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.

**Cell Signaling**

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

M. Gosset 1, F. Berenbaum 1, 2, C. Salvat 1, E. Pecchi 1, A. Sautet 2, M. Holzenberger 1, C. Jacques 1

1 U4R Paris Univs. UPMC, Paris, France; 2 Hosp. Saint-Antoine, Paris, France

**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 inhibited using HNMPA-(AM)₃ and a siRNA strategy was used to investigate the role of IR in visfatin induced PGE₂ release. The role of IGF-1 Receptor (IGF-1R) in visfatin signaling was assessed using primary chondrocytes from IGF-1R knockout mice (IGF-1R−/−) and iMACs treated with an IGF-1R blocking antibody, NAMPT activity was inhibited using APO866. PGE₂ was measured by EIA.

**Results:** (1) Visfatin is known to bind to, and to activate IR in various cell types. However, IR is not considered to be usually present on chondrocytes. We therefore tested the implication of IGF-1R, a close homologue to IR, in visfatin signaling. When stimulated with 5μg/ml visfatin, IGF-1R/IR-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 5μg/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 (100nM - 24h pre-treatment) inhibited visfatin (5μg/ml - 24 h) 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 siRNA1: 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.

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

D.F. Remst 1, E.N. Blaney Davidson 1, 2, E.L. Vitters 1, R.A. Bank 3, W.B. van den Berg 1, P.M. van der Kraan 1

1 Radboud Univ. Med. Ctr., Nijmegen, Netherlands; 2 Leiden Univ. Med. Ctr., Leiden, Netherlands; 3 Univ. Groningen, Groningen, Netherlands

**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 AKL1 (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 Smad2/3 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 LH3 gene expression increased.

**Conclusion:** TGF-β overexpression induced LH2b in human synovial fibroblasts similar to our findings in mice. In addition we investigated which TGF-β route AKL1 (Smad1/5/8) or ALK5 (Smad2/3) is responsible for LH2b regulation in human fibroblasts.

**Figure 1.** Relative RNA expression of LH 1, 2b and 3 in human synovial fibroblasts after 24 h TGF-β stimulation. Error bars represent 95% CI.

**Figure 2.** Relative RNA expression of LH 1, 2b and 3 in human synovial fibroblasts after 24 h pre-incubation with SB/DM prior to 24 h TGF-β stimulation. Error bars represent 95% CI.