with the combination of ASU and EGCG (4 or 40 ng/ml) before exposure to 300-4000 μM H2O2 significantly inhibited PGE2 and NO production (p<0.05). Pre-treatment with ASU and EGCG combination also significantly inhibited caspase 3 activity by about 50%.

**Conclusions:** The present study demonstrates that H2O2 induced oxidative stress in chondrocytes can be significantly inhibited by the combination of ASU and EGCG. This observation indicates that the combination can modulate the signaling pathways and cellular damage associated with oxidative stress in chondrocytes. The finding suggests that the combination of ASU and EGCG may be beneficial for the management of joint conditions associated with oxidative stress.

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**VISFATIN/NAMPT SIGNALING PATHWAYS IN ARTICULAR CHONDROCYTES: IMPLICATION OF THE INSULIN RECEPTOR AND NAMPT ACTIVITY**

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**Purpose:** Because obesity is associated with osteoarthritis (OA) of non weight-bearing joints, we have evaluated the role of visfatin, an adipose tissue-derived hormone, to the pathophysiology of OA. We recently reported on visfatin expression in articular chondrocytes and its role in prostaglandin (PG)E2 release in cartilage. However, the signaling pathways of visfatin remain unclear. Visfatin was first reported as a protein that binds to the insulin receptor (IR). More recently, a nicotinamide phosphoribosyltransferase (NAMPT) enzymatic activity involved in nicotinamide mononucleotide (NMN) production, a precursor of the cofactor NAD+, has been demonstrated. The aim of this study was to decipher the signaling pathways implicated in visfatin induced PGE2 release in chondrocytes.

**Methods:** IR expression from OA human chondrocytes and immature murine articular chondrocytes (iMACs) were assessed using real-time RT-PCR, immunoblotting and immunocytofluorescence. A highly specific monoclonal antibody raised against IR was used. Phosphorylation of IR and AKT was analyzed using Western Blot. IR tyrosine kinase activity was measured using a monoclonal antibody raised against IR. Phosphorylation of IR, IR, in visfatin signaling. When stimulated with 5 µg/ml visfatin, IGF-1R-/- chondrocytes unexpectedly exhibited higher PGE2 release than IGF1R+/+ controls (228 ±4 compared to 86±29 pg/ml, p<0.05), ruling out a direct role of IGF-1R in the visfatin effect. Moreover, visfatin (5µg/ml - 24h) induced PGE2 release in iMACs treated with 2 and 5 µg/ml IGF-1R blocking antibody compared to control cells (respectively a 1.3 and a 1.9 fold, p<0.05 - visfatin treated cells released 110±16 pg/ml PGE2, n= 3). (2) IR was expressed in cultured human chondrocytes from healthy (n=3) and OA patients (n=7) and in iMACs (n=3); (3) Insulin (100NM - 24h) did not trigger PGE2 release in iMACs (control: 118±68 pg/ml and insulin-stimulated cells: 130±79 pg/ml, n=3, NS); (4) Dose-responses of insulin from 0 to 1µM was performed in term of phosphorylation of IR and Akt. Moreover, insulin (100NM) from 0 to 60min triggered IR and Akt phosphorylation. (5) Blocking IR activity using HNMPA-(AM)3 (100M - 24h pre-treatment) inhibited visfatin (5µg/ml - 24h) induced PGE2 release (54% decrease, n=3, p<0.05). Moreover, blocking IR expression by siRNA inhibited visfatin-induced PGE2 release (5µg/ml visfatin: 3576±265 pg/ml versus visfatin + IR siRNA: 1864±515 pg/ml and visfatin + IR siRNA2: 930±216 pg/ml corresponding to a 48% and 74% decrease respectively, n=2, p<0.05). (6) Inhibition of the NAMPT activity of visfatin using APO866 from 0 to 1µM gradually induced a decrease in PGE2 release up to 32% (visfatin treated cells released 95±30 pg/ml PGE2,n=3, p<0.05).

**Conclusions:** In chondrocytes, visfatin exerts pro-inflammatory events by both activation of its cognate receptor and Nampt activity.

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**RELATION BETWEEN TGF-β-INDUCED INDUCTION OF LH2B AND SYNOVIAL FIBROSIS**

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**Purpose:** In knee joints with OA, fibrosis is a major contributor to both joint pain and stiffness. We found that TGF-β overexpression in murine knee joints leads to persistent fibrosis of the joint capsule while overexpression of CTGF, induced transient fibrosis. Strikingly, TGF-β overexpression gave a major increase in lysyl hydroxylase 2b (LH2b) expression, whereas CTGF did not change LH2b gene expression. It has been shown that hydroxylsine-derived cross links, formed by LH2b, are much more resistant to degradation than lysine-associated cross links. Therefore a causal relationship between LH2b and irreversible fibrosis after TGF-β exposure is plausible. We investigated whether TGF-β can also induce LH2b in human synovial fibroblasts similar to our findings in mice. In addition we investigated which TGF-β route ALK1 (Smad1/5/8) or ALK5 (Smad2/3) is responsible for LH2b regulation in human fibroblasts.

**Methods:** Human synovial fibroblasts were isolated from OA synovial tissue, obtained from knee joint arthroplasties. Primary cell cultures derived from five different donors were used to carry out the experiments. The fibroblasts were stimulated with TGF-β to determine the effects on LH1, LH2b, LH3, lysyl oxidase (LOX), collagen type 1A1 (COL 1A1), and CTGF gene expression. After 24h incubation with TGF-β, RNA was isolated and the gene expression was analyzed with RT-PCR. To examine the effect of ALK1 (Smad1/5/8) and ALK5 (Smad2/3) signaling on gene expression separately, we used SB-505124 (SB5) and dorsomorphin (DM) to block Smad2/3 and Smad1/5/8 phosphorylation respectively. Changes in Smad 2/3 and Smad 1/5/8 phosphorylation were determined with Western Blotting.

**Results:** Western Blotting showed that the increase of Smad2P as a result of TGF-β exposure is completely blocked by SB-5 and not by DM. Smad1/5/8 phosphorylation was inhibited by DM whereas SB-5 did not influence Smad1/5/8P levels. TGF-β stimulation induced LH2b, CTGF, COL1A1 and
LOX gene expression in human synovial fibroblasts. No major changes for LH1 and LH3 gene expression were found. Without TGF-β, SB-5 had no notable effect on the measured genes. Remarkably, incubation with DM alone decreased CTGF and ID-1 gene expression, the latter being a Smad1/5/8 activation marker. SB-5 completely blocked the effect of TGF-β on LH2b, CTGF, COL1A1 and LOX gene expression (Fig. 2). TGF-β-induced COL1A1, CTGF and LOX gene expression were also fully blocked by DM while LH2b gene expression was only decreased by DM. Identical results were observed for all five primary fibroblast cell cultures.

**Conclusions:** From the measured lysyl hydroxylases only LH2b gene expression was strongly elevated by TGF-β in human synovial fibroblasts. Increase of LH2b may play an essential role in the persistence of fibrosis, since LH2b induces “hard-to-degrade” cross links. The increase of COL1A1, CTGF and LOX gene expression induced by TGF-β was blocked by SB-5 as well as DM, indicating that both Smad2/3P and Smad1/5/8P are needed for COL1A1, CTGF and LOX regulation. In contrast, DM was not able to fully block the induction of LH2b gene expression by TGF-β, indicating that Smad2/3P is the dominant route for LH2b regulation. Selective blocking of LH2b may prevent persistent fibrosis and this enzyme might be a new target for OA treatment.

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**miR324-5p IS INCREASED IN END-STAGE OSTEOARTHRITIC CARTILAGE AND REGULATES HEDGEHOG SIGNALLING**

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**Purpose:** Currently unknown cellular changes occur in osteoarthritis (OA), which lead to an alteration in the phenotype of the chondrocyte and subsequent cartilage destruction. We hypothesise that these changes may be due in part to microRNAs (miRNAs), small endogenous transcripts of which approximately 1000 miRNAs have been discovered in humans. Each miRNA regulates the expression of a discrete repertoire of genes through base-specific interactions within the target genes 3’untranslated region to block translation. The objectives of this study are to identify miRNAs differentially expressed in OA and to assess the function of one such differentially expressed miRNA, miR-324-5p.

**Methods:** Real-time PCR was used to identify miRNAs differentially expressed in OA and normal cartilage. To assess the function of miR-324-5p in hedgehog (hh) signalling, a mouse mesenchymal progenitor cell line, C3H10T1/2, was transfected with the miRNA and stimulated with recombinant Indian hedgehog (Ihh). Gli1 (a transcription factor for the hh pathway) was measured using real-time PCR and immunoblotting. Stable Gli3 silence labelling with amino acids in cell culture (SILAC) and Mass spectrometry was used to identify proteins whose expression changed following miR324-5p overexpression. For assessment of direct miRNA-mRNA interactions the chondrosarcoma cells line, SW1353 was dual transfected with miR324-5p and plasmids containing Gli1 3’UTR-luciferase construct. Alkaline phosphatase was used as a marker of bone formation.

**Results:** 75 miRNAs were differentially expressed in OA cartilage compared with normal including miR-324-5p. miR324-5p reduced Gli1 mRNA and protein in C3H10T1/2 cells stimulated with Ihh. Luciferase reporter assay has shown miR324-5p directly targets the hedgehog transcription factor Gli1. miR324-5p can also reduce the level of alkaline phosphatase. Using SILAC technology and Quantitative Proteomics, we found that miR-324-5p alters the expression of a number of proteins associated with OA.

**Conclusions:** miR-324-5p regulates the Hedgehog signalling pathway by targeting Gli1. Hedgehog signalling is important in cartilage and bone development and plays a role in OA. This work furthers our understanding of miRNAs in OA, and may lead to a potential miRNA therapy for OA.

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**INVOLVEMENT OF P38 MAP KINASE PHOSPHORYLATION IN CHONDROCYTES APOPTOSIS INDUCED BY HEAT STRESS**


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**Objective:** Activation of p38 MAP kinase has traditionally been associated with the stress response and some apoptotic processes. However, it is still clearly unknown the function of the p38 MAP kinase in chondrocytes. We have shown that mechanical stress induced chondrocyte apoptosis, and inhibition p53 activation prevented chondrocyte from apoptosis induced by mechanical stress [1]. In this study, we analyzed the expression of p38 and phosphorylated p38 in chondrocytes of OA (osteoarthritis) cartilage and normal cartilage. We induced chondrocyte apoptosis by heat stress, and then investigated the relationship between chondrocyte apoptosis and phosphorylation of p38 MAP kinase.

**Methods:** Normal cartilage samples were obtained from femoral head of patients undergoing joint replacement surgery for the neck fracture of the femur. OA cartilage samples were collected from patients during total knee joint replacement surgery. The expression of p38 and phosphorylated p38 in OA cartilage and normal cartilage were analyzed by Western blotting. Heat stress was introduced to NHAC-kn (cell line derived from human normal chondrocyte) by using incubator. Chondrocyte apoptosis was detected by TUNEL staining and Western blotting. The expression of p38 and phosphorylated p38 were detected by Western blotting. In order to evaluate the function of p38, NHAC-kn were pre-treated with SB203580, which is specific inhibitor of p38 MAP kinase. Furthermore, the expression of p38 MAP kinase mRNA was down-regulated by the treatment with p38 specific siRNA transfection.

**Results:** The expression levels of p38 were not changed between OA and normal primary chondrocytes, however those of phosphorylated p38 in OA chondrocytes were significantly higher than in normal chondrocytes (Fig. 1). Heat stress induced apoptosis and increased phosphorylation of p38 in NHAC-kn cells (Fig. 2). The percent of TUNEL positive cells and expression levels of phosphorylated p38 in response to stress were decreased when chondrocytes were incubated with SB203580 or p38 specific siRNA transfection (Fig. 3, Fig. 4).

**Conclusion:** We demonstrated that heat stress increased chondrocyte apoptosis, and p38 and p38 phosphorylation were increased in normal and OA chondrocytes. Inhibition of p38 kinase by SB203580 reduced the phosphorylation of p38 and apoptosis in OA chondrocytes.