with or without pre-treatment with hydrogen peroxide (exogenous ROS) or the anti-oxidants MnTBAP or NAC. IGF-I signaling proteins were evaluated by immunoblotting with phosphospecific and control antibodies. Lentiviral constructs were used to overexpress constitutively active (CA)-Akt, CA-MEK and dominant (DN)-Akt. Proteoglycan synthesis was measured by sulfate incorporation corrected for cell numbers by DNA and aggrecan expression was measured by real-time PCR.

Results: In time course experiments (0,5,15,30 and 60 min) with normal human chondrocytes, IGF-I initiated a strong and sustained phosphorylation of IRS-1 at Tyr-612 and Akt at Ser-473 and Thr-308, and a transient ERK phosphorylation. In contrast, OA chondrocytes possessed an elevated basal ERK and JNK phosphorylation and IGF-I failed to stimulate significant IRS-1 or Akt phosphorylation. In normal human chondrocytes, exogenous ROS triggered strong IRS-1 Ser-312 phosphorylation, which is an inhibitory site, along with ERK and JNK phosphorylation and inhibited IGF-I-induced IRS-I Tyr-612 and Akt phosphorylation. Lentivirus-mediated overexpression of CA-Akt significantly enhanced proteoglycan synthesis in the absence or presence of IGF-I, while expression of either DN-Akt or CA-MEK (which activates ERK) inhibited both basal and IGF-I-induced proteoglycan synthesis (Fig. 1). ROS treatment and CA-MEK expression also significantly inhibited aggrecan mRNA expression in normal chondrocytes. In osteoarthritic chondrocytes, treatment with the antioxidants MnTBAP or NAC induced IRS-1 Tyr-612 and Akt phosphorylation, and promoted IGF-I-mediated proteoglycan synthesis.

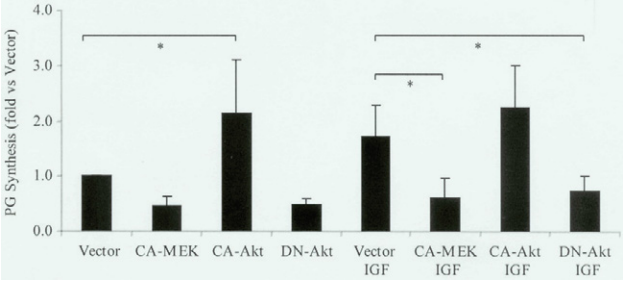

Figure 1. Effects of MEK and Akt activity on chondrocyte proteoglycan synthesis.

Conclusions: These results demonstrate for the first time opposing roles for PI-3 Kinase-Akt and MEK-ERK in the IGF-I regulation of chondrocyte proteoglycan synthesis and suggest that excessive ROS in OA chondrocytes inhibit IGF-I mediated proteoglycan synthesis by altering the balance of Akt to ERK activation.

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EXTRACELLULAR SULFATASES SUPPORT CARTILAGE HOMEOSTASIS BY REGULATING BMP AND FGF SIGNALING PATHWAYS

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Purpose: Cartilage homeostasis is controlled by several major cell signaling pathways. Sulf-1 and Sulf-2 (Sulfs) are the only known extracellular sulfatases and by changing heparan sulfation patterns regulates the activity of several major cell-signaling pathways. This study addressed the role of Sulfs in regulating BMP-7 and FGF2 signaling pathways in articular cartilage.

Methods: Sulf-1 and Sulf-2 expression was inhibited in primary cultured human articular chondrocytes using siRNA. Over expression experiments were conducted with an immortalized human chondrocyte cell line (T/C-28a2). Cells were stimulated with BMP-7 (100ng/ml) or FGF2 (100ng/ml), and SMAD and ERK signaling was monitored via western blotting. Knee joints and articular chondrocytes were isolated from Sulf knock out mice, and BMP and FGF signaling was analyzed using western blotting, immunohistochemistry and Taqman PCR.

Results: Chondrocyte transfection with siSulf-1 or siSulf-2 reduced Sulf protein levels by 50-70%. This was associated with a reduction in basal SMAD1 protein expression and SMAD1/5 phosphorylation. In contrast, under identical conditions basal ERK1/2 protein expression and phosphorylation increased (Fig. 1a). Sulf siRNA also inhibited the BMP-7 induced increase in SMAD1 protein expression and SMAD1/5 phosphorylation (Fig. 1b). The effects of FGF2 on ERK1/2 protein expression and phosphorylation were dramatically accelerated following Sulf knock down (Fig. 1c). Sulf overexpression did enhanced BMP-7-induced SMAD1 protein expression and SMAD1/5 phosphorylation (Fig, 1d and 1e). The FGF2 response was not altered by Sulf overexpression (Fig. 1f). To confirm and extend these in vitro findings, we analyzed Sulf knock out mice. Articular cartilage from Sulf knock out mice showed reduced SMAD1 protein expression and SMAD1/5 phosphorylation, whereas ERK1/2 phosphorylation was increased. mRNA levels of Col2a1, Sox9, Smad1 and Aggrecan were reduced, while increased levels of MMP-13 and Noggin were noted, especially in Sulf-1 knock out chondrocytes.

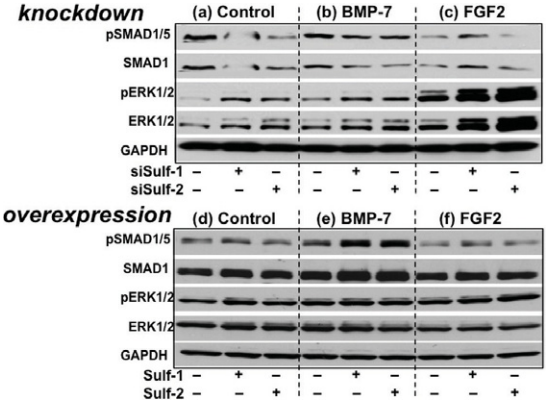

Figure 1. Sulfs regulate BMP-7-induced SMAD1/5 and FGF2 induced ERK1/2 phosphorylation in human chondrocytes.

Conclusions: Sulfs regulate BMP-7 and FGF2 signaling pathways, and contribute to the balance for cartilage homeostasis by regulating these major signaling pathways at the same time. Regulating Sulf expression represents a potentially new therapeutic approach to inhibit cartilage degeneration.

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HYPOXIA CONDITIONS DIFFERENTIALLY MODULATE NORMAL AND OSTEOARTHRITIC HUMAN ARTICULAR CHONDROCYTE PROTEOMES

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Purpose: Osteoarthritis (OA) is a degenerative disease characterized by the degradation of articular cartilage. This tissue is avascular, and it is characterized by the low oxygen tension and poor nutrient availability for its cells, the chondrocytes. Hypoxia conditions have been reported to stimulate chondrogenesis and synthesis of extracellular matrix components. Therefore, we pur-

sued in this work to analyze the effect of hypoxia on normal and osteoarthritic cartilage cells by a proteomic approach.
Methods: Chondrocytes were obtained from osteoarthritis patients undergoing joint replacement, and normal cartilages from autopsies without history of joint disease. Cultured cells were subjected to normoxia or hypoxia conditions during 96 hours. Whole cell proteins were then isolated and resolved by two-dimensional gel electrophoresis. Gels were stained with SYPRORuby fluorescent dye, images were acquired using a CCD camera and image analysis was performed using PDQuest software. Proteins of interest were picked from the gels and identified by MALDI-TOF/TOF mass spectrometry. Database search and visualization of biological pathways were performed using PathwayStudio 6 software. Validation of the results was carried out by real-time PCR, Western blotting and immunofluorescence analyses.
Results: We examined a mean of 500 protein spots that were present in the gels. Both qualitative and quantitative changes of protein patterns between normoxia and hypoxia were studied, considering expression changes within 95% confidence interval ($p<0.05$), and standardized average ratios exceeding 1.5. 32 proteins were found to be modulated by hypoxia in normal chondrocytes, and 16 in OA cells when compared to their normoxia controls. A more extended modulation could be detected in normal cells, although we observed in both cases a hypoxia-dependent decrease in many metabolism-related proteins. We also identified 44 proteins that were altered in OA chondrocytes under hypoxia when compared to normal cells. In this case, the major difference was observed in a group of 7 proteins involved in the glycolysis pathway, which are significantly decreased in OA. On the other hand, cytoskeleton-related proteins such as vimentin, vinculin or gelsolin were found to be increased in OA cells under hypoxia. We confirmed the hypoxia-dependent upregulation of the TNFalpha-receptor associated protein 1 (TRAP1), both at transcriptional and protein levels. Immunohistofluorescence assays revealed its increase specifically in the deep layer of OA cartilage.
Conclusions: Hypoxia conditions induce diverse modifications in the proteomic profile of normal and OA human articular chondrocytes. This probably renders a different capacity of OA and normal cells to react under a hypoxic environment.

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ALTERED WNT/b-CATENIN SIGNALING IN HUMAN OSTEOARTHRITIC SUBCHONDRAL OSTEOBLASTS IS DUE TO DICKKOPF-2 (DKK2) AND PROSTAGLANDIN E2 (PGE2) LEVELS

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Purpose: Altered bone remodeling is considered important in the initiation of osteoarthritis (OA). This is linked with the abnormal phenotype and proliferation of human OA osteoblasts (Ob). The canonical Wnt/β-catenin signaling pathway is crucial for osteogenesis yet there is limited information on its potential role in OA. We recently showed that an antagonist of Wnt signaling, DKK-2, is increased via a TGF-β1 regulation in OA Ob. Here we questioned if high DKK-2 levels in OA Ob could alter Wnt/β-catenin signaling, and if variable levels of PGE_2 in these cells could also impact Wnt/β-catenin signaling.
Methods: We prepared primary human subchondral osteoblasts from tibial plateaus of normal individuals at autopsy and of OA patients undergoing total knee arthoplasty. We evaluated the role of endogenous PGE_2 levels in OA Ob, and of exogenous addition of PGE_2 or inhibition of COX-2 activity via NS-398 on Wnt signaling. We determined the levels of PGE_2 produced by OA Ob using a selective ELISA. Wnt/β-catenin signaling was evaluated using two approches. First, Wnt3a-dependent Wnt signaling was measured using the TOPflash TCF/lef1 reporter assay. Second, we used Western blot analysis to determine the levels of β-catenin and GSK-3β.
Results: Normal Ob showed a classic dose-dependent TCF/lef1 expression response to Wnt3a stimulation. Low and high OA Ob, based on their endogenous PGE_2 levels showed reduced and normal Wnt3a-dependent TCF/lef1 expression compared to normal. However, inhibiting DKK-2 expression using siRNA technique enhanced TCF/lef1 expression in all OA Ob, indicating an overall reduction of Wnt signaling in these cells. Hence, we tested if endogenous PGE_2 levels could explain the difference between low and high OA Ob. Indeed, addition of PGE_2 to low OA Ob directly increased their TCF/lef1 expression while NS-398 had no effect. In contrast, PGE_2 addition to high OA Ob had limited effect on Wnt signaling whereas NS-398 reduced it. Western blot analysis indicated a reduced level of free β-catenin in low OA Ob stimulated by Wnt3a compared to normal yet the addition of PGE_2 increased the level of β-catenin whereas NS-398 reduced it. Phosphorylated GSK-3β levels were elevated in normal Ob yet reduced in OA Ob regardless of the stimulation with Wnt3a.
Conclusions: These results suggest that altered Wnt/β-catenin signaling contributes to abnormal function of OA Ob and is possibly linked to OA pathophysiology. Moreover, elevated DKK-2 levels in OA Ob are responsible for their reduced Wnt/β-catenin signaling even in presence of elevated endogenous PGE_2 levels which partly correct this situation. Hence, high endogenous PGE_2 levels could partly correct abnormal Wnt signaling in OA Ob.