changes towards de-differentiation or hypertrophy. The loss of normal articular chondrocyte phenotype could be a consequence miRNAs expression changes. Our objectives were: i) to identify alteration in miRNAs pattern during osteoarthritis establishment or in a model of phenotype chondrocytes loss (cytokine stress, subculturing); ii) to search for their potential targets by in silico analysis; iii) to confirm the functional role of the miRNA candidate with the highest differential expression.

Methods: A 1200 miRNAs pattern has been established by microRNA chip analysis of human chondrocytes stimulated with IL-1beta or after sub-culturing (P3). The expression of candidates miRNAs (selected after chip analysis) was confirmed by qRT-PCR and their potential targets identified by in silico analysis using Microcosm, targetScan and PicTar tools. Amongst miRNAs of interest, we focused on miR-29b, known to be a regulator of collagen expression in other cell types. Then, we performed qRT-PCR analysis of 18 osteoarthritic patients and 7 healthy age-matched donors. The miR-29b potential seed region identified in the 3′UTR of type II collagen was cloned directly upstream luciferase coding sequence and under constitutive promoter control. Stable transfectants overexpressing miR-29b were generated in ATDC5 chondrogenic cell line and collagen synthesis was followed up during differentiation (Siris red staining). Finally, chondrocytes from 5 patients were transfected with miR-29b overexpression vector and type II collagen neosynthesis was determined by C2CP ELISA.

Results: A subset of 20 miRNAs differentially expressed during experimentally induced loss of chondrocyte phenotype was isolated. Some of these miRNAs (miR-140, mir-455) were previously identified as differentially expressed in OA vs healthy cartilage. Amongst miRNAs of interest, we focused on miR-29b as it was overexpressed in IL-1beta challenged chondrocytes and known to be involved in type I and III collagen expression in myocytes and fibroblasts. We demonstrated a 6-fold induction of miR-29b in chondrocytes of osteoarthritic patients. Reporter gene experiments (luciferase activity) of chimeric constructs (luciferase-Type II collagen 3′UTR) in Hela cells confirmed the presence of miR-29b seed region. Stable overexpression of miR-29b in ATDC5 cell line reduced collagen synthesis during chondrogenic differentiation. Finally, preliminary data suggested that miR-29 overexpression in human chondrocytes reduced C2CP secretion (25%).

Conclusions: MiRNAs are differentially expressed during loss of chondrocytes phenotype and miR-29b is over expressed in osteoarthritic patients. Using molecular (mutation of seed sequence) and functional (Siris red staining and C2CP ELISA) approaches we demonstrated that miR-29b inhibited type II collagen expression.

271 POTENTIAL ROLE OF NUCLEAR ORPHAN RECEPTORS NR4A1 AND NR4A3 IN HUMAN CHONDROCYTES

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Purpose: Chondrocyte apoptosis and abundance of matrix-metalloproteases (MMPs) and aggregecans (ADAMTSs) leads to breakdown of articular cartilage in osteoarthritis (OA). A previous gene expression profiling experiment using chondrocytes treated with the lipid signalling mediator sphingoamine-1-phosphate (S1P) has shown all three members of the nuclear orphan receptor NR4A subgroup among the top ten deregulated genes. This finding was confirmed by quantitative real-time PCR. Nuclear orphan receptors act as ligand-independent transcriptionally activated regulator proteins; they are known to be involved in proliferation, apoptosis and gene-regulation in various tissues. We evaluated the influence of NR4A3 on chondrocyte gene expression. Furthermore we investigated the role of NR4A1 by employing the NR4A1 agonist Cyto sporone B (Cnb).

Methods: siRNA knock-down of NR4A1 and NR4A3 was performed in primary human chondrocytes isolated from OA patients who underwent total knee joint replacement and in the C28I2 chondrocyte cell line. Relative mRNA expression of NR4A1 and NR4A3 receptor, ADAMTS-4 and -5, MMP13, iNOS and collagen II was determined using quantitative real-time PCR. NR4A1 expression was quantified in C28I2 cells exposed to CsnB. Cytotoxicity (MTS assay) and apoptosis (Caspase 3/7 activity) were evaluated after 12 and 72 hours, respectively.

Results: NR4A1/CsnB increases NR4A1 gene expression in a concentration dependent manner. CsnB dependent NR4A1 presence led to increased caspase-3 and -7 activities in human chondrocytes and C28I2 cell line cells, respectively. NR4A3 mRNA was found to be expressed in OA chondrocytes and in the C28I2 cell line. siRNA knock-down achieved 60% (± 7%) reduction of NR4A3 mRNA expression. This decrease in NR4A3 expression led to a 45% (± 5%) decline of MMP-13 expression. Furthermore ADAMTS-5 mRNA expression was reduced 54% (± 22%). Gene expression of ADAMTS-4, iNOS, and collagen II remained unaltered by diminished NR4A3 expression.

Conclusions: Our results implicate a pro-apoptotic role of NR4A1 in human chondrocytes. Furthermore, NR4A3 is involved in the regulation of MMP-13 and ADAMTS-5 expression. NR4A1 and NR4A3 nuclear receptors might be of relevance for future osteoarthritis therapies.

272 STIMULATION OF SUPERFICIAL ZONE PROTEIN/LUBRICIN ACCUMULATION BY HEDGEHOG SIGNALING IN SURFACE ZONE ARTICULAR CHONDROCYTE

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Purpose: Superficial zone protein (SZP), homologous to lubricin, is a large proteoglycan that is synthesized and secreted into synovial fluid by surface zone articular chondrocytes (SZAC) and synovial cells. SZP is known to function as a boundary lubricant of articular cartilage and plays an important role in cartilage homeostasis and degeneration. It is well established that cytokines play important roles in cartilage homeostasis. We have previously reported that TGF-β is a critical regulator of SZP synthesis and accumulation in SZAC. In addition, hedgehog signaling also plays a key role in skeletal development, including synovial joint induction, and has been implicated in cartilage homeostasis and the pathogenesis of osteoarthritis. However, the action of hedgehog signaling on SZAC is unknown. We hypothesized that hedgehog signaling regulates SZP synthesis and accumulation in SZAC. The aim of this study was to investigate the action of hedgehog signaling on SZP accumulation. We also examined interactions between hedgehog signaling and TGF-β1.

Methods: Surface zone articular cartilage was harvested from the femoral condyles of stifl joints from 3-month-old calves and digested with 0.2% collagenase. Isolated chondrocytes were cultured in monolayer at a density of 1x10^5 cells/well in 12-well plates in DMEM/F12 with 10% FBS. After 24-hours equilibration, cells were switched to serum-free DMEM/F12 with ITS+ Premix containing various concentrations of Sonic hedgehog (Shh), Indian hedgehog (Ihh), hedgehog signaling inhibitor cyclopamine, and TGF-β1 (n=6, for all experiments). The medium from the cultures was harvested after the 4-day treatment and quantitatively analyzed for SZP protein by enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody S67.9.

Results: Both Shh and Ihh stimulated SZP accumulation significantly (p<0.01) at all concentration (0.1, 0.3 and 1 μg/ml). Compared to control, 1 μg/ml Shh treatment elicited a maximum increase of 2.4-fold while 1 μg/ml Ihh produced a 1.7-fold increase (Figure 1). The up-regulation of SZP accumulation by 1 μg/ml Shh was reduced in part by 1 μM cyclopamine and completely abolished at a dose of 10 μM (Figure 2). In addition, there were additive effects between Shh (1 μg/ml) and Ihh (1 μg/ml) and TGF-β1 (1,3 and 10 ng/ml) (Figure 3).

Conclusions: The present study showed for the first time the action of hedgehog signaling on SZAC. Our results demonstrate that activation of hedgehog signaling by Shh and Ihh stimulates SZP accumulation. These results might provide insights into understanding normal articular joint homeostasis and the progression of cartilage degeneration, and aid in the potential utility of hedgehog signaling in cartilage regeneration.
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FUNCTIONAL ESTROGEN BIOSYNTHESIS MACHINERY IS EXPRESSED IN HUMAN POSTMENOPAUSIC OSTEOARTHRITIS CHONDROCYTES

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Purpose: High prevalence of osteoarthritis (OA) in postmenopausic women has led to growing interest in the study of estrogen deprivation as a physiopathologic mechanism for the development of OA. In fact, ovariectomized rabbits presented higher cartilage damage than controls. However, a relationship between circulating estrogen levels and the progression of OA has not been found. Therefore local estrogens may be more important in regulating chondrocyte functions in cartilage. This data led us to investigate whether human chondrocytes can produce local estrogen despite of the systemic estrogen depletion.

Methods: Human chondrocytes were obtained from postmenopausic women (mean age 73) undergoing total knee arthroplasty. All patients were evaluated as having OA according to ACR criteria. To determine if chondrocytes produce local estradiol, cells were isolated and cultured to confluence in DMEM phenol red-free supplemented with 10% charcoal-stripped FBS and then incubated with testosterone (100ng/ml) or estrone (135ng/ml) or without any substrate (control) for 8, 24 or 48 hours. Afterwards, the presence of estradiol in chondrocytes was evaluated by immunofluorescence and additionally measured by ELISA in the culture medium. Furthermore, we assessed by semiquantitative real-time PCR the expression levels of aromatase and 17β-hydroxysteroid dehydrogenase (17βHSD) in chondrocytes after 8h and 24h of incubation with the appropriate substrate.

Results: The concentration of estradiol in the media after testosterone or estrone incubation at 8, 24 and 48 hours was higher vs control (p<0.05). Moreover, no significant differences were found between 24h and 48h respect 8h. The highest concentration of estradiol achieved in conditioned media was 81.3pg/ml with estrone and 68.4pg/ml with testosterone. Additionally, the immunofluorescence for estradiol in cultured cells was increased after addition of testosterone or estrone in the media vs. control. Aromatase mRNA expression was induced by testosterone after 8h, returning to basal levels at 24h. However, 17βHSD showed no induction by its substrate.

Conclusions: The findings of this study reveal that chondrocytes acting through the aromatase and 17βHSD can produce estradiol to maintain its local metabolism despite of the systemic estrogen depletion.

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ISOThIOCYANATES FROM THE HABITUAL DIET ARE POTENTIAL CHONDROPROTECTIVE AGENTS.


Purpose: There are currently no effective disease-modifying drugs to treat OA, and drug development in this area is difficult. Our research has therefore focused on exploring the relative efficacy of a range of bioactive compounds obtained from the habitual diet with reported anti-inflammatory and/or anti-oxidant properties. We have investigated possible mechanisms by which they may be able to prevent the onset or slow the progression of OA. We have screened a number of phytochemicals (particularly organosulphur compounds (isothiocyanates and allyl sulphides) and flavonoids) for their ability to inhibit IL-1-induced metalloproteinase expression in human primary chondrocytes (HACs). Sulforaphane (SFN), an isothiocyanate which is found abundantly in broccoli, has been studied in more detail.

Methods: Several isothiocyanates (including SFN), flavonoids and other dietary compounds were screened in vitro and gene expression changes analysed by qRT-PCR. SFN was investigated further for its ability to modulate histone acetylation (a reported mechanism of SFN in other cell types, and a known regulatory mechanism for metalloproteinase expression) by Western blotting. SFN was also assayed for its ability to prevent cartilage destruction in the bovine nasal cartilage (BNC) explant model. Modulation of NFκB signalling was explored using a luciferase reporter assay, Western blotting, electrophoretic mobility assay and immunocytochemistry. Lactate dehydrogenase and caspase assays were used to test for toxicity.

Results: All ITCs tested were able to repress IL-1-induced MMP and ADAMTS mRNA expression in HACs, but differed in their potency. SFN and erinolic were the most potent, followed by iberin and erucin. Phenethyl isothiocyanate (PEITC), allyl isothiocyanate (AITC), and benzyl isothiocyanate (BITC) equally showed the least potency in IL-1-induced metalloproteinase expression in human primary chondrocytes (HACs). Sulforaphane (SFN), an isothiocyanate which is found abundantly in broccoli, has been studied in more detail.

Conclusions: Isothiocyanates represent a group of phytochemicals with potential chondroprotective properties. SFN can dose-dependently attenuate the induction of metalloproteinase expression and protect against...