Results: CEMIP was overexpressed in human and murine OA cartilage and along chondrocytes dedifferentiation. Most of genes deregulated in CEMIP depleted cells were involved in cartilage turnover (e.g. collagens), mesenchymal transition and fibrosis. CEMIP regulated β-catenin protein level. Moreover, CEMIP was essential for chondrocytes proliferation and promoted αSMA expression, a fibrosis marker, and TGFβ signalling towards the p-Smad2/3 (Alk5/PAI-1) pathway. Interestingly, CEMIP was induced by the p-Smad1/5 (Alk1) pathway. αSMA and type III collagen expressions were overexpressed in human OA cartilage and along chondrocytes dedifferentiation. Finally, CEMIP was co-expressed *in situ* with αSMA in all OA cartilage layers.
Conclusions: CEMIP was sharply overexpressed in human and mouse OA cartilage and along chondrocytes dedifferentiation. CEMIP regulated transdifferentiation of chondrocytes into « chondro-myo-fibroblasts » expressing α-SMA and type III collagen, two fibrosis markers. Moreover, these « chondro-myo-fibroblasts" were found in OA cartilage but not in healthy cartilage.

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CONTRIBUTION OF ZINC FINGER PROTEIN 440 TO FACET AND KNEE CARTILAGE DEGENERATION

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Purpose: We recently discovered that zinc finger protein 440 (ZNF440) may be involved in the process of spine (facet joint) osteoarthritis (FJ OA). This study is aimed to investigate the role of ZNF440 in the pathophysiology of cartilage degeneration during not only facet but also knee OA.
Methods: Expression of ZNF440 in facet and knee cartilage was determined by immunohistochemistry, quantitative real-time (q)PCR, and western blot. Human chondrocytes isolated from facet and knee OA cartilage were cultured and transfected with lentivirus carrying ZNF440-GFP-plasmid or control-GFP-plasmid, ZNF440 small interfering RNA (siRNA) or control siRNA, with/without interleukin (IL)-1β, to determine the contribution of ZNF440 on the expression of inflammatory, catabolic, anabolic, and cell proliferative/ death markers by qPCR, western blot and flow cytometry. Drugs that could inhibit the expression of *ZNF440* were determined by *In Silico* screening.
Results: We observed a marked increase in the expression of ZNF440 in both facet and knee OA cartilage compared to normal cartilage. Further, the relative expression of *ZNF440* RNA in facet cartilage significantly correlated with the severity of facet OA based on MRI grading. In vitro, overexpression of ZNF440 in knee OA chondrocytes transfected with lentivirus-GFP-plasmid showed a significant increase in the expression of cartilage catabolic marker MMP13 and cell death markers (Annexin V/7-AAD), and a significant decrease in the expression of type II collagen compared to OA chondrocytes transfected with control-GFP-plasmid. On the other hand, transfection of facet and knee OA chondrocytes with ZNF440 siRNA significantly suppressed the expression of inflammatory (*IL6*/*MCP1*), catabolic (*MMP13*), and cell death (PARP p85) markers stimulated with IL-1β compared with control. *In Silico* Drug screening followed by qPCR validation identified cyclosporine A and scriptaid that downregulated the expression of *ZNF440*. Treated knee OA chondrocytes with these drugs showed a significant alleviation of IL-1β-induced inflammatory, catablolic and cell death markers.
Conclusions: We have identified, for the first time, that the expression of *ZNF440* is significantly increased in the human facet and knee OA cartilage and may regulate inflammatory/ catabolic/ cell death markers during facet and knee OA.

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EXPRESSION OF αVβ5, αVβ3 AND β6 INTEGRINS BY OSTEOARTHRITIC CHONDROCYTES AND VERTEBRAL OSTEOPHYTES

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Purpose: Osteoarthritis is an evolving joint disease characterized by chondrolysis and osteophyte formation. We have previously shown in vivo by PET/CT the fixation of the tracer ^{18}F-PRGD2g in the coxofemoral joint osteoarthritis and peridiscal lumbar osteophytic structures. This RGD pattern is recognized by several heterodimeric integrin receptors whose expression is poorly known in situ. Through this work, our goal is to: (i) determine the tracer affinity for different integrin complexes, (ii) analyze their expression in situ and (iii) test in vitro the effect of osteogenic differentiation on integrin subunit expression.
Methods: The affinity of various RGD ligands suitable in vivo was measured by competition between their binding to heterodimeric integrins and ^{125}I-echistatin. In situ studies are performed on fractured healthy cartilage (n = 4), osteoarthritis (n = 8) and vertebral osteophytes (n = 10). Osteophytes are stained with Masson's Trichrome, hematoxylin/eosin, alizarin red and anti-collagen immunolabeling. The heterodimeric integrins αVβ3, αVβ5 and β6 are visualized by immunohistochemistry. The expression of αV, β3, β5 and β6 is also analyzed by immunodetection in human chondrocytes (n = 20) subjected to osteogenic differentiation. The Cytomine, QuPath and ImageStudioLite programs allowed for quantification.
Results: The complexes recognized with the most affinity by the RGD ligands were in order: αVβ5, αVβ3 and αVβ6. In situ, the percentage of stained cells in healthy cartilage vs osteoarthritic cartilage was calculated for αVβ5 (19.92 ± 4.13 vs 48.22 ± 9.04), αVβ3 (23.73 ± 4.19 vs 50, 9 ± 9.93) as well as β6 (44.78 ± 6.72 vs 67.31 ± 3.79) and was found significantly increased in osteoarthritic cartilage. The average area of the osteophytes analyzed is 42.74 mm^2 with 34 to 94% of connective tissue. Most of them are vascularized (from 10 to 177 vessels per sample). All included mineralized area and positive area for type II collagen. Only one in two was positively stained for type III collagen. Expression of integrins is detected in most of cases at the level of the blood vessels and in the positive zones for collagens type II and III (4/5), as well as type II (9/10). In vitro, the integrin subunits are expressed by dedifferentiated chondrocytes and their expression is significantly increased after osteogenic differentiation.
Conclusions: αVβ3, αVβ5 and β6 integrins are expressed in osteoarthritic cartilage, vertebral osteophytes (vessels and collagenous areas) and upon osteogenic differentiation of chondrocytes. The anatomic zones that bind the RGD tracer in vivo express well in situ the integrins that it recognizes, providing relevance to PET/CT imaging using ^{18}F-PRGD2g.

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THE ROLE OF FOXA2 TRANSCRIPTION FACTOR AS POTENTIAL REGULATOR OF ARTICULAR CARTILAGE HYPERTROPHY AND OA PROGRESSION

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Purpose: The proposed studies are anticipated to establish whether FoxA2 is a potential target for the treatment of OA, and also to provide insights into mechanisms underlying OA initiation.
Methods: The following mouse lines were used: FoxA2$^{flox/flox}$, FoxA3$^{-/-}$, TREtight-FoxA2, and Prg4$^{CreERt2\text{-}GFP}$ mice.
Results: We evaluated the expression of FoxA2 in both healthy and OA cartilage using *FoxA2$^{IRES\text{-}CreERT2/+}$ Tomato* reporter mice. In healthy articular cartilage, the majority of cells positive for FoxA2 expression (red) are located on the tidemark (TM) or below, in the calcified zone of the articular cartilage (Fig.1). We also looked for co-localization of FoxA2 and MMP13 in articular cartilage by performing immunofluorescence for MMP13 on sections from *FoxA2$^{IRES\text{-}CreERT2/+}$;Tomato* mice and found that FoxA2 positive cells (red) and MMP13 positive cells (green) overlap in the hypertrophic zone (overlay yellow) (Fig.1). Next, we looked at expression of FoxA2 in OA articular cartilage using the Destabilization of the Medial Meniscus (DMM) murine model of OA. We performed DMM surgery on *FoxA2$^{IRES\text{-}CreERT2/+}$; Tomato* reporter mice, injected the mice with tamoxifen, and stained the knee joints with green fluorescent dye 5-DTAF (20μg/ml) to visualize cartilage matrix. We found that the number of Tomato FoxA2-positive cells located ABOVE the tidemark in the operated knee articular cartilage is significantly higher than the number of FoxA2-positive cells observed above the tidemark in the control knee (Fig.2). This suggests that surgical destabilization of the knee joint induced expression of FoxA2 above the tidemark, in the non-calcified region of the articular cartilage, a place where it's not normally expressed in healthy articular cartilage. Since FoxA2 expression is induced in murine OA cartilage, we next

asked whether FoxA2 is necessary for cartilage degradation, and examined whether the loss of FoxA2 in superficial articular cartilage would alter the progression of the disease in the DMM model. We employed a newly created mouse line in which tamoxifen-inducible CRE recombinase (Cre-ERT2) is driven by the *Prg4* (Lubricin) regulatory sequences to delete FoxA2 specifically in the chondrocyte population above the tidemark. Tamoxifen treatment of $Prg4^{Cre/+}$; $Rosa26^{LacZ}$ mice induces recombination at the $Rosa26^{lacZ}$ locus and labels the cells in the top layer of the articular cartilage. Using this line, we specifically removed *FoxA2* alone, by injecting 8-week old $Prg4^{Cre/+}$; $FoxA2^{fl/fl}$ mice with either tamoxifen or corn oil (as shown in Fig.3) for 10 days followed by DMM or sham surgery. At 16 weeks post surgery, knee joints were processed for Safranin O/Fast green staining and scored for cartilage degradation using the OARSI histological scale. While control (corn oil treated) $Prg4^{Cre/+}$; $FoxA2^{fl/fl}$ mice (with intact *FoxA2)* displayed significant cartilage destruction following DMM surgery (OARSI score of 3.2), tamoxifen treated $Prg4^{Cre/+}$; $FoxA2^{fl/fl}$; mice had considerably less cartilage destruction (OARSI score of 1.9). These findings demonstrate that loss of *FoxA2* and *FoxA3* in articular cartilage can slow the progression of cartilage destruction following surgically induced joint destabilization. We next asked whether overexpression of FoxA2 in murine articular cartilage cells is sufficient to accelerate cartilage degradation. To drive exogenous FoxA2 expression in articular chondrocytes, we have generated mice containing the FoxA2 transgene driven by a reiterated reverse tetracycline transactivator (rtTA) response element (*TREtight-FoxA2*). We performed DMM surgery on either the triple transgenic *Prg4CRE;rtTA;TgFoxA2* mice or their control *Prg4CRE;rtTA* littermates both treated with Doxycycline. Control mice, lacking the FoxA2 transgene, that have undergone surgery developed mild symptoms of the disease (OARSI score 1.37). In contrast, triple transgenic mice that have undergone surgery developed far more cartilage damage with more lesions on the articular cartilage (OARSI score 2.63).

Conclusions: Osteoarthritis (OA) is a painful joint disease characterized by progressive and irreversible deterioration of articular cartilage. There is no cure for OA and current treatments provide relief from pain and inflammation associated with the more advanced phases of disease but do not target OA progression because the molecular mechanisms are not well understood. Here we seek to establish whether deficiency in the FoxA factors inhibits articular cartilage hypertrophy and OA progression, thus qualifying these factors for therapeutic intervention.

Fig.1

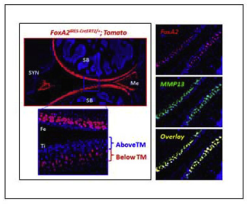


Fig.2

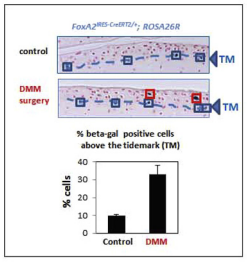
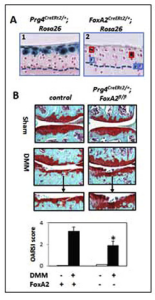


Fig.3



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PARKIN DEFICIENCY IMPAIRS THE CLEARANCE OF DYSFUNCTIONAL MITOCHONDRIA AND AUGMENTS IL-1B INDUCED INFLAMMATION IN HUMAN ARTICULAR CHONDROCYTES

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Purpose: Chondrocyte mitochondrial dysfunction and damage are readily detected in injured, aging, and osteoarthritis (OA) cartilage. The damaged mitochondria can trigger and amplify inflammatory processes in chondrocytes. Increased mitochondrial dysfunction is associated with cartilage matrix breakdown and inflammation in the OA joints, indicating that regulation of mitochondrial homeostasis can be a therapeutic target for the management of OA. However, the mechanism of clearance of dysfunctional mitochondria and its effect on inflammation in chondrocytes has not been explored. Parkin is an E3 ubiquitin ligase, which is recruited to the dysfunctional mitochondria upon mitochondrial membrane potential loss and ubiquitinates the mitochondrial membrane proteins and marking them for mitophagy. Here, we investigated the role of Parkin mediated mitophagy in the regulation of inflammation in primary human articular chondrocytes.

Methods: Human articular chondrocytes and cartilage explants were prepared from the OA knee cartilage obtained from donors undergoing arthroplasty at the Barberton SUMMA Hospital. Human normal cartilage was obtained from NDRI. The expression of Parkin in cartilage was determined either by IHC or immunofluorescence staining (IF) followed by confocal microscopy. The loss of mitochondrial membrane potential (MMP) was determined by JC-1 dye staining and ATP levels were determined using a kit. The accumulation of dysfunctional mitochondria was determined by staining with MitoTracker Deep Red followed by flow cytometry. Role of Parkin was studied by loss of function (suppression of Parkin expression by siRNA) and gain of function (overexpression of Parkin using plasmid) approach. The expression of Parkin, IL-6, COX-2 and iNOS was determined by using gene specific TaqMan assays and immunoblotting with validated antibodies. The levels of secreted cytokines in Parkin depleted chondrocyte's culture supernatant were determined by multiplex assay and at mRNA levels by gene expression analysis using a PCR based array.

Results: Expression of Parkin was downregulated in OA cartilage compared to normal cartilage. We also found the accumulation of dysfunctional mitochondria and reduced ATP production in OA chondrocytes compared to normal chondrocytes. Parkin protein was found to be colocalized with the mitochondrial marker SDHA in the damaged areas of human OA cartilage indicating active Parkin-mediated clearance of dysfunctional mitochondria. Knockdown of Parkin expression in primary human OA chondrocytes increased the expression of several proinflammatory cytokines including IL-6. In addition, Parkin knockdown also increased the expression of COX-2 and iNOS. SiRNA-mediated depletion of Parkin expression in human chondrocytes resulted in the accumulation of dysfunctional mitochondria. We also found that IL-1β stimulation of chondrocytes increased the mitochondrial dysfunction as determined by loss of MMP, reduced ATP production and fission of dysfunctional mitochondria from the mitochondrial network. Cytokine Multiplex assay and PCR array profiling revealed that the expression of several inflammatory genes, cytokines and chemokines was modulated by Parkin expression in chondrocytes. Overexpression of Parkin disease associated mutant, C431N, failed to suppress IL-1β induced mitochondrial membrane potential loss and clearance of dysfunctional mitochondria. Finally, overexpression of $Parkin_{wt}$ in human OA chondrocytes suppressed IL-1β induced loss of mitochondrial membrane potential and increased the clearance of dysfunctional mitochondria.

Conclusions: Our results demonstrate that Parkin-mediated clearance of dysfunctional mitochondria is a critical event in maintaining chondrocyte homeostasis and inhibition of inflammatory mediators and may be targeted for the therapeutic management of OA.

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INHIBITION OF LYSOSOMAL FUNCTION ENHANCES INFLAMMATORY GENE EXPRESSION, INDUCE OXIDATIVE STRESS AND APOPTOSIS IN HUMAN CHONDROCYTES

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Purpose: Lysosomes are the major catabolic organelle of the cells and are essential for the clearance of autophagosomes. Loss of lysosomal function with age has been associated with mitochondrial dysfunction, oxidative stress and apoptosis of the cells. However, the role of lysosomes in chondrocytes homeostasis under pathological conditions has not been explored. Here, we used Chloroquine (CQ) and Bafilomycin A1 (Baf) to inhibit lysosomal function and investigated its consequences on chondrocyte homeostasis and viability *in vitro*.

Methods: Human articular chondrocytes and cartilage explants were prepared from OA knee cartilage obtained from donors undergoing knee arthroplasty at the SUMMA Health Barberton Hospital. Human normal cartilage was obtained from NDRI. The expression of lysosomal associated membrane protein 1 (LAMP1) in normal and OA cartilage was determined by immunohistochemistry. Primary human OA chondrocytes or cartilage explants were maintained in complete DMEM/F12 and treated with CQ or Baf for 24 h or indicated time in DMEM/F12 medium without serum. Induction of apoptosis in chondrocytes treated with CQ or Baf was determined by measuring the activity of LDH