then treated with mixed secondary antibodies, Alexa Fluor488 donkey anti-rabbit IgG and Alexa Fluor594 donkey anti-goat IgG for 30 min.
Results: There were no significant differences in the level of HTRA1 mRNA between control and experimental chondrocytes treated with 42oC, TNFα, or the high hydrostatic pressure. In contrast, the level of HTRA1 mRNA increased by ~5-fold in human chondrocytes treated with TGF-β1. The level of HtrA1 mRNA was also increased in mouse chondrocytes treated with TGF-β1. Expression of p-Smad1 was hardly detected in Col11a1+/- and DMM mice and corresponding control mice. However, the expression of Tgf-β1 and p-Smad2 was increased. The increased expression of p-Smad2 and HtrA1 was co-localized in Col11a1+/- and DMM mice. Interestingly, expressions of p-Smad2 and HtrA1 were hardly detected in Col11a1+/- mice treated with the neutralizing TGF-β1 antibody.
Conclusions: These results indicate that TGF-β1 may induce HTRA1 in chondrocytes. Therefore, current OA therapy using TGF-β1 may be contraindicated as it may do more harm than good in the protection of articular cartilage against OA in matured joints.

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IKKα MODULATES OXIDATIVE STRESS-INDUCED DNA DAMAGE AND REPAIR IN PRIMARY HUMAN OA CHONDROCYTES

S. Neri [1], S. Guidotti [1], D. Platano [1], E. Olivotto [1], A. Facchini [1,2], K. Marcu [1,3], R. Borzì [1]. [1] *Lab. Immunoreumatologia e rigenerazione tissutale/RAMSES, Istituto Ortopedico Rizzoli, Bologna, Italy;* [2] *Dip. Med. Clinica, Università di Bologna, Bologna, Italy;* [3] *Biochemistry and Cell Biology Dept., Stony Brook Univ., Stony Brook, 11794-5215, New York, NY, USA*

Purpose: Functional derangement of osteoarthritic chondrocytes make them more susceptible to stressful conditions such as oxidative stimuli within aging tissue, which can further provoke extrinsic senescence by DNA damage responses (DDR). Our previous observation that IKKα knockdown increased the replicative potential of primary human OA chondrocyte monolayer cultures and the survival of the same cells undergoing hypertrophic-like differentiation, prompted us to investigate whether IKKα knockdown could modulate the stress-induced senescence of OA chondrocytes as assessed by their DDR.
Methods: We assessed the effects of IKKα loss on ROS-induced DDR by exposing control and IKKα KD chondrocytes (derived from 10 patients) in low density in monolayer cultures to 1 hr of 100 μM hydrogen peroxide and then scored them for their relative degrees of microsatellite instability (MSI) and double strand breaks (DSB).
Mismatch repair (MMR), the main post-replicative correction pathway has a fundamental role in maintaining genomic stability and its efficiency can be monitored by assessing MSI. Part of the cells, left untreated or exposed to 100 μM H_2O_2, were collected after 6 hour recovery to evaluate MMR enzymes (i.e., MLH1, MSH2, MSH6, MSH3, PMS1 and PMS2) mRNA expression by quantitative RT-PCR using GAPDH as a reference control. Other cells were further cultured for 72 hours and analyzed for MSI at five different genomic DNA loci (CD4,VWA, FES, TPOX, and P53).
DDR was evaluated in cells immediately after H_2O_2 exposure by flow cytometric analysis for γH2AX foci in chromatin. γH2AX foci serve as a marker of double strand breaks representing sites of H2AX phosphorylation, which can subsequently lead to the recruitment of DNA repair factors.
Results: Real time PCR data show increased expression of MSH3 and PMS1 mRNAs in IKKα KD chondrocytes after H_2O_2 exposure. MSI was detected in control cells but not in their IKKα KD counterparts. Under basal conditions, preliminary flow cytometry results indicate the comparable presence of γH2AX DNA foci in IKKα KD and controls in 6 out of 7 patient chondrocytes. However, after H_2O_2 exposure, preliminary results indicate a higher percentage of γH2AX positive cells and more foci per cell in the absence of IKKα.
Conclusions: Thus far our preliminary data indicate that IKKα controls the intensity of primary human OA chondrocyte DDR induced by oxidative stress. Thus IKKα loss appears to increase cell susceptibility to ROS-mediated DDR as indicated by their augmented DSB induction, perhaps due in part to the faster doubling time of IKKα deficient cells. However, MSI and MMR gene expression data suggest that IKKα deficient primary OA chondrocytes also possess a higher capacity to repair DNA damage. More experiments are underway to confirm and extend our results with cells at comparable passage numbers to determine if H_2O_2-mediated stress induces these effects or exacerbates a pre-existing physiological state reflecting the faster division rate of IKKα compromised cells.

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AN INVESTIGATION OF THE EFFECT OF EXOGENOUS GROWTH FACTOR GDF5 ON PRIMARY OA CHONDROCYTES - IS THERE A PREDICTABLE RESPONSE?

M. Ratnayake [1], F. Plöger [2], M. Santibanez-Koref [1], J. Loughlin [1]. [1] *Newcastle Univ., Newcastle upon Tyne, United Kingdom;* [2] *Biopharm GmbH, Heidelberg, Germany*

Purpose: A genetic deficit mediated by SNP rs143383 and involving reduced expression of the growth differentiation factor 5 gene GDF5 is strongly associated with large-joint OA. We speculated that this deficit could be attenuated by the application of exogenous GDF5 protein and as a first step we have assessed what effect such application has on primary OA chondrocyte gene expression.
Methods: Chondrocytes were isolated from the cartilage of OA patients who had undergone elective surgery of the hip or knee and cultured in the presence of 100ng/ml of wild-type recombinant human GDF5 protein for 6, 12, 24 and 48 hours. We also studied variants of GDF5 that have a higher affinity for the BMP Ia receptor, which is highly expressed by chondrocytes. As a positive control, chondrocytes were treated with TGFβ, which is known to elicit a predictable anti-catabolic response. The expression of genes coding for catabolic, anti-catabolic, and structural proteins of cartilage were measured by quantitative PCR (qPCR).
Results: The expression of the relevant GDF5 receptor genes BMPRII, BMPRIA and BMPRIB was confirmed by qPCR. The capacity of GDF5 to initiate cell signaling in chondrocytes was demonstrated by the phosphorylation of intracellular SMADs, and the ability of the signal to then translocate to the nucleus was demonstrated by the activation of a luciferase reporter construct harbouring SMAD response elements. Chondrocytes cultured with TGFβ demonstrated a consistent down-regulation of the catabolic metalloproteinase genes MMP1 and MMP13, of the cartilage differentiation transcription factor gene SOX9, and of the aggrecan gene ACAN. They also showed a consistent up-regulation of TIMP1, which codes for an inhibitor of MMPs, and of the cartilage type II collagen gene COL2A1. In contrast, chondrocytes cultured with wild-type GDF5, or its variants, did not show any consistent response, with variation observed relative to the length of time of culture, and with an inconsistent response between individuals irrespective of the donor's sex or of the original site of the chondrocytes (hip or knee).
Conclusions: OA chondrocytes do not respond in a predictable manner to culture with exogenous GDF5. This may be a cause or a consequence of the OA disease process and will need to be surmounted if treatment with exogenous GDF5 is going to be advanced as a potential means of alleviating the genetic deficit mediated by OA susceptibility at the gene GDF5.

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THE ROLE OF NOREPINEPHRINE IN HUMAN ARTICULAR CHONDROCYTES

J. Kaps [1,2], R.H. Straub [3], M. Böhm [4], J. Beckmann [2], J. Grifka [2], S. Grässel [1,2]. [1] *Experimental Orthopedics, Ctr. for Med. Biotechnology, BioPark1, University of Regensburg, Germany;* [2] *Orthopaedic Surgery, University of Regensburg, Germany;* [3] *Dept. of Internal Med., exp. Rheumatology and Neuroendocrine-Immunology, University of Regensburg, Germany;* [4] *Dept. of Dermatology, Lab. for Neuroendocrinology of the Skin and Interdisciplinary Endocrinology, University of Münster, Germany*

Purpose: Norepinephrine belongs to the catecholamine family of tyrosine-derived neurotransmitters of the sympathetic nervous system. Tyrosine-hydroxylase positive sympathetic nerve fibers have been identified in bone marrow, in the periosteum and in bone-adherent ligaments indicating that growth and metabolic activity of bone and joint tissues is regulated by sympathetic neurotransmitter. It is known that norepinephrine can regulate cell proliferation or apoptosis in several cell types, such as osteoblasts. It is further described that norepinephrine modulates inflammation during rheumatoid arthritis and gut inflammation. Here, we aim to

understand the role of norepinephrine in human osteoarthritic chondrocytes with regard to inflammation and its impact on metabolic activity.
Methods: Human chondrocytes were isolated from post-surgery discarded human osteoarthritic articular cartilage. Expression of β2-adrenergic receptor on articular cartilage was tested with standard immunohistochemical analysis. Employing 3D cell cultures in fibringel, effects of norepinephrine on interleukin-1β induced gene expression of pro-inflammatory cytokines and matrix metalloproteinases (MMP) were analyzed with quantitative real-time PCR. The impact of norepinephrine on cell proliferation was determined in monolayer culture with BrdU and XCelligence analyses.
Results: ß2-adrenergic receptors are abundantly expressed in human osteoarthritic cartilage. Stimulation with norepinephrine has significantly reduced interleukin-1β induced gene expression of interleukin 8 and MMP-13 in human osteoarthritic chondrocytes cultured in 3D fibringel. Notably, we were unable to detect an impact on interleukin-1β induced gene expression of interleukin-6, MMP-2 and MMP-3. Furthermore, norepinephrine inhibits BrdU incorporation compared to the controls. Additionally, we measured a lower cell spreading, assessed through electrical impedance using the XCelligence System (Roche), starting 6 h after norepinephrine stimulation until analysis was stopped (50 h after stimulation). All effects were observed exclusively with 10-6 M and not with 10-8 M norepinephrine indicating for a signaling via β-adrenergic receptors.
Conclusions: Neurotransmitters of the sympathetic nervous system like norepinephrine presumably mediate an anti-inflammatory / chondroprotective effect in human osteoarthritic chondrocytes via reducing interleukin-8 and MMP-13. Furthermore, norepinephrine is able to modulate the metabolic activity by inhibiting cell proliferation of human osteoarthritic chondrocytes. These findings indicate, together with β2-adrenergic receptor expression in human osteoarthritic articular cartilage, a yet unknown function of catecholaminergic neurotransmitters in adult human cartilage and might have an impact on osteoarthritis pathology.

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EGR-1 MEDIATES THE SUPPRESSIVE EFFECT OF IL-1 ON PPARG EXPRESSION IN HUMAN OA CHONDROCYTES

S. Nebbaki, F. El Mansouri, N. Zayed, M. Benderdour, J. Martel-Pelletier, J.-P. Pelletier, H. Fahmi. *Univ. of Montreal, Montreal, QC, Canada*

Purpose: PPARg has been shown to down-regulate several inflammatory and catabolic responses in articular cartilage and chondrocytes and to be protective in animal models of OA. We have previously shown that IL-1 down-regulated PPARg expression in OA chondrocytes. In the present study we will investigate the mechanisms underlying this effect of IL-1.
Methods: Chondrocytes were stimulated with IL-1, and the level of PPARg and Egr-1 protein and mRNA were evaluated using Western blotting and real-time reverse-transcription polymerase chain reaction, respectively. The PPARg promoter activity was analyzed in transient transfection experiments. Egr-1 recruitment to the PPARg promoter was evaluated using chromatin immunoprecipitation (ChIP) assays. Small interfering RNA (siRNA) approaches were used to silence Egr-1 expression.
Results: We demonstrated that the suppressive effect of IL-1 on PPARg expression requires de novo protein synthesis and was concomitant with the induction of the transcription factor Egr-1. ChIP analyses revealed that IL-1 induced Egr-1 recruitment at the PPARg promoter. IL-1 inhibited the activity of PPARg promoter and overexpression of Egr-1 potentiated the inhibitory effect of IL-1, suggesting that Egr-1 may mediate the suppressive effect of IL-1. Finally, Egr-1 silencing with small interfering RNA blocked IL-1-mediated down-regulation of PPARg expression.
Conclusion: These results indicate that Egr-1 contributes to IL-1-mediated down-regulation of PPARg expression in OA chondrocytes and suggest that this pathway could be a potential target for pharmacologic intervention in the treatment of OA and possibly other arthritic diseases.

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THE FUNCTIONAL EFFECTS OF DIO2 IN AN IN-VITRO MODEL FOR CHONDROGENESIS

N. Bömer [1], Y.F. Ramos [1], S.D. Bos [1,2], B.J. Duijnisveld [3], W. Koevoet [4], G.J. van Osch [4,5], R.G. Nelissen [3], P.E. Slagboom [1,2], I. Meulenbelt [1,2]. [1] *Dept. of Molecular Epidemiology, LUMC, Leiden, Netherlands;* [2] *The Netherlands Genomics Initiative-sponsored Netherlands Consortium for Hlth.y Aging (NGI-NCHA), Leiden, Netherlands;* [3] *Dept. of Orthopedics, LUMC, Leiden, Netherlands;* [4] *Dept. of Otorhinolaryngology, Erasmus MC, Rotterdam, Netherlands;* [5] *Dept. of Orthopedics, Erasmus MC, Rotterdam, Netherlands*

Purpose: Previously, we identified the deiodinase iodothyronine type-2 gene (DIO2) as a relevant susceptibility gene for osteoarthritis (OA). The type 2 deiodinase (D2) is a selenoprotein responsible for catalyzing the conversion of intracellular inactive thyroid (T4) to active thyroid (T3), a process that occurs in specific tissues including the growth plate. In the growth plate, T3 specifically signals terminal maturation of the chondrocytes. Here, we investigate the role of DIO2 during chondrogenesis by interfering with D2 protein function in an in-vitro chondrogenesis model.
Methods: The 'RAAK' study contains human bone marrow derived mesenchymal stem cells (BM-MSCs) which were isolated from OA affected hips joints of subjects undergoing total hip arthroplasty. These BM-MSCs were cultured as micromasses and differentiated into cartilage particles. First, we assessed expression of DIO2 at consecutive weeks of chondrogenesis by RT-qPCR on samples previously isolated by the Rotterdam departments. Next, we tested the RAAK samples for the effect of T3 and Iodopanoic Acid (IOP), a DIO2 inhibitor, on chondrogenesis by adding them to the chondrogenic culture medium of the micromass cultures. These micromass cultures were than maintained up to 49 days. To measure the effects of interfering with D2 protein function we initially assessed pellet sizes at different time points and histochemistry with Alcian blue to determine glycosaminoglycan and Alizarin red to determine calcifications.
Results: DIO2 gene expression significantly increased in untreated BM-MSC micromasses during in-vitro chondrogenesis from 3 weeks onwards of differentiation. Upon inhibition of DIO2 protein function with IOP, the BM-MSC micromasses showed no significant changes in size after 7 weeks of differentiation when compared to untreated controls. In contrast, excess of T3 resulted in significantly smaller micromasses after 7 weeks of differentiation suggesting decreased extracellular matrix deposition. This observation was supported by histological analysis, which showed decreased Alcian blue staining already after 5 weeks of differentiation in T3 treated micromasses compared to untreated controls. In addition, only upon treatment with T3, calcium deposition could be detected by Alizarin Red staining from 5 weeks of differentiation onwards.
Conclusions: In addition to the association between DIO2 and osteoarthritis and the body of literature on the role of DIO2 in endochondral ossification, we are investigating the functional effect of DIO2 on chondrogenesis. Our first results show a significant up-regulation of DIO2 gene expression during chondrogenic differentiation. Furthermore, we show that excess T3 in this in-vitro chondrogenesis model, appears to interfere with the chondrogenesis process reflected by significant decreased pellet sizes and decreased Alcian blue staining accompanied by increased calcium deposition. Currently, we are using both lentiviral-induced DIO2 over expression and siRNA knock-down to explore directly the effect of DIO2 interference during in-vitro chondrogenesis.

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EXPRESSION OF NUCLEOSTEMIN IN SYNOVIAL TISSUES AND IN NORMAL AND OSTEOARTHRITIC CHONDROCYTES

M. Ricketts, D.M. Salter, S.H. Ralston. *Univ. of Edinburgh, Edinburgh, United Kingdom*

Purpose: Osteoarthritis is a common disease with a strong genetic component. Despite this, previous attempts to identify genetic variants that predispose to osteoarthritis have met with limited success. In the past few months, the results of a large genome wide association study for osteoarthritis have been reported and identified a novel susceptibility locus for the disease on chromosome 3 (Panoutsopoulou et al OARSI 2011). The strongest association within this region was found with a SNP located within the coding region of *GNL3* gene which encodes nucleostemin ($p=7.24x10^{-11}$). Nucleostemin is a protein that is found within the nucleolus of stem cells and tumour cells. It is thought to play a role in regulating cell cycle progression by modulating the action of p53, but its role in the joint is unknown. Here we wanted to determine if nucleostemin was expressed in articular chondrocytes and other joint tissues to determine if