and connective tissue growth factor. IL-1 stimulated release of the cartilage degeneration marker MMP-3, as well as proteins with uncharacterized roles in cartilage pathology, such as neutrophil gelatinase-associated lipocalin. RetA stimulated release of extracellular matrix proteins, COMP, link protein and matrilin-3 into the media, accompanied by dramatic reduction in corresponding mRNA transcripts levels. Gelsolin, implicated in cytoskeletal reorganization in arthritic synovial fibroblasts but not previously associated with cartilage pathology, was regulated by IL-1 and RetA.

Conclusions: This first analysis of mouse cartilage degradation and protein release using proteomics has identified proteins and fragments, some of which represent novel candidate biomarkers for cartilage degradation. Applying these proteomic techniques to wild-type and genetically modified mouse cartilage will provide insights into mechanisms of cartilage degeneration.

**476 GLUCOSAMINE SULFATE AND CHONDROITIN SULFATE: THEIR EFFECT ON CHONDROCYTE PROTEOME**

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Purpose: Some symptomatic slow-acting drugs, such as glucosamine sulfate (GS) and chondroitin sulfate (CS), have proved efficacy in relieving the symptoms of mild and moderate osteoarthritis (OA). In vitro and in vivo studies have demonstrated that these drugs are capable to inhibit cartilage destruction and to promote disease suppression. However, their mechanism of action remains poorly characterized. The aim of this study was to evaluate the effects of these compounds on cartilage biology, alone or in combined administration, in normal chondrocytes stimulated with interleukin-1β (anti-inflammatory effect) and in osteoarthritic chondrocytes (disease modifying effect).

Methods: Chondrocytes were obtained from 3 osteoarthritic patients undergoing joint replacement and from 3 healthy donors. Normal chondrocytes were treated with GS 10 mM and/or CS 200 μg/mL, and then stimulated with IL-1β 10 ng/mL. Osteoarthritic chondrocytes were treated with GS 10 mM and CS 200 μg/mL, alone and in combination. Whole cell proteins were isolated 24 hours after cellular stimulation and resolved by two-dimensional electrophoresis (2-DE). The gels were stained with SYPRO Ruby and digitalized using a CCD camera. The image analysis was performed using the PDQuest 7.3.1 computer software. Using PDQuest tools, protein spots were enumerated, quantified and characterized with respect to their molecular mass and isoelectric point by bilinear interpolation between landmark features on each image. Protein expression data from each gel were normalized for the total density present in the gel images. Differentially abundant proteins were identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/TOF).

Results: We examined a mean of 500 protein spots that were present in each gel. Both qualitative and quantitative changes in protein expression patterns between controls and treated cells were studied. In normal chondrocytes, 39 protein forms were modulated by GS treatment, 35 by CS treatment and 48 by GS+CS treatment compared to the untreated cells (stimulated with IL-1β). In osteoarthritic chondrocytes, 11 protein forms were found to be statistically altered in GS treated cells, 9 in CS treated cells and 17 in GS+CS treated cells compared to the untreated cells (p < 0.05). Most of the identified proteins are involved in stress response (HSP7C, HSPB1, ANXA2), cellular metabolism (AK1C2, PGK1, KPYM), protein folding (PDIα1, PDI3α), protein targeting (GRP78) and oxidative stress (SODM, PRDX1).

Conclusions: This study describes the differences between the protein profiles of normal and osteoarthritic chondrocytes treated with glucosamine and/or chondroitin sulfate. We have identified novel molecular targets that might explain the efficacy of these molecules in osteoarthritis treatment. Our results highlight the synergistic effect of the combined administration and the effectiveness of the two molecules as anti-inflammatory drugs (anti-IL-1β) effect.

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**477} HEY-1 INTERACTS WITH SOX9 TO MEDIATE PASSIVE REPRESSION OF CHONDROGENESIS**

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Purpose: Notch signaling induces bHLH proteins (Hes-1 and Hey-1) that actively repress Col2a1 transcription by binding N-box domains located in intron 1 of Col2a1. bHLH proteins also participate in passive repression by binding activator proteins. In this current study, we tested whether Hey-1 directly binds to Sox9 using luciferase reporter and GST-pull down assays.

Methods: Luciferase Assays. Chondrogenic ATDC5 cells were co-transfected using FuGENE HD (Roche). The 12x48-pGL3P-Luc luciferase reporter plasmid that does not contain bHLH binding domains was used. Readings were normalized by Renilla (pRL-SV40; Promega). Sox9 and bHLH interaction was tested by over expression using pCDNA-FLAG-Sox9 in combination with pCMV-FLAG-Hes-1 and pcDNA3-FLAG-Hey-1. Luciferase activities were analyzed 48 hours following transfection using the Dual-Luciferase Reporter Assay System (Promega), GST-pull down assays. Sox9 and Hey-1 interaction was tested using GST-Hey-1 and 35S-met-Flag-Sox9. GST-Hey-1 and 35S-Flag-Hes-1 were also utilized. The pcDNA3-met-Flag expression plasmid was transfected in BL21 cells (Strategene), lysed and bound to Glutathione Sepharose (GE Healthcare). A Reticulocyte system (Promega) was used to produce 35S-met-labelled proteins (Sox9 and Hes-1). Appropriate combinations of GST and 35S-met-labelled protein were mixed overnight at 4°C. Beads were extensively washed, placed into SDS loading buffer and loaded on SDS-page gels (Invtrogen). Dried gels were exposed to film for detection of radioactive signal.

Results: Luciferase assays show that Sox9 increased reporter expression levels 70-fold relative to control (Fig. 1A). Co-transfection with Sox9/Hes-1 and Sox9/Hey-1 led to an 81% and 64% reduction in reporter levels, respectively (Fig. 1A). Detection of a band at 60 kDa in the GST pull down assays indicated that Sox9 and Hey-1 bind (Fig. 1B). Interaction between Hey-1 and Hes-1 (positive control) was also observed (Fig. 1B).

Figure 1. (A) Luciferase assay showing Sox9 induction of Col2a1 reporter and competitive inhibition of reporter signal by Hes-1 and Hey-1. (B) GST-Pulldown assay demonstrating that Hey-1 and Sox9 proteins (lane 3) and Hes-1 and Hey-1 proteins (lane 8) interact. (lane 1 = 20% input 35S-met-Sox9; lane 2 = GST-Hey beads only; lane 3 = GST-Hey beads + 35S-met-Sox9; lane 4 = GST-null beads + 35S-met-Sox9; lane 5 = plain beads + 35S-met-Sox9; lane 6 = 20% input 35S-met-HA-Hes; lane 7 = GST-null beads only; lane 8 = GST-Hey+35S-met-HA-Hes; lane 9 = GST-null beads + 35S-met-HA-Hes; lane 10 = plain beads + 35S-met-HA-Hes).

Conclusions: These data indicate that Hey-1 passively represses Col2a1 transcription by directly binding to Sox9. Since a reduced luciferase signal was observed with Hes-1/Sox9 co-transfection, we suggest that this bHLH also binds to Sox9. We propose that high bHLH levels repress Col2a1 transcription both actively and passively. The elevated bHLH levels may induce Sox9 expression by competitively binding with Sox9. During chondrogenesis, Notch signaling and bHLH levels are reduced, which permits a shift away from a repression environment to allow Sox9 to drive transcription.

Acknowledgements: Plasmids were generously supplied by Dr. de Crombrugghe, University of Texas (Sox9); Dr. Stifani, McGill University (Hes-1) and Dr. Kedes, USC (Hey-1).