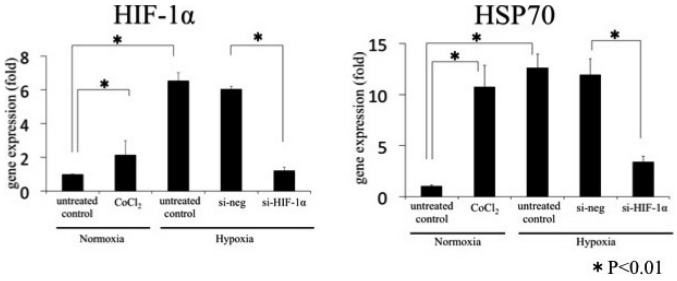
Results: The cells without siRNA or CoCl2 treatment were designated as the untreated control cells. The untreated control cells under hypoxia revealed a significant increase in the HIF-1α mRNA levels compared with cells cultured under normoxia. HIF-1α mRNA expression was significantly increased in chondrocytes cultured under simulated hypoxia in comparison with the untreated cells under normoxia. Under condition of hypoxia, si-HIF-1α reduced HIF-1α mRNA levels to about 15.2% at 24hours after transfection, compared to non-targeting scramble siRNA (si-neg) treated cells. The mRNA levels of HSP70, PG, and ColII under hypoxia and simulated hypoxia were significantly increased in comparison with chondrocytes cultured under normoxia. Under condition of hypoxia, the mRNA levels of HSP70, PG, and ColII, were significantly suppressed by si-HIF-1α transfection.
Conclusions: Up-regulation of HSP70 under hypoxia is part of the low oxygen stress response seen in Drosophila, and mammalian tissues. Up-regulation of heat shock factor (HSF), by which HSP70 are known to be regulated, under hypoxia requires the activity of HIF-1α, the effector of the low oxygen response. In this study, HIF-1α and HSP70 mRNA expressions in cultured chondrocytes were significantly increased under condition of hypoxia and simulated hypoxia. These expressions were not increased in chondrocytes by transfection with si-HIF-1α. These results suggest that HSP70 might be transcriptionally regulated by HSF through HIF-1α in cultured chondrocytes under hypoxia. We previously reported that the expression of HSP70 had an important role for PG and ColII synthesis with heat stimulation in rabbit articular cartilage. In this study, the expression of HSP70, PG, and ColII in cultured chondrocytes were significantly increased under condition of hypoxia, and were increased with CoCl2 under normoxia. But these expressions were not increased under hypoxia in which HIF-1α was inactivated by si-HIF-1α transfection. These findings suggest that PG and ColII gene might be regulated via the HIF-1α and HSP70 pathway under hypoxia.



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DICKKOPF-3 CAN REGULATE CARTILAGE DEGRADATION AND CHONDROCYTE CELL SIGNALLING

S.J. Snelling[1], R.K. Davidson[1], T.E. Swingler[1], P.A. Hulley[2], P.J. Andrew[2], I.M. Clark[1]. *[1]Univ. of East Anglia, Norwich, United Kingdom; [2]Univ. of Oxford, Oxford, United Kingdom*

Purpose: To investigate the role of Dickkopf-3 (Dkk3) in Osteoarthritis (OA).
We have previously shown that Dkk3 expression is increased in OA cartilage and synovium. Additionally levels of Dkk3 in synovial fluid are in individuals with tricompartmental OA after arthroscopy. The factors regulating Dkk3 expression in cartilage and the effect of Dkk3 on chondrocyte function are poorly ascribed.
Correct regulation of cell signalling pathways is integral to cartilage homeostasis and thus the prevention of OA pathogenesis. Dkk3 is a member of the Dkk family of Wnt antagonists and therefore may impact on chondrocyte biology through interaction with the Wnt pathway. Dkk3 has also been found to influence TGFbeta signalling in other cell systems
Methods: Expression of Dkk3 was assessed in primary human articular chondrocytes (HACs) following treatment with interleukin-1 (IL1) and oncostatin-M (OSM). The effect of Dkk3 on IL1/OSM-induced proteoglycan and collagen release from bovine nasal cartilage explants and primary human chondral explants was assessed using the DMMB and hydroxyproline assays. SW1353 chondrosarcoma cells were treated with Dkk3 +/− Wnt3a or TGFbeta and TOPFlash and CAGA luciferase reporters used to measure Wnt and TGFbeta signalling respectively. RNA was extracted from primary HACs treated with Dkk3 +/− TGFbeta or Wnt3a. ADAM12 and TIMP3 expression were measured to assess TGFbeta signalling and AXIN2 expression measured to assess Wnt signalling.
Results: Dkk3 expression was decreased in primary HAC following IL1/OSM treatment. In bovine nasal cartilage explants, IL1/OSM-induced proteoglycan release was inhibited by Dkk3. Dkk3 antagonized canonical Wnt signalling, decreasing Wnt3a-induced AXIN2 expression and Wnt3a-induced luciferase expression from the TOPFlash reporter. Interestingly, Dkk3 appears to enhance TGFbeta signalling, increasing TGFbeta-induced TIMP3 and ADAM12 expression and TGFbeta-induced luciferase from the CAGA-luc reporter
Conclusions: OA pathogenesis is likely regulated by a multitude of factors relating to cell signalling including the balance of cytokines present in the articular joint. Dkk3 expression is increased in OA but we have found that it can still be regulated by OA-relevant cytokines IL1 and OSM. This suggests a balance of Dkk3 effects depending upon the biological stimuli within the cartilage. Dkk3 may act in a protective role in the presence of inflammatory cytokines as exemplified by its ability to inhibit matrix loss from chondral explants. Furthermore the ability of Dkk3 to antagonize Wnt signalling and enhance TGFbeta signalling implies that Dkk3 could influence multiple OA-relevant processes and be important in cartilage development and homeostasis.

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CYTOKINE INDUCED MULTIMERIZATION OF SYNDECAN-4 IS MEDIATED BY GAG SIDE CHAINS

L. Godmann[1], A. Stratis[1], C. Cromme[1], F. Echtermeyer[2], T. Pap[1], J. Bertrand[3]. *[1]IEMM, Muenster, Germany; [2]Univ. Hosp. Hannover, Hannover, Germany; [3]Queen Mary Univ., London, United Kingdom*

Purpose: Syndecan-4 (SDC4) is a transmembrane heparan sulphate proteoglycan, which can act as a membrane receptor constituting binding sites for an array of ligands, such as TGF beta. Although it has been shown that SDC4 binds a variety of extracellular ligands, the exact mechanisms concerning SDC4 signaling are still unknown. As we could show in our previous study, the antibody-mediated blockade of SDC4 function as well as its complete loss leads to protection of osteoarthritis induction and cartilage degradation in mice. Thus, we investigated the underlying mechanisms leading to reduced cartilage destruction in osteoarthritis.
Methods: In order to elucidate SDC4 signaling, different side chain mutants were designed using overlap PCR. We mutated the serine residues, which constitute the heparan sulphate attachment sites to alanine, to abolish side chain attachment. Laser scanning fluorescence microscopy of transient transfected Cos-7 cells was performed to ensure membrane localization of generated mutants. The multimerization pattern of different mutants was analysed using crosslinking upon cytokine stimulation and subsequent western blot analysis.
Results: All side chain lacking SDC4 mutants exhibited normal intracellular trafficking into the cell membrane. While wildtype SDC4 shows normal multimerization, the side chain mutants exhibited an impaired tetramer formation. We showed that SDC4 is known to be expressed as a monomere and shows dimer formation upon cytokine stimulation. Interestingly, the side chain lacking mutants were less able to form dimers than the wildtype.
Conclusions: We could show that side chains are essential for multimerization. Moreover, our results demonstrate that cytokine stimulation promotes the multimerization process. Therefore, SDC4 multimerization might be an important step in signal transduction during osteoarthritic cartilage damage.

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FIBRONECTIN FRAGMENT ACTIVATES AKT LEADING TO NF-κB UP-REGULATION IN OSTEOARTHRITIC CHONDROCYTES

T. Yasuda. *Tenri Univ., Tenri, Japan*

Background: Increased fibronectin fragments are thought to contribute to joint destruction in osteoarthritis (OA). However, the mechanism whereby fibronectin fragments cause catabolic activities is not totally understood. While COOH-terminal heparin-binding fibronectin fragment (HBFN-f) has been shown to activate nuclear factor (NF)-κB pathway, intracellular upstream events that cause NF-κB up-regulation in response to HBFN-f remain unclear.
Purpose: This study was aimed to elucidate the involvement of phosphoinositide-3-OH kinase (PI3K)/Akt pathway in NF-κB activation by HBFN-f in OA chondrocytes.
Methods: Chondrocytes isolated from articular cartilage from OA knee joints were cultured in monolayer with HBFN-f. Secreted levels of nitric

oxide (NO) in conditioned media were determined. Activation of NF-κB and Akt pathways was assessed with enzyme-linked immunosorbent assay (ELISA). Involvement of CD44 in HBFN-f action was evaluated using anti-CD44 antibody and high molecular weight hyaluronan (HA).
Results: In chondrocyte monolayer cultures, HBFN-f stimulated NO production in association with up-regulation of NF-κB and Akt. Inhibition studies using BAY11–7085 confirmed that NO production by HBFN-f was dependent on NF-κB pathway. Inhibition studies using LY294002 revealed the requirement of PI3K/Akt pathway for NF-κB activation by HBFN-f. Anti-CD44 treatment with anti-CD44 antibody and HA resulted in significant inhibition of HBFN-f actions on activation of Akt and NF-κB, and on NO production.
Conclusions: This is the first study demonstrating that HBFN-f activates PI3K/Akt pathway leading to up-regulation of NF-κB through interaction with CD44. Elucidation of intracellular pathways activated by fibronectin fragments may be helpful to understand the pathological process in OA cartilage destruction.

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PGE2 AND FGF-2 UPREGULATE ACTIVITIES OF THE HUMAN F-SPONDIN PROMOTER
M. Attur[1], Q. Yang[2], J. Wang[3], K. Mix[4], G. Palmer[1], S.B. Abramson[1]. *[1]NYU Hosp. for Joint Diseases, New York, NY, USA; [2]NYU Sch. of Med., New York, NY, USA; [3]NYU Cancer Inst., NYU Ctr. for Hlth.Informatics and Bioinformatics, New York Univ. Sch. of Med., New York, NY, USA; [4]Dept. of Biological Sci., Loyola Univ. New Orleans, New Orleans, LA, USA*

Purpose: The extracellular matrix protein, F-spondin is a marker of both hypertrophic and osteoarthritic cartilage. Understanding the mechanisms that regulate its expression could therefore identify key pathways that regulate chondrocyte activity in development and disease. In this study we investigated transcriptional regulation of human F-spondin via the cloning and characterization of its 5′ regulatory (promoter) region.
Methods: Genomic sequence containing the 5′ regulatory region of F-spondin was obtained by PCR of human total genomic DNA (Clontech) using upstream primers designed from F-spondin genomic sequence available at the gene database www.ncbi.nlm.nih.gov, gene ID: 10418. PCR fragments were cloned into TA cloning vectors (Invitrogen) for sequencing and subcloned into pGL3 Luciferase Reporter Vectors (Promega) for functional analysis. Luciferase assays were performed 36 h after transfection in the human chondrosarcoma cell line, SW1353.
Results: An upstream 2.4 kb genomic sequence of human F-spondin was obtained by PCR, subcloned and sequenced bidirectionally. DNA sequence fidelity was confirmed using the NCBI blast alignment search tool. DNA sequence analysis revealed the presence of TATA box at position −74 and identification of transcription factor binding sites was performed using the JASPAR CORE database (http://jaspar.genereg.net/). High scoring putative binding sites within the 2.4 kb promoter region included NURR1 (NR4A2-nuclear receptor), NFAT, SOX-10 and CREB1. Functional promoter activity was assessed by transient transfection of pGL3 luciferase vectors encoding 2.4 kb (pFS-2.4Luc) and 0.5 kb (pFS-0.5Luc) of F-spondin upstream genomic sequence. In human chondrocytes, both pFS-2.4Luc and pFS-0.5Luc significantly increased luciferase activity above a pGL3 promoterless control vector, 20- and 70-fold, respectively. Since we have previously observed that F-spondin mRNA levels are induced by FGF-2 and PGE2 in OA chondrocytes, we examined their effects on promoter activity. PGE-2 (10 uM) stimulated F-spondin luciferase activity 10–20-fold for both 2.4 and 0.5 kb promoter constructs ($p < 0.001$) above unstimulated controls. Cotransfection of a cDNA encoding NURR1, a transcription factor induced by PGE2 in human chondrocytes, also increased luciferase activity (100-fold) irrespective of promoter length. This is consistent with the presence of multiple putative NURR1 binding sites (~16) throughout the 2.5 kb promoter region. Conversely, FGF-2 (25 ng/ml) significantly increased luciferase activity of pFS-0.5Luc (2-fold; $p < 0.05$) but not pFS-2.4Luc. This finding suggests that regulation of F-spondin via FGF-2 occurs via regulatory regions within its proximal 0.5 kb region.
Conclusions: Our results indicate that both developmental (FGF-2) and proinflammatory (PGE2) factors induce F-spondin expression in chondrocytes via discrete regions in its promoter. Sequence analysis and transfection studies suggest that NURR1 may mediate PGE2 induction of F-spondin via multiple binding sites in the promoter region. The current findings are consistent with previous observations demonstrating overlapping pro-inflammatory/catabolic effects of PGE2, NURR1 and F-spondin in OA chondrocytes.

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EXPRESSION OF HUMAN ENDOGENOUS RETROVIRUS HERV-K18 IN OSTEOARTHRITIS PATIENTS
J. Lamas, M. Garcia-Montojo, J. Hoyas, I. Dominguez-Mozo, E. Villafuertes, P. Tornero-Esteban, A. Arias-Leal, L. Abasolo-Alcazar, R. Alvarez-Lafuente, B. Fernandez-Gutierrez. *Hosp. Clinico San Carlos, Madrid, Spain*

Purpose: To evaluate the possible involvement of HERV-K18 in OA, through the analysis of the expression levels of this endogenous retrovirus in OA patients in comparison with healthy controls, and through the analysis of different qualitative and quantitative clinical variables.
Methods: 113 OA patients and 62 controls were included in the study. To analyze HERV-K18 mRNA expression, quantitative RT-PCR was performed; the transcriptional expression was expressed in a relative manner as a normalization ratio (NR), and for controls were assigned a NR = 1. The WOMAC (Western Ontario and McMasters Universities Osteoarthritis Index), Lequesne Index, and the Stanford Health Assessment Questionnaire (HAQ) were analyzed in relation to the expression levels of HERV-K18.
Results: In our study, 54 of 113 OA patients (47.8%) and 22 of 62 controls (35.5%) showed detectable expression levels of HERV-K18. We found statistical significant differences when we compared the results of the Womac Index, Lequesne Index for knee and hip, and HAQ between OA patients with higher expression (NR > 10) vs. OA patients without HERV-K18 expression ($p = 0.0003$, $p = 0.0005$, $p = 0.002$, and $p = 0.05$, respectively), and also when the comparison was made between OA patients with higher expression (NR > 10) vs. OA patients with low expression of HERV-K18 (NR = 1) for the Womac Index, and Lequesne Index for knee and hip ($p = 0.002$, $p = 0.013$, and $p = 0.006$, respectively).
Conclusions: It seems to be a relation between the health status measurement systems and severity index for OA with the levels of expression of HERV-K18. These results suggest a possible involvement of HERV-K18 in the etiopathogenesis of the disease.

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THE α5 and αV INTEGRINS ARE REGULATED DURING CHONDROCYTE DIFFERENTIATION AND OSTEOARTHRITIS FOR GDF-5 AND BMP-7
H.I. Aguirre-Sánchez[1], R.F. Abarca-Buis[1], J.B. Kouri[2], M.C. Velasquillo[1], J.C. Ibarra[1], D. Garciadiego-Cázares[1]. *[1]Intituto Natl. de Rehabilitación, Ciudad de México, Mexico; [2]CINVESTAV, Ciudad de México, Mexico*

Purpose: In this study we proposed that β1 integrins family regulate chondrocyte differentiation in the articular cartilage, as well as in the scar and blood vessels formation during OA. This is suggested on the basis of the integrins pattern expression on different stages of osteoarthritis in a experimental OA rat model.The effect of GDF-5 (growth differentiation factor 5) and BMP-7 (bone morphogenetic protein) on chondrocytes differentiation is also analyzed here.
Methods: The experimental OA animal model, rats were subject to partial meniscectomy on the right knee and they exercised daily to induce OA. After the 5, 10 and 20 days, the knees were dissected and fixed in 4% PFA overnight. Then, tissues were dehydrated and paraffin-embedded for preparing histological sections. Expression pattern of Integrin α5 and αV, Gdf-5, Bmp-7, and chondrocyte differentiation markers were determinate by Immunohistochemistry and In situ hybridization. In vitro assay for chondrogenesis were made by micromasses cultures, the cells formed cartilaginous nodules and the effect of GDF-5 and BMP-7 on micromasses was then tested. By immunohistochemistry were determine the expression of different integrins subunits and MEC molecules typical of articular cartilage. Assay for endochondral ossification were made by mouse knee cultures, 20 days old mice were used. The knees were longitudinally dissected and placed on air liquid inter-phase using BGJb medium with 0.1% bovine serum albumin. Organ cultures then were kept for 4 days at 37°C in a 5% CO2 incubator. Hedgehog signaling was blockade with 10 nM Cyclopamine.