Methods: Primary chondrocytes were isolated from the joint cartilages of OA patients undergoing total knee arthroplasty (grade 4, mean age: 68 years, BMI: 31.4 ± 6.0 kg/m2). Cells were treated with different concentrations (*Zey*EX®) for 24 h. The alterations in cell proliferation (MTT), adhesion profile (RTCA-iCELLigence System), reactive oxygen species generation (ROS), lipid hydroperoxide levels (LPO), HNE-protein adduct levels (HNE), AGE-protein adduct levels (AGE), 3-nitrotyrosine levels (3-NT) and inflammatory progenitors ICE/caspase-1, IL-1β, IL-6 and TNF-α levels (ELISA) were determined. Moreoever, toll-like receptor-4 (TLR-4) and Receptor for AGE (RAGE) levels were quantified by Western blot analysis.
Results: Cell viability of OACs were significantly increased at lower concentrations (1-10 nM) of ZeyEX® and seemed to be non-toxic at nanomolar concentrations (Figure 1A). In addition to that, adherence capacity of OACs were dramatically increased at 1 nM concentration (Figure 1B). ZeyEX® treatment reduced the ROS, AGE and HNE levels slightly ($p<0.05$-0.01) in concominant with the drastic reduction in LPO and 3-NT levels ($p<0.01$-0.001) (Figure 1C). Treatment with ZeyEX® (10 nM- 10 μM) not only downregulated the inflammatory progenitor ICE/caspase-1 levels, but also IL-1β and IL-6 were decreased significantly in all concentrations ($p<0.001$) (Figure 1D). However, Tnf- α levels were dramatically increased only at 100 nM concentration ($p<0.05$) (Figure 1D). Moreover, RAGE and TLR-4 levels were decreased tremendously after ZeyEX® (>100 nM) treatment (Figure 1E, 1F) ($p<0.05$ to $p<0.001$).
Conclusions: Our results suggest that lower concentrations of ZeyEX® ameliorates oxidative-nitrosative stress mediated pro-inflammatory cytokine release along with the induction of proliferation and adherence capacity in human primary chondrocytes, *in vitro*.
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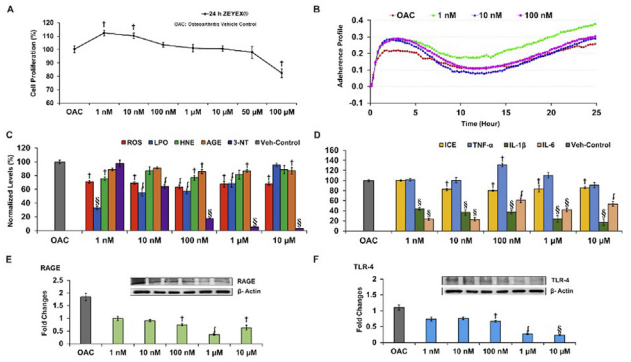

Figure 1. Cell proliferation of human osteoarthritic chondrocytes (A) and cell adhesion profile after *Zey*EX® treatment (24 h) (B) ROS, LPO, HNE, AGE, 3-NT levels (C) ICE/caspase-1, Tnf-α, IL-1β, IL-6 levels after *Zey*EX® treatment (24 h) (D). Western blot analysis of RAGE (E) and TLR-4 (F) proteins. $^{\$}p<0.05$, $^{\$}p<0.01$, $^{\$}p<0.001$ vs OAC (Kruskal Wallis analysis)

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PROTEOGLYCAN 4 EXPRESSION BY SUPERFICIAL ZONE CHONDROCYTES IS REGULATED IN PART BY JNK AND P38 IN VITRO

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Purpose: Osteoarthritis (OA) is a degenerative disease that affects joint homeostasis leading to cartilage degeneration. Inflammatory mediators, including interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), are key factors in this catabolic process that are known to activate the mitogen-activated protein kinases (MAPKs) and have been shown to play a central role in mediating this degradative response by OA chondrocytes. However, these studies typically use chondrocytes from full-thickness cartilage. A study by Rosenzweig et al. showed that the temporal and spatial expression of phosphorylated MAPKs in response to cartilage injury was zone-specific, and observed a chondroprotective effect of activation of the MAPK jun amino-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38) in the superficial zone (SZ). The purpose of this study was to investigate the role of JNK and p38 in regulating key aspects of the SZ phenotype including cell morphology, actin polymerization status, expression of the chondroprotective SZ molecule proteoglycan 4 (PRG4), and localization of yes-associated protein and transcriptional co-activator with PDZ-binding motif, which we have previously shown to regulate PRG4 expression in SZ chondrocytes (SZC).
Methods: Bovine chondrocytes were isolated from the SZ of the metacarpo-phalangeal joint of calves aged 6-9 months by sequential digestion of the tissue in 0.5% protease (45 minutes) followed by 0.1% collagenase (14-17 hours). SZC were seeded in monolayer in high glucose DMEM supplemented with ITS, 100nM dexamethasone, 40μg/mL proline, 100mM pyruvate, 100μg/mL ascorbic acid. After 24 hours of culture, SZC were treated with either SP600125 (JNK inhibitor (JKNi); 10μM) or SB203580 (p38 inhibitor (p38i); 5μM) and the effects on the SZ phenotype (cell shape, actin polymerization status, and PRG4 expression, YAP/TAZ localization) were assessed after 24 hours of treatment. Cell shape was evaluated by measuring cell circularity and area using Image J software analysis. Actin polymerization status was assessed by visualizing globular and filamentous actin by confocal microscopy and determining the ratio of globular to filamentous actin, which was extracted by differential triton extraction and separated by SDS-PAGE. PRG4 mRNA levels was determined by RT-PCR. YAP/TAZ localization was assessed by fractionating the cellular cytoplasmic and nuclear components (NE-PER Nuclear and Cytoplasmic Extraction Kit; ThermoFisher Scientific) and evaluating them by SDS-PAGE followed by western blot. Experiments were repeated with SZC from 3 independent cell isolations. Statistical analysis was performed using Student's T-test with significance assigned as $p<0.05$.
Results: SZC at 24 hours maintain their phenotype as demonstrated by their spindle cell shape and secretion of PRG4. To determine whether JNKi and p38i affect chondrocyte morphology and actin organization, SZC were treated with JNKi and p38i and cell shape and actin polymerization status was assessed. Treatment with JNKi did not significantly affect cell area ($N=3$; $p=0.43$) but resulted in an increase in cell circularity ($N=3$; $p=0.047$). No changes in actin organization were observed with confocal microscopy, and no significant changes in the G-/F-actin ratio were observed ($N=4$; $p=0.5$) with JNKi treatment. Treatment with p38i did not affect cell area ($N=3$; $p=0.13$) or circularity ($N=3$; $p=0.91$). Similarly, no changes in actin organization were observed with confocal microscopy, and no significant changes in the G-/F-actin ratio ($N=4$; $p=0.49$) were observed with p38i treatment. Despite no change in actin polymerization status with treatment, a decrease in nuclear TAZ was observed with JNKi ($N=5$; $p=0.03$) and p38i ($N=4$; $p=0.009$) treatment. No change in levels of cytoplasmic YAP was observed with JNKi treatment; however, treatment with the p38i resulted in an increase in cytoplasmic YAP ($N=4$; $p=0.02$). PRG4 mRNA levels decreased with JNKi ($N=3$; $p<0.0001$) and p38i ($N=3$; $p<0.001$) treatment.
Conclusions: The data suggests that JNK and p38 regulates TAZ nuclear localization and PRG4 expression without affecting actin cytoskeleton status. Regulation of the SZ phenotype is complex and likely results from the convergence of several major regulatory pathways. While actin polymerization and nuclear localization of YAP/TAZ have been shown to regulate PRG4 expression, JNK and p38 also appear to regulate PRG4 expression and YAP/TAZ localization; however it has yet to be determined whether JNK and p38 act downstream of actin polymerization or independent of actin polymerization. Understanding the regulatory mechanisms governing the SZ phenotype and the expression of the chondroprotective molecule PRG4 may provide insight into therapeutic treatments to delay the onset or progression of OA.

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A MULTICOMPONENT MEDICATION PROMOTES CHONDROGENESIS AND REDUCES MMP-13 IN PRIMARY ARTICULAR CHONDROCYTES FROM KNEE OSTEOARTHRITIS PATIENTS *IN VITRO*

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Purpose: HE-1100 is a multicomponent medicinal product. Initial preclinical data potentially suggest a preventive effect on cartilage degradation. This study aims to understand the mode of action of HE-1100 on OA chondrocytes *in vitro*.
Methods: Primary chondrocytes were obtained from 10 knee osteoarthritis (OA) patients undergoing knee replacement surgery. The cultures were treated with 20% (v/v) HE-1100 or placebo. Samples were collected for subsequent RNA extraction using standard methods. The reads were generated with Illumina NextSeq5000 sequencer and

aligned to the human reference genome (UCSC hg19) to generate the transcriptome. Differential expression analysis between HE-1100 and placebo was made in R using the DESeq2 package to identify the differentially expressed genes in the OA-associated regulatory pathways. The protein production of the selected genes was quantified by ELISA in 10 independent human OA chondrocytes cultures.

Results: According to the DESeq2 analysis, HE-1100 significantly modified the expression of 13 genes in OA chondrocytes by at least 10% with an adjusted p-value < 0.05 : EGR1 (+93%), FOS (+87%), NR4A1 (+43%), DUSP1 (+18%), ZFP36 (+18%), ZFP36L1 (+14%), NFKBIZ (+16%) and CYR61 (+14%) were upregulated and ATF7IP (-10%), TXNIP (-11%), C10orf10 (-12%), CLEC3A (-12%) and MMP13 (-18%) were downregulated after 24h HE-1100 treatment. HE-1100 significantly increased (2.3 fold +/-1.2 after 24h, p=0.0444 and 2.3-fold +/-1.0 after 72h, p=0.0239) the CYR61 protein production by human OA chondrocytes. After 72h, HE-1100 slightly but not significantly increased aggrecan production by 14 ± 19 % (p=0.1117) and significantly increased type II collagen pro-peptide production by 27 ± 20 % (p=0.0147). For both time points CYR61 production by OA chondrocytes was positively and significantly correlated with aggrecan (r=0.66, p=0.0004) and type II collagen pro-peptide (r=0.64, p=0.0008) production. In alginate beads culture, pro-MMP-13 was significantly decreased by HE-1100 treated cultures from day 7 to day 14 (from -16 to -25 %, p<0.05) and from day 17 to 21 (-22 %, p=0.0331) in comparison to controls.

Conclusions: HE-1100 significantly modified the expression of DUSP1, C10orf10, ZFP36/L1 and CLEC3A, which are pathway mediators involved in MMP-13 expression and activation. Further, long-term (28 days) treatment with HE-1100 significantly reduced the production of pro-MMP-13, the inactive precursor of the metalloproteinase MMP-13 involved in type II collagen degradation. HE-1100 also promoted extracellular matrix formation probably through CYR61 production, a growth factor well correlated with type II collagen and aggrecan production.

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AN IN *VITRO* CHONDROGENIC DIFFERENTIATION MODEL USING KS483 MOUSE MESENCHYMAL PROGENITOR CELL LINE

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Purpose: KS483 cells are multipotent mouse mesenchymal progenitor cells that are able to differentiate into chondrocytes, adipocytes and osteoblasts and are a well-characterized model for the study of osteoblast differentiation and bone formation. We have studied the use of KS483 cell line in an *in vitro* model of chondrogenic differentiation.

Methods: The cells were cultured in three-dimensional (3D) pellet culture system in serum-free differentiation medium in the presence and absence of BMP-6 for 17-28 days. The pellets were cultured in 96-well plates and the medium was changed in 2-3 day intervals. The pellets were embedded in paraffin, sectioned and stained for Safranin O and Alcain blue for assessment of sulfated glycosaminoglycan (sGAG) content, and processed for immunohistochemistry to visualize type II collagen. For biochemical analysis, the pellets were digested with proteinase K and assayed for sGAG at days 17, 24 and 28. Type II collagen carboxyterminal propeptide (CP-II) was determined in the culture medium collected at day 17.

Results: Based on histological analysis, BMP-6 showed concentration-dependent stimulatory effects on chondrogenic differentiation, increased sGAG content of the pellets, and increased CP-II released into the culture medium.

Conclusions: These results suggest that the KS483 cell line can be used for setting up an *in vitro* model for studying chondrogenic differentiation and for identifying novel compounds with anabolic effects on chondrogenesis. As these cells can rapidly proliferate, they provide an unlimited source of easily expanded progenitor cells for conducting large number of replicates and studies.

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GENERATION OF AN INDUCIBLE TISSUE SPECIFIC DIO2 OVER-EXPRESSION MOUSE

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Purpose: Osteoarthritis (OA) is one of the most common chronic musculoskeletal disorders, for which the current therapeutic strategies are limited. Progressive damage to the articular cartilage and bone leads to pain and loss of joint function. The development of OA is very complex, due to the activation of several signaling pathways in the different tissues composing the joint, and is influenced by both genetic and environmental risk factors. GWAS studies identified an association between OA and the *DIO2* gene. DIO2 is an enzyme belonging to the iodothyronine deiodinase family. It intracellularly activates thyroid hormone by converting T4 to T3. OA is characterized by the loss of the stable phenotype of chondrocytes and progression towards hypertrophy. Our hypothesis is that *DIO2* needs to be reduced in aged cartilage, to maintain the stable phenotype of the articular chondrocytes. Indeed, we previously documented that *Dio2* knockout mice are protected against cartilage damage in a treadmill-running OA model and this was also reflected in a specific gene expression profile, marking either a protective effect in *Dio2* knockout or an OA-triggering effect in wild-type cartilage.

Methods: We have generated a new transgenic mouse model, overexpressing *DIO2*, under the control of an inducible Aggrecan promoter using the *Cre*-recombinase system. We have introduced the *DIO2* cDNA in the ROSA locus, preceded by a floxed STOP-cassette. We first determined the functionality of different tagged and modified versions of the DIO2 protein using a Thyroid-responsive luciferase reporter assay. Next, the necessary targeting vector was constructed, using the Gateway recombination system. After homologous recombination in ES cells, correctly recombined ES cell colonies were identified using PCR and Southern Blot analysis; and these were used in blastocyst injection experiments to obtain transgenic mice. This mouse was intercrossed with an *Acan-Cre* mouse, driving Tamoxifen-dependent expression of *Cre* recombinase in fully differentiated cartilage chondrocytes (generating *'DIO2 KI'* mice). A transcriptome analysis of the articular cartilage of *DIO2 KI* mice was performed. RNA was isolated from articular cartilage of the tibial plateau of the knee and used in an RNA sequencing study to compare gene expression with cartilage from normal wild-type C57Bl/6 mice (Illumina NextSeq500).

Results: *Dio2 KI* mice are currently being challenged in a spontaneous OA model of ageing and in a treadmill-running OA model. Articular cartilage of *DIO2 KI* mice was analyzed in an RNA sequencing transcriptomics analysis. Our initial pathway analysis of these data suggests a dysregulation of lipid metabolism due to *DIO2* gain-of-function.

Conclusions: Our preliminary results demonstrate that this mouse is a useful model to study the development of OA, and confirm the involvement of *DIO2* herein.

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TNF ALPHA-STIMULATED GENE 6 (TSG6) IS CHONDROPROTECTIVE IN VIVO BUT OVEREXPRESSION CANNOT COMPENSATE FOR LOSS OF FGF2

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Purpose: TNF alpha stimulated gene 6 (TSG6) is expressed in response to tissue injury and has purported tissue-protective and anti-inflammatory effects. High levels of TSG6 have been detected in the synovial fluid of patients with OA and following acute knee injury. Our published work shows that TSG6 is regulated after joint destabilisation in mice, in a highly mechanosensitive and fibroblast growth factor 2 (FGF2)-dependent fashion. As $FGF2^{-/-}$ mice develop accelerated OA, and injury-induced FGF2-dependent genes such as *Timp1* and *inhibin* were downregulated in TSG-$6^{-/-}$ mouse joints, we speculated that the chondroprotective role of FGF2 may be mediated through TSG6. We asked whether overexpressing TSG-6 could reduce the severity of OA in wild type or $FGF2^{-/-}$ mice.

Methods: OA was induced by cutting the medial menisco-tibial ligament (DMM) in 10-12 week old male and female $TSG6^{-/-}$, $TSG6^{tg}$, $FGF2^{-/-}$ mice with their respective controls (C57BL/6 and Balb/c), and male $FGF2^{-/-}$;$TSG6^{tg+/+}$ or $FGF2^{-/-}$;$TSG6^{tg-/-}$ mice. Focal cartilage injury was also performed in male and female $TSG6^{tg}$ mice. 8 and 12 weeks post-surgery, joints were sectioned and the severity of cartilage degradation was assessed according to a modified OARSI score. FGF2 levels were measured in cartilage injury conditioned medium from $FGF2^{-/-}$, Balb/c, TSG-$6^{tg+/+}$, TSG-$6^{tg-/+}$, TSG-$6^{tg-/-}$ hips by V-PLEX bFGF kit from Meso Scale Discovery (MSD, Rockville, MD).