Conclusions: These data showed that xenestrogens as well as the vinclozolin metabolite M2 modified the rate of early chondrogenic differentiation by transiently maintaining COL2A expression. Our hypothesis is that COL2A induced persistence, might provide matrix weakening in the long term and/or sequester growth factors, eventually modifying the course of cartilage degenerative diseases such as osteoarthritis. EDC also modified COL2A expression in dedifferentiated chondrocytes such as found in aging or osteoarthritic tissues. These data are in accordance with the strong COL2A expression reported in OA cartilage but its role remains to be studied.

193 QUANTITATIVE PROTEOMICS OF ARTICULAR CARTILAGE USING SEQUENTIAL EXTRACTION OF TRANSVERSE CRYOSECTIONS

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Purpose: Articular cartilage is a highly cross-linked tissue. Because the insoluble collagen-rich residues are left behind after commonly used guanidine-HCl (Gu-HCl) extraction, the composition of the insoluble residue has not been fully elucidated. Moreover, articular cartilage has a variety of microenvironments varying by distance from the surface (superficial, middle, and deep layers) or distance from the chondrocyte (pericellular, territorial and interterritorial zones). We hypothesize that during joint damage, the catabolic activities and the rate of protein synthesis vary within these subregions. The goal of this study was to quantify the amount of extractable and non-extractable protein and to elucidate the differences between subregions in different joints (hip and knee) and under different physiological conditions (healthy and osteoarthritic).

Methods: We collected hip and knee cartilage samples (healthy and osteoarthritic) as surgical waste from surgical repair of trauma or joint replacement. Transverse cryosections were generated by distance from the surface. To avoid the confounding background by intracellular proteins, chondrocytes were depleted by performing one rapid freeze/thaw cycle in hypotonic solution. We performed Gu-HCl extraction to obtain extractable proteins from knee cartilage sections and in situ trypsin digestion to extract proteins from the insoluble residue. An adjacent set of cartilage sections from knee and hip cartilage were acquired for toluidine blue staining to distinguish different zones. By laser capture microscopy (LCM, Carl Zeiss PALM Microbeam), we precisely sampled (~2 mm² area samples) the cartilage matrix from the territorial (T) and interterritorial (IT) matrix zones from each layer. We performed in situ trypsin digestion of these LCM-harvested samples to extract all proteins including portions of the insoluble residue remaining after Gu-HCl extraction. All batches of extracts were quantified using multiple reaction monitoring (MRM) by a triple quadrupole instrument (TSQ-VENTAGE, Thermo Scientific) and calculated the ratio of extractable to total protein (sum of extractable and non-extractable) and defined this value as extractability.

Results: As expected, aggrecan core protein was readily extracted by Gu-HCl methods. It was noteworthy that the extractability of cartilage intermediate layer protein (CILP) 1–1 increased with cartilage depth in articular cartilage, but decreased in healthy cartilage. However, the extractability of CILP 1–2 increased with cartilage depth in both types of cartilage. In articular cartilage, a significant increase of extractability was identified in several proteins, including fibronectin (FNIC) and cartilage acidic protein 1 (CRAC1). We also investigated the protein distribution in distinct and precisely sampled subregions of cartilage. Similar to previous work focused on aggrecan core protein, the amount of all three aggrecan subdomains increased in the deeper regions. The aggrecan G3 domain accumulated in the territorial regions while the G1 and G2 domains accumulated in the interterritorial regions. These patterns were the same in both healthy knee and hip cartilage.

Conclusions: Characterization of the precise cartilage matrix architecture and functional subdomains will provide new insight into protein network signaling as well as a refined understanding of the catabolic and anabolic responses to arthritis. Interestingly, the extractability by Gu-HCl of some matrix proteins was increased in articular cartilage suggesting either increased amounts of protein and/or altered protein interactions to make them more readily dissociated from the cartilage meshwork. Our LCM-capture strategy with modern proteomic tool was validated by the comparability of our results for aggrecan domains by subregion with what is known in the literature. Ongoing analysis of other targeted proteins will be expected to provide a compelling new and comprehensive understanding of their distribution within cartilage and a global understanding of matrix architecture in health and disease.

194 IMPAIRED LONG BONE GROWTH IN CHONDROCYTE-SPECIFIC ERPS7 KNOCKOUT MICE IS INDUCED BY ER STRESS

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Purpose: During long bone growth by endochondral ossification chondrocytes produce large amounts of extracellular matrix molecules. Before folding and entering the secretory pathway, these molecules are subject to extensive post-translational modification, orchestrated by chaperone complexes in the endoplasmic reticulum (ER). ERPs7 is a member of the protein disulfide isomerase (PDI) family and facilitates correct folding of newly synthesized glycoproteins by rearrangement of native disulfide bonds. Here, we analyzed the functional role of Erp57 in bone development and growth in vivo.

Methods: Cartilage specific Erp57 knockout (KO) mice were generated by mating Erp57 fl/fl mice with mice expressing alpha1 Collagen II driven cre-recombinase. Size, weight, and bone length was determined in KO and wild type (WT) littermates at post-natal days 1, 7, 12 and at 1 and 2 months. Paraffin-embedded legs were analyzed by extensive histological, immunohistological and TUNEL staining. Tibiae of 1 month old mice were investigated by micro-computed tomography.

Results: The mice were viable, had a normal lifespan, and were fertile. However, their post-natal weight gain and growth of long bones was retarded, suggesting that Erp57 function is required for normal skeletal development and growth. The differences between KO and WT animals in male mice were maximal at the time of their pubertal growth spurt. Loss of Erp57 triggered ER stress, unfolded protein response, reduced proliferation and accelerated apoptotic cell death of chondrocytes. Together this resulted in a delay of long bone growth with the following characteristics: 1. enlarged growth plates; 2. expanded hypertrophic zones; 3. retarded osteoclast recruitment; 4. delayed remodeling of the proteoglycan-rich matrix and 5. delayed formation of trabecular bone in the primary and secondary ossification centers. All the growth plate abnormalities, however, became attenuated after the pubertal growth spurt, when protein synthesis is decelerated and, hence, ERp57 chaperone function is less essential.

Conclusions: Erp57-dependent protein disulfide isomerase activity is crucial for skeletal development and growth. Loss of Erp57 chaperone function results in ER stress and delays bone growth especially during the pubertal growth spurt when matrix deposition is particularly intensive.

195 TGF-BETA BLOCKS CHONDROCYTE HYPERTROPHY AND MAINTAINS CELL VIABILITY IN CULTURED CARTILAGE EXPLANTS BUT DOES NOT PROTECT AGAINST PROTEOGLYCAN LOSS

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Purpose: Degradation of the extracellular matrix of cartilage is a key aspect of osteoarthritis. In part, this degradation is due to chondrocytes gaining a hypertrophy-like phenotype, characterized by increased production of matrix metalloproteinase 13 (MMP13) and collagen type X (Col10a1). TGFβ1 is considered to be a protective factor for articular cartilage. It is able to block hypertrophy and able to maintain or increase production of glycosaminoglycans (GAG), an essential matrix component. These functions of TGFβ1 have mainly been identified using either stem cells or very young cartilage. However, TGFβ1-signaling is different in mature cartilage compared to young cartilage. Therefore, we investigated the effect of TGFβ1-signaling on healthy mature cartilage.
in regard of chondrocyte phenotype, viability and total amount of GAGs in long term ex vivo culture.

**Methods:** Bonevivo articular cartilage explants were isolated from the metacarpalphalangeal joint of healthy 5-year-old adult cows within 3 h post mortem. Subsequently, explants were cultured in DMEM/F12 alone or supplemented with either 10% Fetal Calf Serum (FCS), rhTGFβ1 or Insulin-like growth factor 1 (rhIGF1) for the duration of 2 weeks. Medium was refreshed every 72 h. To investigate via which TGFβ1 receptor, ALK1 or ALK5, the observed TGFβ1 effects run, the ALK5-kinase inhibitor SB-505124 was used at a concentration of 5 μM. In these experiments, DMSO was used as vehicle control. Sulfated-glycosaminoglycans (GAGs) were measured using dimethylmethylene blue (DMB) (Farndale assay). Cellular viability was measured with the use of XTT. To correct for the amount of cells, total DNA content of explants was measured using Picogreen. Furthermore, gene expression of chondrocyte hypertrophy markers was measured using qPCR with validated, cDNA-specific, primers.

**Results:** Compared to freshly isolated samples, expression of the chondrocyte-hypertrophy markers Col10a1 and Mmp13 was profoundly upregulated 64-fold in explants cultured for 2 weeks ex vivo and Sox9 expression was decreased 2-fold. The addition of 10% FCS to the medium did not inhibit the upregulation of these genes, and even induced expression of Alkaline phosphatase (Alpl), another marker of chondrocyte hypertrophy. In contrast, addition of TGFβ1 (1 ng/ml or 10 ng/ml) almost fully blocked induction of Col10a1 and Mmp13 expression, and lowered expression of Alpl 4-fold. Additionally, TGFβ1 induced Col2a1 expression 16-fold. Furthermore, TGFβ1 also maintained chondrocyte viability, because after 2 weeks mitochondrial activity corrected for DNA content was 50% higher in TGFβ1-treated samples compared to 10% FCS. All these effects of TGFβ1 were blocked by 5 μM of the ALK5-kinase inhibitor SB-505124. Apart from induction of chondrocyte hypertrophy marker genes, long term ex vivo explant culture also resulted in GAG loss; total GAG content was reduced from approximately 4% of wet weight in freshly isolated tissue to 1% of wet weight in the course of 2 weeks. As expected, addition of 10% FCS fully prevented GAG loss over time. However, addition of TGFβ1 to the medium remarkably did not inhibit GAG loss over time. Replacement of 10% FCS with 20 ng/ml of IGF1 also prevented GAG loss, but surprisingly, when IGF1 was combined with TGFβ1, TGFβ1 inhibited the positive effect of IGF1 on total GAG content.

**Conclusions:** Based on our results, we conclude that in adult cartilage TGFβ1 is a potent inhibitor of chondrocyte hypertrophy and that this inhibition runs via ALK5. Additionally, TGFβ1 profoundly induced Col2a1 production, and maintained chondrocyte viability in long term culture. In contrast, TGFβ1 was not able to counteract GAG depletion over time and even inhibited the beneficial effect of IGF1 on total GAG content in cartilage, demonstrating that TGFβ1 does not positively regulate GAG content in adult cartilage directly.

196

**DIFFERENTIAL REGULATION OF AUTOPHAGY IN ARTICULAR CARTILAGE CHONDROCYTES AND SYNOVIAL FIBROBLASTS DURING OSTEOARTHRITIS**

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**Purpose:** Osteoarthritis (OA) is the most common form of arthritis. Two major joint structures that typically play a key role in the initiation and progression of OA include articular cartilage and synovium. Chondrocytes are the only cell types present in the articular cartilage. During OA episode, chondrocytes have the ability to undergo accelerated cell death. Contrastingly, synovial fibroblasts exhibit opposite proliferative and replication phenotype than chondrocytes. During OA, synovial fibroblasts tend to proliferate faster and exhibit higher rate of cell survival. Understanding the exact cell death and survival mechanisms in chondrocytes and synovial fibroblasts within the joint structure is essential to devise strategies to achieve joint homeostasis. We have previously shown that process of autophagy, a cell survival mechanism, is significantly compromised in the articular chondrocytes during OA resulting in enhanced chondrocyte apoptosis and increased catabolic activity leading to degeneration of the articular cartilage, we investigated the expression and regulation of autophagy markers in OA synovial fibroblasts.

**Methods:** Normal and human synovial fibroblasts were cultured and subjected to qPCR and western blot analysis to determine the expression of autophagy markers in human OA fibroblasts compared to normal fibroblasts.

**Results:** Our results showed that synovial fibroblasts exhibit opposite autophagy regulation and activity compared to chondrocytes during OA. Specifically, we observed a significant up-regulation in the expression of critical autophagosome and autophagy activity markers including LC3B, ULK1, AMPK1, and ATG2 in synovial fibroblasts from OA patients compared to healthy controls. Furthermore, we determined the expression of TGF-β1, major profibrotic factor involved in fibroblast survival and proliferation. Compared to healthy control fibroblasts, OA fibroblasts exhibit increased expression of TGF-β1 and phosphorylation of its downstream SMAD2/3 signalling pathway. Treatment with TGF-β1 resulted in increased expression of both LC3B and ULK1 that correlated with increased rate of cell proliferation.

**Conclusions:** These results show that two joint structures (articular cartilage and synovium) exhibit differential autophagy regulation during OA: (a) Loss of autophagy in articular chondrocytes results in accelerated chondrocyte cell death. (b) Increased autophagy results in enhanced synovial fibroblast survival and proliferation. We now plan to investigate the regulation of autophagy in subchondral bone in OA. Understanding the exact autophagy mechanisms and associated cell survival mechanisms will help us understand joint homeostasis and delay or stop the progression of OA.

197

**LOOSE BODIES IN THE KNEE JOINT — A RECAPITULATION OF ENDOCHONDRAL OSSIFICATION**

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**Purpose:** Loose bodies are free floating elements of bone and cartilage in the knee or any joint affected. Loose bodies can be caused by degenerative joint disease, a bone fragment resulting from fracture or possibly a torn piece of cartilage. They can cause symptoms such as: pain and swelling, the inability to straighten the knee, intermittent locking of the knee. The hypothesis of the study was that loose bodies develop via endochondral ossification.

**Methods:** Loose bodies were harvested during surgery. They were fixed in 4% PFA after overnight and embedded in paraffin. Bone architecture was analysed using X-Ray and μCT analysis. For analysis of histological composition sections were stained using safranin-orange and alizarin-red/alcian blue staining. Immunohistological stainings for collagen II, collagen VI, collagen X and MPP13 were performed. Osteoclasts were detected using TRAP staining and osteoblasts using osterix.

**Results:** X-Ray and μCT analyses revealed a trabecular structure in some of these bodies. We found 4 stages of loose body differentiation: a fibrous, a cartilage, a bone/cartilage and a bone stage. We found that a synovium like tissue surrounds stages 1–3, but is not present any more at the bone stage. All stages are marked by expression of specific marker proteins. Stage 1 expresses high levels of collagen II, no collagen X and MPP13. Only a weak staining for collagen VI is present, indicating nearly no hyaline cartilage. Stage 2 expresses high levels of collagen II, VI as well as X and MPP13. These staining indicate that the loose body is formed mainly of hyaline cartilage, which chondrocytes differ- entiating to hypertrophic state. Stage 3 also expresses all collagens, showing increased amounts of MPP13 and collagen X. Furthermore, osterix positive cells can be found indicating a differentiation to an osteoblastic phenotype. Stage 4 expresses only at the superficial layer collagen II, nearly no collagen X and VI, as well as MPP13. Some osterix positive cells can be found. No osteoclasts were found in all stages.

**Conclusions:** Loose bodies seem to derive from a cartilage like source and then undergo endochondral ossification. A transdifferentiation into a more fibroblast like phenotype, as well as an osteoblast like phenotype can be observed. The differentiation to osteoclasts, however, seems not to be possible.