which may account for their transient phenotype.
© 2011 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.

## Introduction

The generation of phenotypically stable hyaline repair cartilage is still a challenge for regenerative medicine. The aim of therapeutic strategies is not confined to the induction of chondrogenesis but also includes the perpetuation of a stable, permanent cellular phenotype that is typical for articular chondrocytes. Unfortunately, cartilage repair approaches that focus on the recruitment of bone marrow stem cells are not only confronted with insufficient chondrogenesis but also with the problem of chondrocyte hypertrophy and inadvertent endochondral ossification[1–4]. Thus, the cellular differentiation programme within cartilage repair tissue recapitulates cellular processes that are similar to the fetal growth plate, fracture callus or forming osteophytes[5,6]. All these tissues have in common that their cartilaginous tissue is only an intermediate state toward bone formation and their cells only adopt

* Address correspondence and reprint requests to: K. Gelse, Department of Orthopaedic Trauma Surgery, University Hospital Erlangen, Krankenhausstr. 12, 91054 Erlangen, Germany. Tel: 49-9131-8542121; Fax: 49-9131-8533300.
*E-mail address:* kolja.gelse@web.de (K. Gelse).

1063-4584/$ – see front matter © 2011 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.joca.2011.12.004

a transient chondrocytic phenotype. In contrast, chondrocytes of healthy articular cartilage are characterized by a postmitotic status and retain their unique phenotype for a period of many decades. To date, the mechanisms that stabilize the articular chondrocyte phenotype have not been fully identified. We hypothesize upon a distinction between transient and permanent chondrocytes, and the aim of this study was to investigate the differences in their gene expression profile.

Since phenotypically instable transient cartilage repair tissue is available only to a limited degree and characterized by a heterogeneous composition, we focussed on the cartilaginous cap of mature osteophytes which can be considered a prototype for cartilage repair tissue that arises in a more predictive and uniform pattern. Osteophytic chondrocytes originate from mesenchymal stem or precursor cells, undergo chondrogenic differentiation leading to the formation of cartilaginous tissue, that is, however, prone to be replaced by endochondral ossification in the mature osteophyte[5,7–11]. Therefore, osteophytic chondrocytes represent a well-suited substrate to investigate the transcriptional differences between transient chondrocytes and permanent chondrocytes from articular cartilage.

## Materials and methods

### *Tissue samples and preparation*

Human articular and osteophytic cartilage probes were obtained as matched pairs from the same respective knee joints of 15 individual patients [with a mean ± standard deviation (SD) age of 68.2 ± 6.7 years] undergoing total knee arthroplasty for osteoarthritis (OA) at the University Hospital Erlangen. The diagnosis of primary OA was based on clinical and radiographic evaluations according to standard criteria and patients with any secondary OA or rheumatoid arthritis were excluded.

Articular cartilage was isolated from the dorsal part of the femoral condyles with a macroscopically intact joint surface characterized by an Outerbridge score of 0 or 1. Osteophytic cartilage was isolated from the same respective joints from the cartilaginous cap of the osteophyte outgrowths located at the edges of the femoral condyles. Osteophytes were distinguished from the marginal transition area of the joint surface by the existence of a concave ridge toward the joint surface. In order to selectively isolate the cartilaginous cap of the osteophyte and to exclude any abrasion of bone trabeculae and calcified tissue of the deepest cartilaginous zone, only minimal forces were applied with a scalpel by cutting tangentially to the surface to yield thin consecutive slices of less than 1 mm thickness. Informed consent was obtained from each patient prior to surgery, and the institutional ethics committee approved the study protocol.

### *Microarray*

Total RNA was isolated from articular or osteophytic cartilage tissue as described previously in detail[11]. The quality of isolated RNA was assessed using the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA), and the concentration was determined with a Spectrophotometer (ND 1000; NanoDrop Technologies, Rockland, DE). RNA preparations from a total of 15 different donors were used. Three paired sets of RNA preparations of articular and osteophytic cartilage each of five donors in equal quantities were used for analysis. The appropriate pooling of RNA samples had been shown to be statistically valid for microarray experiments.[51] Gene expression profiling was performed with the Affymetrix Human Genome (HG) U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. The Genechip U133 Plus 2.0 is a comprehensive whole human genome expression array with over 47,000 transcripts. Genes highly expressed by articular and osteophytic chondrocytes were identified by comparing gene expression levels between the two groups.

### *Quantitative reverse transcriptase polymerase chain reaction (RT-PCR)*

To confirm the validity of the microarray, the individual RNA preparations of 15 donors were additionally analyzed by real-time RT-PCR using an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA) and Verso One-Step QRT-PCR Rox Kit (Abgene, Hamburg, Germany) by quantifying the expression levels of functionally relevant genes including ACAN (aggrecan), BGLAP (bone gamma-carboxyglutamate protein/osteocalcin), COL1A2 (collagen type I, alpha 2), COL10A1 (collagen type X, alpha 1), FRZB (frizzled-related protein), GADD45ß (growth arrest and DNA damage-induced gene 45ß), GREM1 (Gremlin-1), MMP13 (matrix metallopeptidase 13), PTHLH (parathyroid hormone-like hormone), RUNX2 (runt-related transcription factor 2), SOX9 (sex-determining region Y-box 9), and ß2M (ß 2-microglobulin). The relative quantification of gene expression was performed using the standard curve method. For each sample, the relative amount of target mRNA was determined and normalized to that of ß2m mRNA. Primer and probe sets are shown in Table I.

### *Immunohistological analysis*

Osteochondral specimens from the boundary region of articular cartilage and osteophyte were fixed in 4% paraformaldehyde for

**Table I**
Primer/probe sets of target genes used for quantitative RT-PCR (TaqMan Gen Expression Assays, Applied Biosystems)

| Genes | Gene expression assay ID | Reference sequence |
|---|---|---|
| ACAN | Hs00153936_m1 | NM_013227 |
| SOX9 | Hs00165814_m1 | NM_000346 |
| RUNX2 | Hs00231692_m1 | NM_004348 |
| COL1A2 | Hs00164099_m1 | NM_000089 |
| COL10A1 | Hs00166657_m1 | NM_000493 |
| PTHLH | Hs00174969_m1 | NM_002820 |
| MMP13 | Hs00233992_m1 | NM_002427 |
| FRZB | Hs00173503_m1 | NM_001463 |

| | Forward | Reverse | Probe |
|---|---|---|---|
| GREM1 | GTGACGGAGCGCAAATACC | CCTTCCTCGTGGATGGTCT | AGACTGGTGCAAAACCCAGCCG |
| BGLAP | CGGTGCAGAGTCCAGCAAA | AGCGCCTGGGTCTCTTCACT | TGCAGCCTTTGTGTCCAAGCAGGAG |
| GADD45ß | ATTGACGAGGAGGAGGAGGA | CGTTGTCACAGCAGAAGGAC | ATCGCCCTGCAAATCCACTTCACG |
| ß2M | TGACTTTGTCACAGCCCAAGATA | AATCCAAATGCGGCATCTTC | TGATGCTGCTTACATGTCTCGATCCCA |

12 h, followed by decalcification in 0.5 M ethylene diamine tetra-acetic acid (EDTA) for 3 months. After standard processing, the samples were embedded in paraffin. Specimens were cut in serial transverse 5 μm sections and stained with Toluidine blue and Safranin-O/fast green for morphological assessment.

For immunohistochemical detection of GREM1 and BGLAP, deparaffinized sections were pretreated either with 10 mM citrate buffer (pH 6) at 90°C for 20 min, or with 0.2% hyaluronidase (Roche, Mannheim, Germany) (pH 5.5) at 37°C for 60 min, respectively, followed by Protease XXIV (0.02 mg/ml) (Sigma–Aldrich, Hamburg, Germany) for 60 min. The sections were then left to react overnight at 4°C either with rabbit anti-human GREM1 antibodies (AP6133a; Abgent, San Diego, CA) diluted 1:50, or with monoclonal mouse anti-human BGLAP antibodies (8H12; Acris, Herford, Germany) diluted 1:10, respectively. Negative control sections for GREM1 and BGLAP were incubated with isotype normal mouse IgG (Santa Cruz). The sections were incubated with biotinylated anti-rabbit or anti-mouse secondary antibodies (Dianova, Hamburg, Germany), respectively. Bound antibodies were visualized by exposure to a complex of streptavidin and biotinylated alkaline phosphatase (Vectastain, ABC-AP, Vector Laboratories, Burlingame, CA, USA). The sections were developed with fast red and counterstained with hematoxylin.

Immunohistochemical detection of type I and type II collagen was performed as described previously in detail[12].

### *Statistical analysis*

The microarray data of $N = 3$ paired preparations were analyzed with Partek Genomics Suite software (St. Louis, MO). Gene expression between the two cartilaginous tissues was considered to be significant if the false discovery rates were less than 0.005 and the fold-changes were three-fold or higher between the different microarrays. Quantitative gene expression of paired individual preparations from $N = 15$ different donors was analyzed by GraphPad InStat3 (La Jolla, CA) using a paired $t$-test with $P < 0.05$ considered significant. All data are presented as mean with 95% confidence intervals (CIs) (lower, upper).

## Results

### *Gene microarray analysis*

Applying the comprehensive microarray analysis with 47,000 screened transcripts, 515 transcripts were significantly higher expressed (more than three-fold) in osteophytic chondrocytes, and 85 transcripts were significantly higher expressed (more than three-fold) in articular chondrocytes ($P < 0.005$) respectively. Among those genes, 34 transcripts were characterized by differential expression that reached a high level of significance with 31 transcripts being expressed more than 20-fold higher in osteophytic chondrocytes, and three transcripts being expressed more than 20-fold higher in articular chondrocytes (Fig. 1, Table II).

### *Genes significantly higher expressed in osteophytic cartilage*

Based on the functional clustering of the differentially expressed genes, we detected several signaling factors with higher expression in osteophytic cartilage which have a role in terminal chondrocyte differentiation including bone morphogenetic protein-8B (BMP8B), RUNX2, SOST (sclerostin), TWIST1 (twist homolog 1) and GADD45ß (Table III). Quantitative RT-PCR of selected relevant genes confirmed the validity of the microarray analysis with significantly elevated transcript levels for RUNX2 (12.67-fold) and GADD45ß (2.66-fold) in osteophytic cartilage (Fig. 2).

Osteophytic chondrocytes were also characterized by a significantly greater expression of genes that encode molecules which are involved in the endochondral ossification process, including BGLAP, COL1A2, and COL1A1 (collagen type I, alpha 1). Quantitative RT-PCR confirmed significant upregulation of BGLAP (52.78-fold) and COL1A2 (14.18-fold) in osteophytic cartilage (Fig. 2, Table III). The striking differential expression of BGLAP was primarily a result of its nearly absent expression in articular cartilage (0.56% of ß2M levels), since the expression in osteophyte cartilage was only moderate (29.7% of ß2M levels) (Fig. 2). Upregulation of COL10A1, a marker gene for chondrocyte hypertrophy, did not reach the level of significance in specimen from osteophytes as detected by quantitative RT-PCR ($P = 0.063$).

Osteophytic chondrocytes exhibited a higher expression of VCAN (versican), a proteoglycan which is ubiquitously expressed in various tissues and not specific for hyaline cartilage (Table III).

Furthermore, osteophytic chondrocytes were characterized by a significantly higher expression of enzymes which mediate tissue resorption and remodeling, including MMP9, MMP13, as well as HAS1 (hyaluronan synthase 1). Quantitative RT-PCR confirmed a 12.8-fold upregulation of MMP13 in osteophytic cartilage (Fig. 2, Table III).

### *Genes significantly higher expressed in articular cartilage*

Microarray analysis revealed that a number of genes which have a role in chondrogenesis and maintenance of the cartilage matrix had a significantly higher expression in articular cartilage. Functional clustering demonstrated upregulation of genes that encode for factors involved in growth factor-signaling, including GREM1, STC2 (stanniocalcin-2), WISP3 (WNT1 inducible signaling pathway protein-3), S100B (S100 calcium binding protein B), S100A1 (S100 calcium binding protein A1), GDF5 (growth and differentiation factor-5), FRZB, SOX9 and PTHLH (Tables II and IV). GREM1 was the gene with the most prominent differential expression between articular and osteophytic cartilage with a 24.77-fold upregulation in articular cartilage (Fig. 2, Tables II and IV). In articular cartilage, GREM1 transcript levels amounted to 76.5% of ß2M levels (compared to 3.3% of ß2M levels in osteophytic cartilage). Quantitative RT-PCR also confirmed a 10.02-fold upregulation of FRZB in articular cartilage. SOX9, a key transcription factor for chondrogenesis and cartilage-specific genes, was moderately (2.59-fold), yet significantly ($P = 0.026$) higher expressed in articular cartilage. Quantitative RT-PCR could also demonstrate a significantly (5.42-fold) higher expression of PTHLH in articular cartilage.

The microarray analyses demonstrated significantly greater expression of the matrix molecules ACAN and SDC1 (syndecan 1) in articular cartilage. A 11.30-fold higher expression of ACAN could be detected in articular chondrocytes by quantitative RT-PCR (Fig. 2, Table IV).

Furthermore, HS3ST3A1 (heparan sulfate 3-O-sulfotransferase 3A1) was found to be expressed significantly higher in articular cartilage (Fig. 2, Table IV).

### *Histological differences between osteophytic and articular cartilage*

Immunohistological analysis of tissue sections revealed similarities but also distinct differences between osteophytic and articular cartilage isolated from osteoarthritic joints. While articular cartilage is typically characterized by a homogeneous proteoglycan-rich matrix indicated by strong Safranin-O- and Toluidine blue-staining throughout all of its tissue layers, osteophytic cartilage shows a stratified pattern [Fig. 3(A) and (B)]. In contrast to articular cartilage, the superficial half of the osteophytic cartilage layer is characterized by less intense Safranin-O-staining

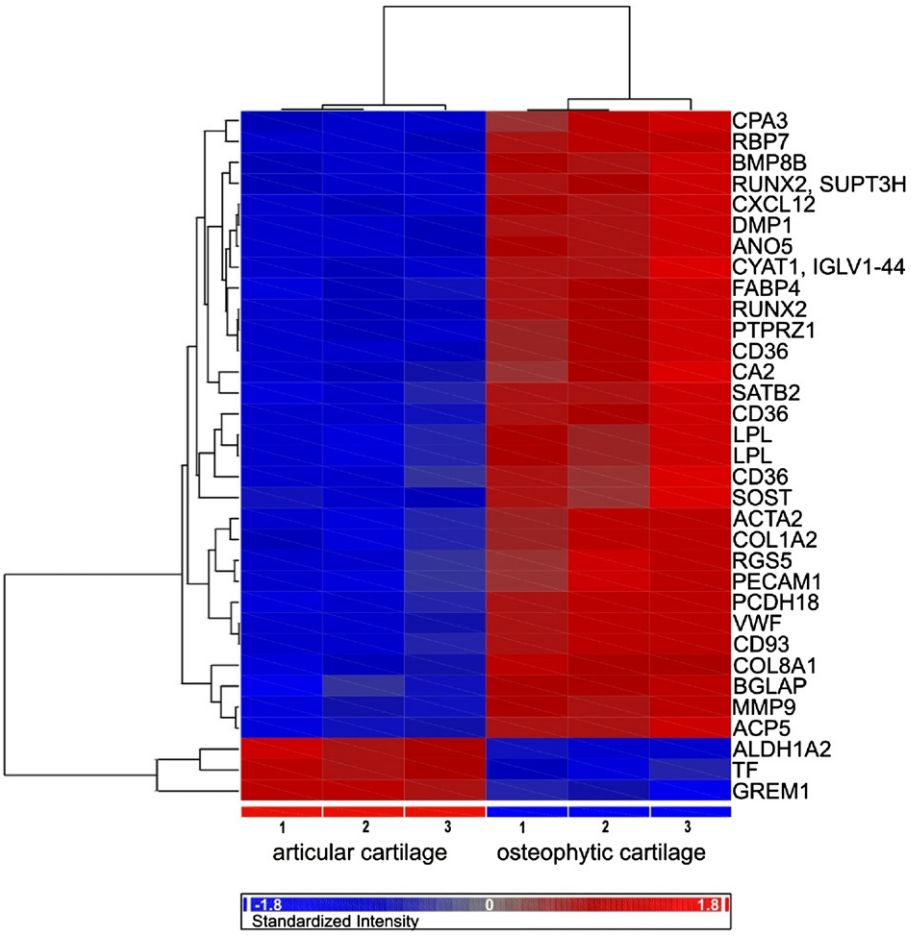


**Fig. 1.** Gene expression profile by cDNA array-analysis. Heat map visualizing hierarchical clustering analysis based on the expression levels of genes that are differentially expressed in osteophytic cartilage compared to articular cartilage. RNA preparations from $N = 15$ different donors were pooled to yield $N = 3$ paired sets of articular and osteophytic cartilage. The genes with a differential expression greater than 20-fold are shown. $P$-value $< 0.005$. (Red = higher expressed genes; blue = lower expressed genes).

and positive staining for type I collagen (Col1), which indicates fibrocartilaginous tissue. Instead, the lower half of osteophytic cartilage strongly stains for Safranin-O and Toluidine blue indicating a high content of proteoglycans. This layer also strongly stains for type II collagen (Col2) but is negative for Col1, which are basically typical features of hyaline articular cartilage. Thus, gross observation suggests similarities between this hyaline layer of osteophytic cartilage and articular cartilage, however, higher magnifications reveal definite differences. Most obviously, the deepest cell layer of osteophytic cartilage is characterized by large hypertrophic chondrocytes [Fig. 3(C)] which are absent in intact articular cartilage [Fig. 3(C)]. However, these hypertrophic chondrocytes are confined to the very deepest cell layer of osteophytic cartilage and not present in the overlying hyaline zone which consists of chondrocytes characterized by a non-hypertrophic phenotype [Fig. 3(C), middle zone]. Furthermore, in contrast to articular cartilage, osteophyte cartilage lacks a tide mark and a continuous plain subchondral bone plate, but exhibits a zone of irregular bone trabeculae.

Immunohistochemical detection of GREM1 [Fig. 3(D)] and BGLAP [Fig. 3(E)], the two most prominently differentially expressed genes, confirmed striking differences between osteophytic and articular cartilage. Articular chondrocytes were characterized by strong intracellular staining for GREM1 [arrows in Fig. 3(D)]. However, in osteophytic cartilage, GREM1 could neither be detected within the matrix, nor intracellularly within the osteophytic chondrocytes [open arrowheads in Fig. 3(D)]. GREM1 could be detected in the endothelial cell layer of invading bone marrow structures, but not in the bone trabeculae of osteophytes or the subchondral bone plate underneath articular cartilage.

BGLAP could immunohistochemically strongly be detected in osseous tissue both in the forming bone trabeculae of the osteophyte and the subchondral bone plate underneath the articular cartilage. Interestingly, most osteophytic chondrocytes, even non-hypertrophic chondrocytes within the middle zone, also showed positive intracellular staining for BGLAP, whereas articular chondrocytes and hyaline articular cartilage matrix were completely negative for BGLAP [Fig. 3(E)].

## Discussion

This was the first study to compare the genome-wide expression analysis of osteophytic and articular cartilage. Applying microarray analysis, we could demonstrate significant differences between these two cartilaginous tissues. The fundamental difference is based on the fact that osteophytic chondrocytes adopt a rather transient than permanent phenotype, which is underlined by its tendency to recapitulate the chondrogenic differentiation cascade cumulating in chondrocyte hypertrophy and endochondral ossification. Such phenotypic instability is also typical for cartilage repair tissue that had been induced by bone marrow-stimulating techniques, and although the cell origin may be

**Table II**
Differentially expressed genes in osteophytic cartilage (OC) and articular cartilage (AC) (change > 20-fold; *P*-value < 0.005)

| GenBank accession number | Gene symbol | Gene title | Fold-change (OC vs AC) | *P*-value |
|---|---|---|---|---|
| 206956_at | BGLAP | Bone gamma-carboxyglutamate (gla) protein | 91.22 | 0.003 |
| 203936_s_at | MMP9 | Matrix metallopeptidase 9 | 65.53 | 0.001 |
| 204638_at | ACP5 | Acid phosphatase 5, tartrate resistant | 47.38 | 0.001 |
| 200974_at | ACTA2 | Actin, alpha 2, smooth muscle, aorta | 42.86 | 0.002 |
| 209301_at | CA2 | Carbonic anhydrase II | 37.98 | 0.003 |
| 226237_at | Col8a1 | Collagen, type VIII, alpha 1 | 36.03 | 0.001 |
| 203548_s_at | LPL | Lipoprotein lipase | 34.57 | 0.001 |
| 203666_at | CXCL12 | Chemokine (C-X-C motif) ligand 12 | 33.31 | 0.0003 |
| 203549_s_at | LPL | Lipoprotein lipase | 32.90 | 0.002 |
| 202878_s_at | CD93 | CD93 molecule | 32.29 | 0.001 |
| 208175_s_at | DMP1 | Dentin matrix acidic phosphoprotein 1 | 32.00 | 0.0004 |
| 204469_at | PTPRZ1 | Receptor-type tyrosine-protein phosphatase zeta | 31.29 | 0.001 |
| 203980_at | FABP4 | Fatty acid binding protein 4, adipocyte | 31.26 | 0.0003 |
| 235275_at | BMP8B | BMP8B | 28.71 | 0.0001 |
| 223869_at | SOST | Sclerostin | 26.76 | 0.004 |
| 202112_at | VWF | Von Willebrand factor | 25.50 | 0.0004 |
| 229313_at | ANO5 | Anoctamin 5 | 24.18 | 0.0003 |
| 238066_at | RBP7 | Retinol binding protein 7, cellular | 23.90 | 0.0001 |
| 213435_at | SATB2 | SATB homeobox 2 | 23.50 | 0.002 |
| 205624_at | CPA3 | Carboxypeptidase A3 (mast cell) | 22.88 | 0.001 |
| 209555_s_at | CD36 | CD36 molecule (thrombospondin receptor) | 22.88 | 0.0046 |
| 228766_at | CD36 | CD36 molecule (thrombospondin receptor) | 22.66 | 0.0004 |
| 236858_s_at | RUNX2 | Runt-related transcription factor 2 | 22.46 | 0.0001 |
| 236858_s_at | SUPT3H | Transcription initiation protein SPT3 homolog | 22.46 | 0.0001 |
| 208981_at | PECAM1 | Platelet/endothelial cell adhesion molecule | 21.74 | 0.003 |
| 229218_at | COL1A2 | Collagen, type I, alpha 2 | 21.27 | 0.002 |
| 214677_x_at | CYAT1 | Cyclosporin A transporter 1 | 21.08 | 0.001 |
| 214677_x_at | IGLV1-44 | Immunoglobulin lambda variable 1-44 | 21.08 | 0.001 |
| 206488_s_at | CD36 | CD36 molecule (thrombospondin receptor) | 20.96 | 0.0005 |
| 236859_at | RUNX2 | Runt-related transcription factor 2 | 20.84 | 0.0003 |
| 225975_at | PCDH18 | Protocadherin 18 | 20.15 | 0.001 |
| 209071_s_at | RGS5 | Regulator of G-protein signaling 5 | 20.09 | 0.004 |
| 214063_s_at | TF | Transferrin | −21.95 | 0.001 |
| 218469_at | GREM1 | Gremlin-1 | −22.81 | 0.002 |
| 207016_s_at | ALDH1A2 | Aldehyde dehydrogenase 1 family, member A2 | −24.85 | 0.0001 |

different, osteophyte formation may therefore also be regarded as a model system for endogenous cartilage repair tissue[1,10]. So far, the molecular mechanisms, which distinguish between the transient and permanent chondrocyte phenotype, have not fully been identified. This study further contributes to the knowledge of the control of chondrogenesis and revealed striking differences in the expression pattern between osteophytic and articular cartilage.

**Table III**
Selected genes with roles in chondrogenesis and osteogenesis with higher expression in osteophytic cartilage (OC) in relation to articular cartilage (AC)

| GenBank accession number | Gene symbol | Gene title | Fold-change (OC vs AC) | *P*-value* | qRT-PCR | |
|---|---|---|---|---|---|---|
| | | | | | Fold-change (OC vs AC) mean (95% CI) | *P*-value* |
| **Signaling factor/receptors** | | | | | | |
| NM_001720 | BMP8B | BMP8B | 28.71 | 0.0001 | | |
| NM_025237 | SOST | Sclerostin | 26.76 | 0.004 | | |
| NM_004348 | RUNX2 | Runt-related transcription factor 2 | 20.84 | 0.0003 | 12.67 (−14.0–39.4) | 0.012 |
| NM_000474 | TWIST1 | Twist homolog 1 (Drosophila) | 6.29 | 0.006 | | |
| NM_015675 | GADD45B | Growth arrest and DNA damage-inducible, beta | 3.61 | 0.225 | 2.66 (−0.5–5.8) | 0.004 |
| **Matrix molecules** | | | | | | |
| NM_199173 | BGLAP | Bone gamma-carboxyglutamate (gla) protein (osteocalcin) | 91.22 | 0.003 | 52.78 (16.2–89.4) | 0.017 |
| NM_000089 | COL1A2 | Collagen type I, alpha 2 | 21.27 | 0.002 | 14.18 (−0.6–28.9) | 0.040 |
| NM_000088 | COL1A1 | Collagen, type I, alpha 1 | 12.51 | 0.003 | | |
| NM_004385 | VCAN | Versican | 8.12 | 0.002 | | |
| NM_000493 | COL10A1 | Collagen, type X, alpha 1 | 4.56 | 0.037 | 7.24 (−18.9–33.4) | 0.063 |
| **Enzymes** | | | | | | |
| NM_004994 | MMP9 | Matrix metallopeptidase 9 (gelatinase B) | 65.53 | 0.001 | | |
| NM_002427 | MMP13 | Matrix metallopeptidase 13 (collagenase 3) | 16.68 | 0.032 | 12.80 (−1.8–27.4) | 0.048 |
| NM_001523 | HAS1 | Hyaluronan synthase 1 | 7.75 | 0.004 | | |

* All *P* values were significant (<0.005 for microarray analysis) or (<0.05 for quantitative RT-PCR), except for the *P* values for genes TWIST1 and Col10A1.

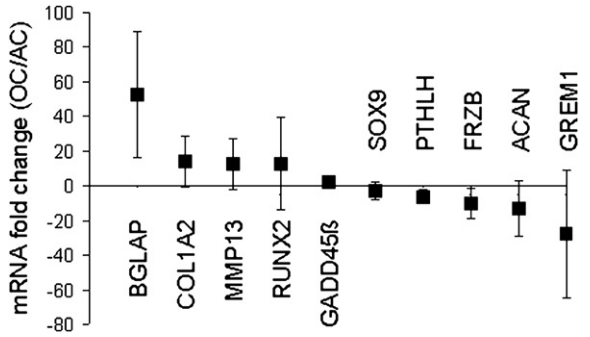


**Fig. 2.** Genes involved in chondrogenesis and endochondral ossification that are characterized by striking differential expression in osteophytic chondrocytes (OC) and articular chondrocytes (AC). The values represent the mean and 95% CI (error bars) of the fold-change in mRNA expression between osteophytic and articular chondrocytes of $N = 15$ different donors detected by quantitative RT-PCR.

It is well-known that the process of osteophyte formation is induced and supported by the action of BMPs, members of the TGFß superfamily that are involved in morphogenesis, bone formation and skeletal development[5,13–15]. Indeed, we could detect significantly greater expression of BMP8B in osteophytic chondrocytes. Its high homology to the bone-inducing factor BMP7, and its functional roles for embryonal cell differentiation and bone formation[16,17], underline its role for osteophyte formation.

Interestingly, the data of this study suggest a contrary pattern for articular cartilage in which the action of BMPs seems suppressed. Indeed, GREM1, a BMP-antagonist, was the gene which expression was most prominently higher in articular chondrocytes compared to osteophytic chondrocytes. Immunohistochemistry for GREM1 confirmed strong staining within articular chondrocytes but absent staining in osteophytic chondrocytes. GREM1 binds and blocks the action of BMP-2, -4 and-7, both on extra- and intracellular levels[18]. Intracellular binding was shown to exert more potent antagonistic effects[19]. In fact, we detected GREM1 predominantly intracellularly rather than extracellularly within the matrix of articular cartilage. During early embryogenic development, GREM1 is centrally involved in a self-regulatory feedback system that controls limb formation and supports the maintenance of the chondrocyte phenotype in early skeletal development[20]. In later developmental stages, GREM1 plays a central role in epiphyseal development. A recent genome wide microarray analysis identified GREM1 to be the most prominently higher expressed gene in the region of the epiphyses compared to the hypertrophic physeal zone of developing bones of 7-day-old mice[21]. The authors concluded that GREM1 helps to maintain the chondrocyte phenotype within the epiphyses, while the action of BMPs propagates endochondral ossification in the physes. In another study, GREM1 was predominantly detected in the resting zone of the growth plate of new-born rats, which may help to maintain the cells in a quiescent, non-hypertrophic state[22]. In our study, we could confirm the relevance of GREM1 in adult human tissue. GREM1 was significantly higher expressed in human articular chondrocytes, a cell population that maintains its phenotype and resists terminal differentiation, whereas GREM1 was not relevantly detected in osteophytic chondrocytes. The levels of GREM1 in articular cartilage were shown to be even further increased in OA [23]. The biological function of this phenomenon is still unclear, but may be secondarily triggered by elevated BMP-2/-4 levels present in osteoarthritic joints[23]. Interestingly, other previous studies demonstrated that overexpression of GREM1 inhibited BMP-induced osteophyte formation[14] and that elevated GREM1-levels were measured in non-union fracture callus in which cartilaginous callus failed to undergo ossification[24].

GREM1 may also have a role in other tissues such as endothelial structures[25]. Immunohistochemically, we could also detect GREM1 in the endothelium of ingrowing vessels, however, we could not detect GREM1 within bone trabeculae of osteophytes or the subchondral bone plate underneath the articular cartilage.

In articular cartilage, the microarray analysis also revealed a significantly higher expression of STC2, WISP3, and FRZB. Similar to GREM1, these factors also act as inhibitors and modulators of growth factor-signaling. STC2 was shown to exhibit potent growth-suppressive effects. Constitutive overexpression of STC2 in mice resulted in pre- and postnatal growth restriction and delayed intramembraneous and endochondral bone development[26]. WISP3 was not only shown to inhibit BMP- and Wnt-signaling but also to stabilize the chondrocyte phenotype by inhibiting IGF-1-induced cellular maturation and hypertrophy[27–29]. FRZB predominantly antagonizes the signaling of Wnt ligands and thus

**Table IV**
Selected genes with roles in chondrogenesis and osteogenesis with higher expression in articular cartilage (AC) in relation to osteophytic cartilage (OC)

| GenBank accession number | Gene symbol | Gene title | Fold-change (AC vs OC) | *P*-value* | qRT-PCR | *P*-value* |
|---|---|---|---|---|---|---|
| | | | | | Fold-change (AC vs OC) mean (95% CI) | |
| **Signaling factors/receptors** | | | | | | |
| NM_013372 | GREM1 | Gremlin-1 | 22.81 | 0.002 | 24.77 (11.4–38.2) | 0.011 |
| NM_003714 | STC2 | Stanniocalcin-2 | 8.34 | 0.001 | | |
| NM_003880 | WISP3 | WNT1 inducible signaling pathway protein-3 | 7.12 | 0.002 | | |
| NM_006272 | S100B | S100 calcium binding protein B | 6.00 | 0.001 | | |
| NM_006271 | S100A1 | S100 calcium binding protein A1 | 4.50 | 0.003 | | |
| NM_000557 | GDF5 | Growth differentiation factor-5 | 4.45 | 0.005 | | |
| NM_001463 | FRZB | Frizzled-related protein | 3.78 | 0.004 | 10.02 (1.4–18.7) | 0.030 |
| NM_000346 | SOX9 | SRY (sex-determining region Y)-box 9 | 2.25 | 0.066 | 2.59 (−2.5–7.7) | 0.026 |
| NM_002820 | PTHLH | Parathyroid hormone-like hormone | 1.39 | 0.216 | 5.42 (3.8–7.0) | 0.016 |
| **Matrix molecules** | | | | | | |
| NM_001135 | ACAN | Aggrecan | 4.23 | 0.003 | 11.30 (9.2–13.4) | 0.003 |
| NM_002997 | SDC1 | Syndecan 1 | 3.82 | 0.001 | | |
| **Enzymes** | | | | | | |
| NM_006042 | HS3ST3A1 | Heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1 | 16.50 | 0.003 | | |

* All *P* values were significant (<0.005 for microarray analysis) or (<0.05 for quantitative RT-PCR).

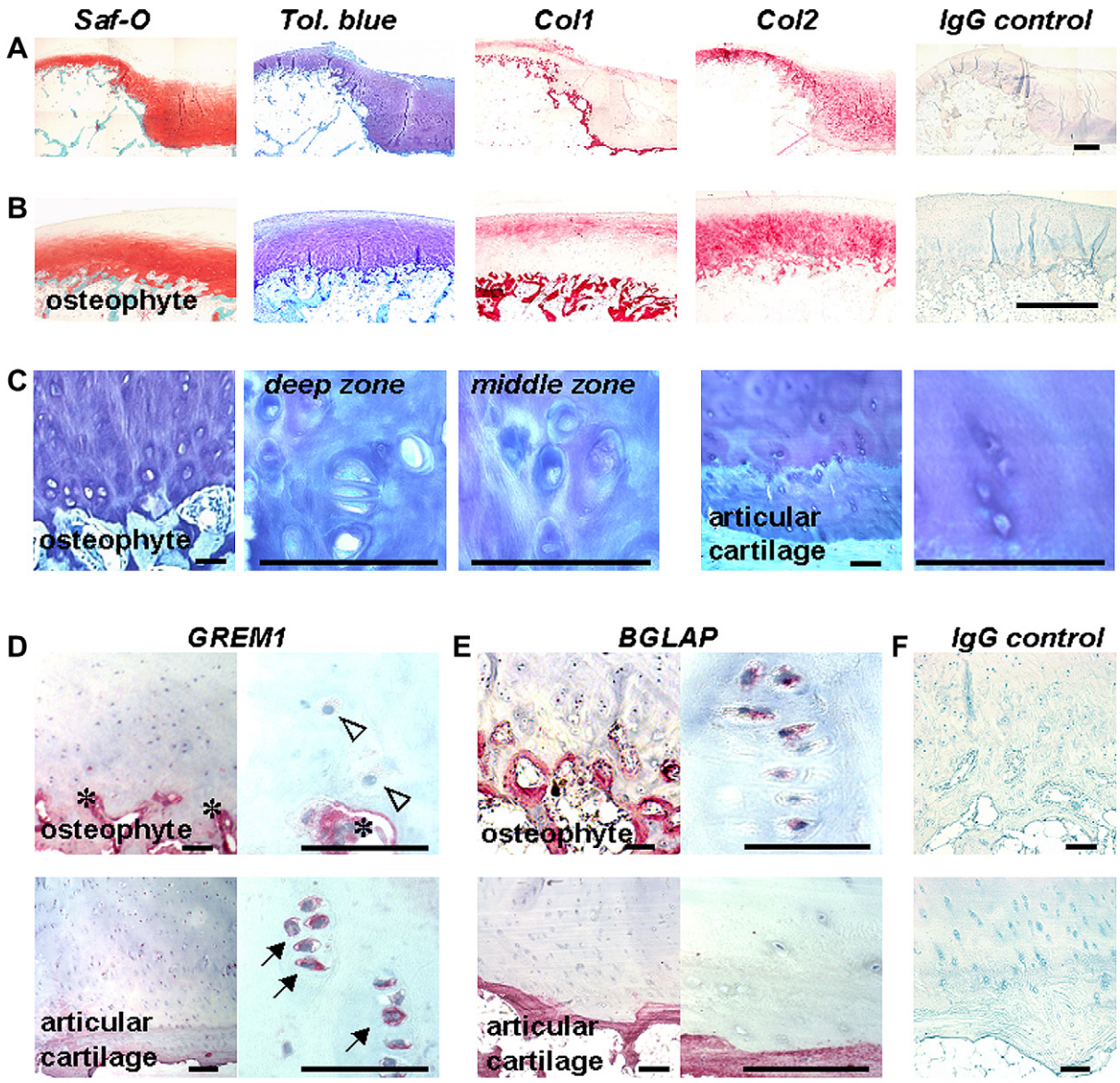


**Fig. 3.** Immunohistochemical analysis of osteophytic and articular cartilage of the border zone of an osteoarthritic joint. (A): Overviews of the transition area of osteophyte and articular cartilage. Insert with high magnification depicts the non-hypertrophic phenotype of articular chondrocytes in deep zones. (B): Higher magnifications of osteophyte cartilage demonstrate a superficial Col1-positive fibrocartilaginous layer, a deeper Col2-positive hyaline cartilage layer, and underlying osseous tissue. (C): High magnifications (Toluidine blue staining) of the lower half of osteophyte cartilage depict hypertrophic chondrocytes only in the deepest cell layer but not in the overlying middle zone. The deep zone of articular cartilage is characterized by a clear tide mark without cellular hypertrophy within the overlying non-calcified cartilage. (D): GREM1 immunohistochemistry shows positive staining for endothelial structures (*) but negative staining for subchondral bone. Higher magnifications demonstrate negative staining for osteophytic chondrocytes (open arrowhead) but strong intracellular staining for articular chondrocytes (arrow). (E): BGLAP immunohistochemistry shows strong positive staining for subchondral bone. Osteophytic chondrocytes are characterized by intracellular staining for osteocalcin, whereas articular chondrocytes are completely negative. (F): No non-specific staining was evident in negative controls incubated with isotype normal mouse IgG. Bar = 1 mm in A, B; Bars = 50 μm in C, D, E, F. Safranin-O–Fast green staining; Toluidine blue staining.

negatively regulates trabecular bone development. In developing bones, FRZB was prominently expressed within the epiphyses and delays transformation to hypertrophy and endochondral ossification[21,30].

S100A and S100B are factors that are abundantly present in articular cartilage. Although their roles have not completely been defined yet, they were shown to inhibit terminal chondrocyte differentiation and, thus, may stabilize the permanent chondrocyte phenotype[31]. S100A and S100B are under the transcriptional control of SOX9, which is the key transcription factor for cartilage-specific genes[32]. The present study confirmed significant higher expression of SOX9 in permanent articular cartilage compared to transient osteophytic cartilage. SOX9 is known to inhibit chondrocyte hypertrophy and calcification, which is an effect that may, at least in part, be mediated by transcriptional activation of PTHLH[33], which delays or inhibits terminal chondrocyte differentiation and endochondral ossification during skeletal development and repair[34,35]. Thus, it was not surprising to find PTHLH significantly higher expressed in permanent articular cartilage.

Articular cartilage is generally characterized by a low turnover rate of cellular components and the collagen network, with the exception of the high turnover rate of the proteoglycan content. The inherent need to maintain the integrity of the extracellular cartilage matrix was reflected by high expression of ACAN and HS3ST3A1 in articular chondrocytes. On the contrary, osteophytic cartilage is characterized by a high expression of collagens including the types

COL1A1 and COL1A2, which are characteristic of fibrous tissue and may primarily be ascribed to the superficial fibrocartilaginous zone.

It is important to clearly distinguish the transient chondrocyte phenotype from the hypertrophic chondrocyte phenotype. While transient chondrocytes display many features of articular chondrocytes such as production of a hyaline-like matrix, hypertrophic chondrocytes are terminally differentiated cells in a pre-apoptotic status and immediately prone to be replaced by osseous tissue. Although, transient osteophytic cartilage finally underlies endochondral ossification, this process is prolongated in osteophytes. Previous histochemical studies demonstrated a restriction of positive COL10-staining to the very deepest cell layer of osteophyte cartilage, while the overlying zones were negative for COL10[10,36]. Since COL10 is a typical marker for chondrocyte hypertrophy and terminal differentiation, such restricted staining pattern indicates that most osteophytic chondrocytes have not attained the status of hypertrophy yet. This is also confirmed by gene expression analyses of the present study with only marginal, but not significant, upregulation of COL10A1. Thus, the osteophytic specimen of this study contained for the utmost part non-hypertrophic cells. Nevertheless, their transient character was underlined by greater expression of matrix metalloproteinases, including MMP9, and MMP13, that are responsible for matrix remodeling and resorption.

In osteophytic chondrocytes, BGLAP was the most striking differentially expressed gene with a 91.22-fold higher expression. Originally, this protein was defined as an osteoblastic marker protein and its expression in chondrocytes has not been published so far. The positive intracellular staining of BGLAP even in non-hypertrophic osteophytic chondrocytes may suggest an osteogenic fate of these cells. However, the relevance of BGLAP should be interpreted with care since the expression levels in osteophytic chondrocytes were only moderate and the dramatic difference between the articular and osteophytic cartilage was primarily a result of extremely low or even absent transcript levels of BGLAP in articular chondrocytes (with less than 0.6% of the transcript levels of ß2M). Nevertheless, the osteogenic fate of osteophytic chondrocytes was underlined by significantly elevated transcript levels of RUNX2, an important transcription factor that induces terminal chondrocyte differentiation, supports osteoblast differentiation and is essential for bone formation and maturation[37]. In osteophytic chondrocytes, we could also detect higher levels of GADD45ß, a target of RUNX2. In recent studies, GADD45ß could be detected in osteophytes and was also shown to be an essential mediator of COL10A1 and MMP13 expression in hypertrophic chondrocytes in the mouse embryo[38,39].

Osteophytic chondrocytes were also characterized by higher expression of TWIST1 which is a transcription factor that plays both positive and negative roles in the regulation of skeletal development and differentiation of mesenchymal cells. As a target of the canonical Wnt-signaling, TWIST1 was shown to suppress the expression of chondrocyte-specific genes[40] and promote osteoblast differentiation and osteogenesis[41].

Microarray analysis also revealed upregulation of SOST in osteophytic chondrocytes. Originally, SOST was considered as a product of osteocytes with inhibitory effects on osteoblasts and formation of novel bone[42]. However, recent studies also detected expression of SOST in hypertrophic chondrocytes of the growth plate and chondrocytes of osteoarthritic cartilage[43,44]. Since SOST expression was shown to be suppressed by PTH/PTHLH[45], the higher SOST expression in osteophytes may be a consequence of decreased PTHLH levels. The functional role of SOST in osteophytic cartilage has yet to be further investigated in detail, but it may exert a modulatory role and decelerate the process of endochondral ossification[42,43].

On a transcriptional level, osteophytic cartilage shares similarities with growth plate cartilage and fracture callus. Thus, a number of genes that were significantly greater expressed in osteophytic cartilage, including BGLAP, RUNX2, GADD45B, MMP9, and MMP13, are also expressed in the hypertrophic zone of growth plate cartilage[6,37,39,46–48] and involved in the process of fracture healing[49]. However, fracture callus and growth plate cartilage are characterized by a considerably higher prevalence of hypertrophic chondrocytes and a far more rapid endochondral ossification process than osteophyte tissue which develops over months or years[10,49]. Thus, osteophytic cartilage contains transient, yet predominantly non-hypertrophic chondrocytes, and is therefore better suited for comparison with permanent articular cartilage.

Despite the careful harvesting technique of osteophytic cartilage in the present study, some contamination with terminally differentiated chondrocytes may not be completely excluded, however, this drawback would exert rather an irrelevant statistical impact on overall gene expression. Another limitation of this study is the isolation of articular cartilage from osteoarthritic joints. However, according to a previous comprehensive microarray analysis, articular cartilage of early degenerative osteoarthritic cartilage is characterized by no or only marginal differences in its gene expression profile compared with healthy articular cartilage[50]. Thus, articular chondrocytes from macroscopically intact cartilage, as used in this study, should represent a valid population of normal articular chondrocytes.

On a transcriptional level, the bradytrophic permanent articular cartilage significantly differs from osteophytic cartilage that is characterized by cell proliferation and tissue remodeling including anabolic and catabolic processes. Thus, articular chondrocytes are characterized by a stable phenotype and their status of cellular quiescence is reflected by the expression of a number of inhibitory factors. The maintenance of the permanent articular cartilage may not only be supported by the activity of pro-chondrogenic factors, such as SOX9, but may also essentially depend on the activity of antagonistic and inhibitory factors. In this respect, GREM1, WISP3 and FRZB, which modulate BMP- and Wnt-signaling pathways may lock the chondrocyte phenotype in articular cartilage and prevent the terminal chondrocyte differentiation process. These data may also provide novel concepts for regenerative strategies which may not only focus on anabolic agents but which may also have to consider the role of modulating antagonistic factors.

## Role of the funding source

This work was supported by the IZKF (Interdisciplinary Centre for Clinical Research of the University of Erlangen-Nürnberg) Grant A36 and the German Research Foundation (DFG) Grant GE 1975/2-1.

## Contributions

All authors have made *substantial contributions to all three of sections* (1), (2) *and* (3) *below*: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data (2) drafting the article or revising it critically for important intellectual content (3) final approval of the version to be submitted.

## Conflict of interests

There are no competing interests.

## Acknowledgments

We thank Petra Rothe, Melanie Pflügner and Herbert Rohrmüller for expert technical assistance.

## References


1. Blanke M, Carl HD, Klinger P, Swoboda B, Hennig F, Gelse K. Transplanted chondrocytes inhibit endochondral ossification within cartilage repair tissue. Calcif Tissue Int 2009;85(5): 421–33.
2. Henderson IJ, La Valette DP. Subchondral bone overgrowth in the presence of full-thickness cartilage defects in the knee. Knee 2005;12(6):435–40.
3. Kreuz PC, Steinwachs MR, Erggelet C, Krause SJ, Konrad G, Uhl M, *et al*. Results after microfracture of full-thickness chondral defects in different compartments in the knee. Osteoarthritis Cartilage 2006;14(11):1119–25.
4. Mithoefer K, Williams 3rd RJ, Warren RF, Potter HG, Spock CR, Jones EC, *et al*. The microfracture technique for the treatment of articular cartilage lesions in the knee. A prospective cohort study. J Bone Joint Surg Am 2005;87(9):1911–20.
5. van der Kraan PM, van den Berg WB. Osteophytes: relevance and biology. Osteoarthritis Cartilage 2007;15(3):237–44.
6. Goldring MB, Tsuchimochi K, Ijiri K. The control of chondrogenesis. J Cell Biochem 2006;97(1):33–44.
7. van Beuningen HM, Glansbeek HL, van der Kraan PM, van den Berg WB. Differential effects of local application of BMP-2 or TGF-beta 1 on both articular cartilage composition and osteophyte formation. Osteoarthritis Cartilage 1998;6(5): 306–17.
8. van der Kraan PM, Vitters EL, van Beuningen HM, van de Putte LB, van den Berg WB. Degenerative knee joint lesions in mice after a single intra-articular collagenase injection. A new model of osteoarthritis. J Exp Pathol (Oxford) 1990;71(1): 19–31.
9. Nakase T, Miyaji T, Tomita T, Kaneko M, Kuriyama K, Myoui A, *et al*. Localization of bone morphogenetic protein-2 in human osteoarthritic cartilage and osteophyte. Osteoarthritis Cartilage 2003;11(4):278–84.
10. Gelse K, Soder S, Eger W, Diemtar T, Aigner T. Osteophyte development–molecular characterization of differentiation stages. Osteoarthritis Cartilage 2003;11(2):141–8.
11. Aigner T, Zien A, Gehrsitz A, Gebhard PM, McKenna L. Anabolic and catabolic gene expression pattern analysis in normal versus osteoarthritic cartilage using complementary DNA-array technology. Arthritis Rheum 2001;44(12):2777–89.
12. Gelse K, Muhle C, Franke O, Park J, Jehle M, Durst K, *et al*. Cell-based resurfacing of large cartilage defects: long-term evaluation of grafts from autologous transgene-activated periosteal cells in a porcine model of osteoarthritis. Arthritis Rheum 2008;58(2):475–88.
13. Gelse K, Jiang QJ, Aigner T, Ritter T, Wagner K, Poschl E, *et al*. Fibroblast-mediated delivery of growth factor complementary DNA into mouse joints induces chondrogenesis but avoids the disadvantages of direct viral gene transfer. Arthritis Rheum 2001;44(8):1943–53.
14. Blaney Davidson EN, Vitters EL, van Beuningen HM, van de Loo FA, van den Berg WB, van der Kraan PM. Resemblance of osteophytes in experimental osteoarthritis to transforming growth factor beta-induced osteophytes: limited role of bone morphogenetic protein in early osteoarthritic osteophyte formation. Arthritis Rheum 2007;56(12):4065–73.
15. Balemans W, Van Hul W. Extracellular regulation of BMP signaling in vertebrates: a cocktail of modulators. Dev Biol 2002;250(2):231–50.
16. Ying Y, Qi X, Zhao GQ. Induction of primordial germ cells from murine epiblasts by synergistic action of BMP4 and BMP8B signaling pathways. Proc Natl Acad Sci U S A 2001;98(14): 7858–62.
17. Ozkaynak E, Schnegelsberg PN, Jin DF, Clifford GM, Warren FD, Drier EA, *et al*. Osteogenic protein-2. A new member of the transforming growth factor-beta superfamily expressed early in embryogenesis. J Biol Chem 1992;267(35):25220–7.
18. Merino R, Rodriguez-Leon J, Macias D, Ganan Y, Economides AN, Hurle JM. The BMP antagonist Gremlin regulates outgrowth, chondrogenesis and programmed cell death in the developing limb. Development 1999;126(23):5515–22.
19. Sun J, Zhuang FF, Mullersman JE, Chen H, Robertson EJ, Warburton D, *et al*. BMP4 activation and secretion are negatively regulated by an intracellular gremlin-BMP4 interaction. J Biol Chem 2006;281(39):29349–56.
20. Benazet JD, Bischofberger M, Tiecke E, Goncalves A, Martin JF, Zuniga A, *et al*. A self-regulatory system of interlinked signaling feedback loops controls mouse limb patterning. Science 2009;323(5917):1050–3.
21. Shapiro F, Flynn E, Calicchio ML. Molecular differentiation in epiphyseal and physeal cartilage. Prominent role for gremlin in maintaining hypertrophic chondrocytes in epiphyseal cartilage. Biochem Biophys Res Commun 2009;390(3):570–6.
22. Nilsson O, Parker EA, Hegde A, Chau M, Barnes KM, Baron J. Gradients in bone morphogenetic protein-related gene expression across the growth plate. J Endocrinol 2007;193(1):75–84.
23. Tardif G, Hum D, Pelletier JP, Boileau C, Ranger P, Martel-Pelletier J. Differential gene expression and regulation of the bone morphogenetic protein antagonists follistatin and gremlin in normal and osteoarthritic human chondrocytes and synovial fibroblasts. Arthritis Rheum 2004;50(8):2521–30.
24. Fajardo M, Liu CJ, Egol K. Levels of expression for BMP-7 and several BMP antagonists may play an integral role in a fracture nonunion: a pilot study. Clin Orthop Relat Res 2009;467(12): 3071–8.
25. Sha G, Zhang Y, Zhang C, Wan Y, Zhao Z, Li C, *et al*. Elevated levels of gremlin-1 in eutopic endometrium and peripheral serum in patients with endometriosis. Fertil Steril 2009;91(2): 350–8.
26. Gagliardi AD, Kuo EY, Raulic S, Wagner GF, DiMattia GE. Human stanniocalcin-2 exhibits potent growth-suppressive properties in transgenic mice independently of growth hormone and IGFs. Am J Physiol Endocrinol Metab 2005; 288(1):E92–E105.
27. Yang Y, Liao E. Mutant WISP3 triggers the phenotype shift of articular chondrocytes by promoting sensitivity to IGF-1 hypothesis of spondyloepiphyseal dysplasia tarda with progressive arthropathy (SEDT-PA). Med Hypotheses 2007; 68(6):1406–10.
28. Nakamura Y, Weidinger G, Liang JO, Aquilina-Beck A, Tamai K, Moon RT, *et al*. The CCN family member Wisp3, mutant in progressive pseudorheumatoid dysplasia, modulates BMP and Wnt signaling. J Clin Invest 2007;117(10):3075–86.
29. Cui RR, Huang J, Yi L, Xie H, Zhou HD, Yuan LQ, *et al*. WISP3 suppresses insulin-like growth factor signaling in human chondrocytes. Mol Cell Endocrinol 2007;279(1–2):1–8.
30. Bodine PV, Zhao W, Kharode YP, Bex FJ, Lambert AJ, Goad MB, *et al*. The Wnt antagonist secreted frizzled-related protein-1 is a negative regulator of trabecular bone formation in adult mice. Mol Endocrinol 2004;18(5):1222–37.
31. Saito T, Ikeda T, Nakamura K, Chung UI, Kawaguchi H. S100A1 and S100B, transcriptional targets of SOX trio, inhibit terminal differentiation of chondrocytes. EMBO Rep 2007;8(5):504–9.
32. de Crombrugghe B, Lefebvre V, Behringer RR, Bi W, Murakami S, Huang W. Transcriptional mechanisms of chondrocyte differentiation. Matrix Biol 2000;19(5):389–94.
33. Amano K, Hata K, Sugita A, Takigawa Y, Ono K, Wakabayashi M, *et al*. Sox9 family members negatively regulate maturation and

calcification of chondrocytes through up-regulation of parathyroid hormone-related protein. Mol Biol Cell 2009;20(21):4541–51.
34. Minina E, Wenzel HM, Kreschel C, Karp S, Gaffield W, McMahon AP, *et al*. BMP and Ihh/PTHrP signaling interact to coordinate chondrocyte proliferation and differentiation. Development 2001;128(22):4523–34.
35. Fischer J, Dickhut A, Rickert M, Richter W. Human articular chondrocytes secrete parathyroid hormone-related protein and inhibit hypertrophy of mesenchymal stem cells in coculture during chondrogenesis. Arthritis Rheum 2010;62(9):2696–706.
36. Aigner T, Dietz U, Stoss H, von der Mark K. Differential expression of collagen types I, II, III, and X in human osteophytes. Lab Invest 1995;73(2):236–43.
37. Komori T. Regulation of bone development and extracellular matrix protein genes by RUNX2. Cell Tissue Res 2010;339(1):189–95.
38. Ijiri K, Zerbini LF, Peng H, Otu HH, Tsuchimochi K, Otero M, *et al*. Differential expression of GADD45beta in normal and osteoarthritic cartilage: potential role in homeostasis of articular chondrocytes. Arthritis Rheum 2008;58(7):2075–87.
39. Ijiri K, Zerbini LF, Peng H, Correa RG, Lu B, Walsh N, *et al*. A novel role for GADD45beta as a mediator of MMP-13 gene expression during chondrocyte terminal differentiation. J Biol Chem 2005;280(46):38544–55.
40. Reinhold MI, Kapadia RM, Liao Z, Naski MC. The Wnt-inducible transcription factor Twist1 inhibits chondrogenesis. J Biol Chem 2006;281(3):1381–8.
41. Connerney J, Andreeva V, Leshem Y, Mercado MA, Dowell K, Yang X, *et al*. Twist1 homodimers enhance FGF responsiveness of the cranial sutures and promote suture closure. Dev Biol 2008;318(2):323–34.
42. Moester MJ, Papapoulos SE, Lowik CW, van Bezooijen RL. Sclerostin: current knowledge and future perspectives. Calcif Tissue Int 2010;87(2):99–107.
43. Chan BY, Fuller ES, Russell AK, Smith SM, Smith MM, Jackson MT, *et al*. Increased chondrocyte sclerostin may protect against cartilage degradation in osteoarthritis. Osteoarthritis Cartilage 2011;19(7):874–85.
44. van Bezooijen RL, Bronckers AL, Gortzak RA, Hogendoorn PC, van der Wee-Pals L, Balemans W, *et al*. Sclerostin in mineralized matrices and van Buchem disease. J Dent Res 2009;88(6):569–74.
45. Keller H, Kneissel M. SOST is a target gene for PTH in bone. Bone 2005;37(2):148–58.
46. Gerstenfeld LC, Shapiro FD. Expression of bone-specific genes by hypertrophic chondrocytes: implication of the complex functions of the hypertrophic chondrocyte during endochondral bone development. J Cell Biochem 1996;62(1):1–9.
47. Sims NA, White CP, Sunn KL, Thomas GP, Drummond ML, Morrison NA, *et al*. Human and murine osteocalcin gene expression: conserved tissue restricted expression and divergent responses to 1,25-dihydroxyvitamin D3 in vivo. Mol Endocrinol 1997;11(11):1695–708.
48. Ortega N, Wang K, Ferrara N, Werb Z, Vu TH. Complementary interplay between matrix metalloproteinase-9, vascular endothelial growth factor and osteoclast function drives endochondral bone formation. Dis Model Mech 2010;3(3–4):224–35.
49. Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. J Cell Biochem 2003;88(5):873–84.
50. Aigner T, Fundel K, Saas J, Gebhard PM, Haag J, Weiss T, *et al*. Large-scale gene expression profiling reveals major pathogenetic pathways of cartilage degeneration in osteoarthritis. Arthritis Rheum 2006;54(11):3533–44.
51. Peng X, Wood CL, Blalock EM, Chen KC, Landfield PW, Stromberg AJ. Statistical implications of pooling RNA samples for microarray experiments. BMC Bioinformatics 2003;4:26.