IL-6, MCPIP1 or other mRNAs were assessed using TaqMan assays (Life Technologies, Foster City, CA). TargetScanS was used to identify miRNA target sites in the MCPIP1 mRNA (NM_025079).
Results: TargetScanS algorithm identified a potential miR-139 "seed sequence" in the 3'UTR of MCPIP1 mRNA. This prompted us to investigate the dynamics of miR-139 expression in damaged cartilage. Expression of miR-139 was higher in 8/9 damaged cartilage samples compared to the levels in smooth cartilage. Furthermore, expression of miR-139 was significantly induced in chondrocytes upon stimulation with IL-1b. OA chondrocytes transfected with miR-139 inhibitor and stimulated with IL-1b exhibited 35% increased expression of MCPIP1 and almost 30% reduction in the expression of IL-6. However this effect was reversed in OA chondrocytes with overexpression of miR-139. Since MCPIP1 is also targeted by miR-9, co-transfection of miR-9 and miR-139 mimics or inhibitors showed cumulative effect on the expression of MCPIP1 and IL-6. To see the effect on catabolic and anabolic gene expression we performed Taqman Assay and found that expression of ADAMTS4 and MMP13 was significantly changed in chondrocytes transfected with miR-139 mimics or inhibitors and this effect was further enhanced upon co-transfection with miR-9. Co-transfection of reporter vector containing the 3'UTR of MCPIP1 mRNA with miR-139 reduced the luciferase activity by 20% and this reduction was further increased to 35% upon co-transfection with miR-139 and miR-9. Mutation of miR-139 or miR-9 "seed sequence" abolished the inhibitory effect of these microRNAs on luciferase activity. Treatment with IL-6 increased the expression of miR-139 in a dose dependent manner in OA chondrocytes.
Conclusions: We for the first time have demonstrated the overexpression of miR-139 in OA cartilage, suggesting its role in OA pathogenesis. We also show that miR-139 overexpression suppress MCPIP1 expression resulting in IL-6 over-production. IL-6 also induce miR-139 expression and this positive feed back loop promotes its overexpression. Therefore targeting miR-139/9 could be therapeutically beneficial in the management of OA.

235
ADVANCED GLYCATION END PRODUCTS (AGES) INDUCE STRESS GRANULE ASSEMBLY IN HUMAN OA CHONDROCYTES THAT CAPTURES MRNAS ASSOCIATED WITH OSTEOARTHRITIS PATHOGENESIS

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Purpose: Stress granules are RNA containing cellular bodies induced in cells under stress and function as storage site for mRNAs disassembled from polysomes. Different environmental stresses such as oxidative stress or heat shock or viral infection cause phosphorylation of eIF2α which is the signal for translational arrest and assembly of stress granules. Several factors including cytokines and advanced glycation end products (AGEs) perturb chondrocytes homeostasis and induce stress and phosphorylation of eIF2α. Here we studied the effect of AGEs on stress granule assembly in human chondrocytes and analyzed the mRNA repertoire captured by stress granules in human OA chondrocytes.
Methods: Primary human chondrocytes were prepared by enzymatic digestion of the non-affected cartilage obtained from OA patients who underwent total knee arthoplasty. AGE-BSA was prepared by reacting BSA (Sigma Chemical Company, St Louis, MO) with glycoaldehyde (Sigma) and the reaction was terminated by removing non-reacted glycoaldehyde by dialyzing extensively against PBS. Human OA chondrocytes (1.2x106 cells/ml) were seeded in 24 well plates in complete DMEM/Ham's F-12 medium (Lonza, Walkersville, MD) and serum starved for 12h/overnight. Serum starved chondrocytes were treated with 100μg/ml AGE-BSA or control BSA 0-6 h. Stress granule formation was analyzed by immunofluorescence staining of stress granule markers, TIA1 or G3BP1. To analyze the mRNAs captured in stress granules, chondrocytes (50x106 cells) were treated with AGE-BSA as above and stress granules were immunoprecipitated with anti-TIA1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and RNA was isolated by Trizol-Chloroform method and analyzed by TaqMan assays for the presence of selective mRNAs in stress granules.
Results: Treatment with AGE-BSA induced the formation of stress granules in human OA chondrocytes with intense immunofluorescence staining of stress granule markers TIA1 and G3BP1 at 4 h post-treatment. No staining of stress granules was observed in untreated control OA chondrocytes (Figure-1). To find out the role of stress granules in translational regulation of specific mRNAs in OA chondrocytes, stress granules were immunoprecipitated with anti TIA1 antibody and stress granules associated RNA was purified and analyzed by TaqMan assays. Our results showed that mRNAs of COX-2 and MMP9 which are known to play important role in the pathogenesis of osteoarthritis were specifically enriched in stress granules in response to AGE-BSA treatment.
Conclusions: In the present study we demonstrate that treatment with AGE-BSA induces stress granules assembly in human OA chondrocytes. Analysis of mRNAs captured in stress granules showed selective capture of COX-2 and MMP-9 mRNAs in stress granules. The future plan of the study is to determine the role of stress granules in translational regulation of mRNAs in human OA chondrocytes and its relation to the pathogenesis of osteoarthritis.

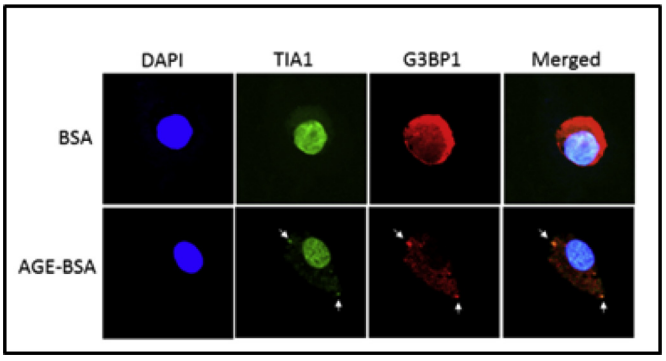

Figure 1. AGE-BSA-induced stress granules formation in human OA chondrocytes.

236
AMP-ACTIVATED PROTEIN KINASE (AMPK) LIMITS MITOCHONDRIAL DNA DAMAGE IN HUMAN KNEE OA CHONDROCYTES BY UPREGULATION OF SIRT3 AND THE DNA REPAIR ENZYME OGG1

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Purpose: Mitochondrial dysfunction in chondrocytes is associated with osteoarthritis (OA), and impacts pathways implicated in cartilage degradation by promoting oxidative stress and increasing matrix catabolism. Mitochondrial DNA (mtDNA) damage can cause mitochondrial respiratory chain dysfunction and augment production of reactive oxygen species (ROS). OA chondrocytes exhibit mtDNA damage and reduced mtDNA repair capacity. AMPK, a master regulator of cellular energy homeostasis, plays an important role in cell stress resistance. We previously discovered that AMPK activity (indicated by phosphorylation of AMPKα Thr172), becomes reduced in human and mouse knee OA chondrocytes/cartilage and aged mouse knee cartilage. Pharmacologic activation of AMPK in chondrocyte prevents de-phosphorylation of AMPKα, inhibits excessive oxidative stress by increasing expression of antioxidant enzymes and limiting mitochondrial ROS generation, and attenuates catabolic responses to IL-1β and TNFα. In this study, we tested the hypothesis that sustained chondrocyte AMPK activity is necessary for limiting mtDNA damage and increasing mtDNA repair capacity by upregulation of OGG1, a DNA repair enzyme, and SIRT3, a mitochondrial sirtuin previously shown to contribute to mtDNA repair through interaction with OGG1.
Methods: Primary human knee chondrocytes from either normal or OA (grade 4) donors were stimulated with the direct AMPK

pharmacological activator A-769662 (0.125 and 0.25 mM) for 24 hours. In some cases, these cells were treated with the ROS generation inducer menadione (100 μM) for 1 hour after A-769662 stimulation. Total cellular DNA was isolated and used for mtDNA damage analysis by regular PCR for the presence of the mt 4977-bp DNA deletion (viewed on agarose gels). Cell lysates were prepared and subjected to Western blot analyses for phosphorylation and expression of AMPKα, expression of OGG1 and SIRT3. Mitochondria-targeted OGG1 was overexpressed in chondrocytes via plasmid transfection. The transfected cells were tested for mtDNA damage in response to menadione, as well as nitric oxide (NO) release after stimulation with IL-1β (10 ng/ml) for 18 hours.

Results: Human knee OA (grade 4) chondrocytes, compared with normal chondrocytes, exhibited reduced phosphorylation of AMPKα and expression of SIRT3 and OGG1, and robust levels of the mt 4977-bp DNA deletion. In addition, the same mtDNA deletion mutant was observed in chondrocytes treated with menadione, suggesting ROS-induced mtDNA damage. However, A-769662 inhibited generation of the mt 4977-bp DNA deletion in either human knee OA chondrocytes or chondrocytes treated with menadione, and concurrently increased expression of SIRT3 and OGG1. Interestingly, overexpression of OGG1 in chondrocytes not only reduced generation of the mtDNA 4977-bp deletion induced by menadione, but also significantly inhibited NO IL-1β-induced NO release by about 41% ($p<0.0001$).

Conclusions: Mitochondrial DNA damage in human knee OA chondrocytes is associated with decreased AMPK activity. Conversely, pharmacologic activation of AMPK limits ROS-induced mtDNA damage and reverses significant amount of mtDNA damage pre-existing in the OA chondrocytes, mediated at least in part by inhibiting oxidative stress and increasing DNA repair capacity via upregulation of SIRT3 and OGG1. These findings identify a novel molecular mechanism of activation of AMPK on mitochondrial DNA integrity in articular chondrocytes, and further support the translational potential of targeted activation of AMPK for OA.

237
DYSREGULATED ATP EFFLUX MECHANISMS IN OSTEOARTHRITIC CHONDROCYTES

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Purpose: Extracellular ATP (eATP) plays a key role in cartilage health and disease. Low levels of eATP regulate mechanotransduction, while high levels produce arthritogenic calcium crystals, signal via purinergic receptors to elicit catabolic cytokines and prostaglandins , contribute to inflammation through innate immune system signaling, and produce pain. ATP efflux is regulated in an osmomechano-sensitive manner which is reflected in vitro by a surge in [eATP] after exposure to mechanical or osmotic stress. We recently showed that basal eATP levels in normal adult primary porcine articular chondrocyte cultures are critically regulated by levels of the gene product of the progressive ankylosis gene (ANK) , while the transient receptor potential vanilloid 4 (TRPV4) is necessary for increased ATP efflux with osmotic stress. We sought to determine if similar patterns of eATP efflux were observed in primary OA chondrocytes.

Methods: After obtaining informed consent and with local institutional review board approval, human articular hyaline cartilage was obtained at the time of knee joint replacement for OA. Normal human chondrocytes were obtained from Lonza and grown and differentiated according to manufacturer's directions. All cells were plated at 4 x10^5/cm^2 in short term high density primary cultures. For osmotic challenge, 30 % of the media was removed and replaced with sterile water. Controls were performed by removing 30% of the media and replacing it with fresh media. After 10 minutes, eATP levels were measured in the media using a standard bioluminescent assay (Sigma ATP Assay Mix (FLAAM)). Cultures were exposed to pharmacologic agonists and antagonists of ATP efflux pathways and modulators of calcium flux and TRPV4 for 30 minutes before basal and osmotically-challenged levels of eATP were measured.

Results: Basal levels of eATP varied considerably in the OA chondrocytes with average values of 4.76 ± 4 nM/mg protein and 6.8±3 nM/mg protein in normal chondrocytes . Both cell types responded to osmotic stress by increasing [eATP], but the magnitude of this increase in OA chondrocytes was less than that observed with normal chondrocytes (2.52 ± 1.4 fold in OA (n=23) vs 4.52± 2.1 fold increase in normal chondrocytes (n=4) ($p= 0.04$). The ANK channel inhibitor, probenecid, decreased levels of ATP under both basal and osmotically-challenged conditions in OA and normal chondrocytes (n= 9, $p<0.03$). No statistically significant responses were seen in normal or OA chondrocytes with exposure to other ATP efflux inhibitors including those blocking pannexin and connexin hemichannels, VARC/VSOAC or maxianion channels, or vesicular transport. In contrast to normal chondrocytes, OA chondrocytes demonstrated no increase in [eATP] after exposure to a calcium ionophore and did not alter their [eATP] after an osmotic challenge if BAPTA was used to stabilize intracellular calcium levels ($p=0.5$). Responses to the TPRV4 agonist GSK1016790A and antagonist HC067047 were also blunted in OA compared to normal chondrocytes. TPV4 western blotting confirmed reduced TRPV4 protein levels on OA compared to normal chondrocytes.

Conclusions: OA chondrocytes display a blunted response to osmotic stress which is likely related to low levels of TRPV4. Probenecid, an ANK inhibitor, potently reduces [eATP] in both normal and OA chondrocytes , and ANK remains an important pharmacologic target in cartilage diseases associated with excess eATP elaboration.

238
THE REGULATORY ROLE OF THE C-TERMINAL DOMAIN OF CONNEXIN 43 IN ARTICULAR CARTILAGE

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Purpose: Gap junction (GJ) channels are composed by a family of structurally-related transmembrane proteins called connexins. GJs were first discovery in myocardium and nerve because their properties of electrical transmission between adjacent cells. However nowadays is well know that connexins play clinical roles in the function of multicellular organisms. The ability to synchronize groups of cells for coordinated electrical, mechanical, metabolic and chemical communication make these proteins essential for tissue function. Mutations in connexin-encoding genes lead to developmental of a wide variety of diseases. Chondrocytes in adult cartilage and growth plate express Connexin 43 (Cx43) and gap junction intercellular communication (GJIC) has been demonstrated to occur in human primary chondrocytes and cartilage in vitro and in vivo. The inhibition of GJIC in micromass culture of chondrocytes reduces chondrocytes differentiation and defective Cx43 functions cause skeletal defects. Recent results from our group and others have convincingly demonstrated the involvement of the overexpression of Cx43 in OA pathogenesis. Cx43 has multiple GJ-independent functions that affect signalling pathways, cell growth, and cell proliferation. The carboxy terminal domain (CTD) of Cx43 is located in the cytoplasmic side and is key for protein functions. The aim of this study was to investigate the role of the CTD of Cx43 in cartilage structure and function.

Methods: Global knockout of Cx43 is embryonic lethal and homozygous K258stop animals, which Cx43 lacks the last 125 amino acid residues of the CTD, died shortly after birth. Cartilage and primary chondrocytes of 8 wild type, Cx43/KO, 6 K258stop/Cx43, 6 K258stop/KO and 6 K258stop/K258stop (21-day gestational section age mice) were subjected to the study. Mouse genotyping was achieved by PCR using DNA extracted from ear tissue and western-blot. The entire knee joints

[1] www.mayan-lab.com