204 CARTILAGE DEGRADATION IN OA: A LIMITED ROLE FOR IL-1b
C. Bougault1, M. Gossot1, X. Houard1, J. Sellami1,2, C. Salvat1, F. Berenbaum1,2, C. Jacques1, 1UR, Univ. Pierre & Marie Curie Paris VI, Sorbonne Universités, Aging, Stress and Inflammation Lab., Paris, France; 2UFR Pierre & Marie Curie Paris VI, Dept. of Rheumatology, Saint-Antoine Hosp., Paris, France

Purpose: The main feature of osteoarthritis (OA) is degeneration and loss of articular cartilage. The cartilage matrix breakdown is due to both abnormal biomechanical stress and activation of catabolic processes involving metalloproteinases (MMPs). IL-1b is currently thought to have a prominent role in shifting the metabolic balance toward degradation. IL-1b is first synthesized as an inactive precursor, which needs a cleavage to turn into the secreted active form. This maturation process mainly occurs in a molecular scaffold called "inflammasome", where initiators (including NLRP3) and adaptor molecules (ASC) oligomerize and recruit pro-caspase-1. The complex formation induces caspase-1 activation, which in turn processes IL-1b precursor. Given the primary role of inflammasome in IL-1b maturation and the putative role of IL-1b in OA pathology, we aimed to clarify the role of both inflammasome and IL-1b cytokine in cartilage breakdown.

Methods: First, we investigated the expression of inflammasome components and we measured soluble IL-1b concentration by ELISA in the conditioned media obtained after 24 hours incubation with cartilage explants of 15 OA patients. Second, in primary mouse articular chondrocytes cultures, we used lipopolysaccharide (LPS, 0.01 to 1 µg/mL) to induce a pro-degradative phenotype, characterized by an increase in gene expression (real-time PCR) and in protein release of MMP-3 (ELISA). MMP-9 (zymography) and MMP-13 (Western-blots). We studied the effects of a deficiency in NLRP3 using chondrocytes from NLRP3−/− mice, of an inhibition of caspase-1 using Z-YVAD-FMK (10 µM) and of a blockade of IL-1b using an IL-1b receptor antagonist (IL-1RA, 100 ng/mL). At last, we triggered degradation in mouse cartilage explants by dynamic compression (0.5 Hz and 1 MPa of magnitude for 24 hours) to investigate the role of NLRP3 and IL-1b in load-induced cartilage degeneration.

Results: Despite the expression of the inflammasome components NLRP3, ASC and caspase-1 in OA chondrocytes, OA cartilage was not able to produce soluble active IL-1b itself. In mouse articular chondrocytes, LPS treatment dose-dependently increased MMP-3, MMP-9 and MMP-13 (538-fold induction in [MMP-3] in culture supernatant by LPS 1 µg/mL). Surprisingly, this catabolic response was similar to the levels in mild OA cartilage, however, XBP1 splicing was lower than the splicing levels stimulated by 1 nM tunicamycin (Table 1). Tunicamycin dose-dependently increased MMP-3, MMP-9 and MMP-13 in NLRP3−/− chondrocytes (mean [MMP-3] ± SEM: 447 ± 99 in WT versus 676 ± 104 ng/mL in NLRP3−/− chondrocytes) and was unchanged with the caspase-1 inhibitor (mean [MMP-3] ± SEM: 1162 ± 188 without versus 1229 ± 223 ng/mL with Z-YVAD-FMK). These results demonstrate that the LPS-induced pro-degradative phenotype was inflammasome-independent. Being aware that other pro-caspases can mediate IL-1b maturation and production, we used IL-1RA to block IL-1b activity. Once again, the LPS-induced catabolic degradation was unchanged (mean [MMP-3] ± SEM: 808 ± 219 without versus 808 ± 226 ng/mL with IL-1RA). Likewise, we characterized mouse cartilage explants degradation in response to dynamic compression, and we show again this load-induced catabolic response was NLRP3- and IL-1b-independent. Taken together, these results suggest that chondrocytes are able to acquire a pro-degradative phenotype without any contribution of IL-1b.

Conclusions: Our results challenge the view that IL-1b is a key mediator for cartilage degeneration in OA and may explain why previous trials with IL-1b inhibitors were all negative.

205 ENHANCED APOPTOTIC AND REDUCED PROTECTIVE RESPONSE IN CHONDROCYTES FOLLOWING ENDOPLASMIC RETICULUM STRESS IN OSTEOARTHRITIC CARTILAGE
K. Takada, J. Hirose, H. Mizuta. Kumamoto Univ., Kumamoto, Japan

Purpose: Endoplasmic reticulum (ER) stress has been shown to participate in many disease pathologies. Although recent reports have demonstrated that ER stress in chondrocytes is present in human osteoarthritis (OA), its role in the pathology of cartilage degeneration, such as chondrocyte apoptosis, remains unclear. The purpose of the present study was to investigate the association between ER stress and chondrocyte apoptosis in degenerative cartilage, and to clarify the involvement of ER stress in the pathology of OA.

Methods: Articular cartilage samples were obtained at total knee arthroplasty from the tibial plateaus of 11 patients suffering from knee OA. The histological severity of cartilage degeneration of each sample was evaluated by the Mankin scoring system. To evaluate ER stress in OA cartilage, the expression of phosphorylated PERK (pPERK), ubiquitin (Ub), GRP78, CHOP and phosphorylated JNK (pJNK) and the mRNA splicing of XBP1 (XBP1 splicing) in human OA cartilage by immunohistochemistry and RT-PCR, respectively. Chondrocyte apoptosis in OA cartilage was evaluated by immunohistochemistry for cleaved caspase-3 (C-CASP3). Additionally, human chondrocytes were treated with various concentrations of tunicamycin (0.5, 1, 5, 10 µg/mL), an ER stress inducer, to assess the impact of ER stress on the mRNA expression of CHOP, XBP1 splicing and apoptosis, as determined by real-time PCR, RT-PCR and ELISA analysis, respectively.

Results: In human OA cartilage, the percentages of chondrocytes positive for pPERK, Ub and CHOP positively correlated with cartilage degeneration (Table 1) and the percentage of chondrocytes positive for C-CASP3 (Table 2). XBP1 splicing and GRP78 expression in severe OA cartilage containing the greatest number of C-CASP3-positive chondrocytes were similar to the levels in mild OA cartilage, however, XBP1 splicing was higher in moderate OA cartilage than in mild and severe OA cartilage (Table 1). Tunicamycin dose-dependently increased CHOP expression and apoptosis of cultured chondrocytes (Figure 1). Tunicamycin increased XBP1 mRNA splicing of chondrocytes, while the levels of the splicing in chondrocytes stimulated with 10 µg/mL concentration of tunicamycin were lower than the splicing levels stimulated by 1 µg/mL of tunicamycin (Figure 1).

Table 1. The relationship between ER stress and chondrocyte apoptosis

<table>
<thead>
<tr>
<th>ER Stress</th>
<th>Mild (n = 5)</th>
<th>Moderate (n = 7)</th>
<th>Severe (n = 8)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPERK (%)</td>
<td>23.0 ± 10.4</td>
<td>37.0 ± 5.0</td>
<td>40.0 ± 7.6</td>
<td>0.06</td>
</tr>
<tr>
<td>Ub (%)</td>
<td>9.1 ± 2.8</td>
<td>29.6 ± 6.6</td>
<td>59.3 ± 3.7</td>
<td>0.01</td>
</tr>
<tr>
<td>GRP78 (%)</td>
<td>16.2 ± 2.9</td>
<td>31.2 ± 19</td>
<td>24.2 ± 2.8</td>
<td>0.51</td>
</tr>
<tr>
<td>CHOP (%)</td>
<td>8.2 ± 5.2</td>
<td>15.9 ± 13.7</td>
<td>35.4 ± 9.2</td>
<td>0.74</td>
</tr>
<tr>
<td>pJNK (%)</td>
<td>8.6 ± 7.5</td>
<td>32.7 ± 14.9</td>
<td>48.6 ± 13.8</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table 2. The relationship between ER stress and cartilage degeneration

<table>
<thead>
<tr>
<th>ER Stress</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
<th>Mankin score</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>XBP1 splicing</td>
<td>0.18 ± 0.00</td>
<td>0.42 ± 0.04</td>
<td>0.26 ± 0.03</td>
<td>0.30</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1.
associated with an enhanced apoptotic response and a reduced protective response by the cells. This study suggests that ER stress has an important role in the pathology of cartilage degeneration.

### 206 HYPOXIA INHIBITS HYPERTROPHIC DIFFERENTIATION AND ENDOCHONDRAL OSSIFICATION IN EXPLANTED TIBIAE

**J. Leijten, N. Georgi, E. Landman, L. Moreira Teixeira, C. van Blitterswijk, M. Karperien. Univ. Twente, Enschede, Netherlands**

**Purpose:** During development, mesenchymal progenitor cells first condense and then differentiate into chondrocytes to form a cartilaginous template of the future long bone. Most of these chondrocytes eventually undergo hypertrophic differentiation leading to cartilage matrix mineralization, chondrocyte apoptosis, and in growth of blood vessels. As a result, hypertrophic cartilage is alleviated from the otherwise hypoxic conditions normally present in the avascular cartilage. In the current study, we addressed the question whether this change in oxygen level from hypoxia towards normoxia is a mere consequence of angiogenesis or a driving factor in hypertrophic differentiation and subsequent endochondral ossification.

**Methods:** Fetal mouse tibiae (E17.5) were explanted from timed-pregnant mice and cultured up to 21 days either under normoxic conditions (21% oxygen) or under hypoxic conditions (2.5% oxygen) with or without 50 or 500 ng/ml recombinant Grem1, Frzb and Dkk1 protein. Previously we have shown that these secreted Wnt- and BMP antagonists were able to prevent hypertrophy in chondrogenically differentiating MSCs. Growth kinetics was analyzed using microscopical evaluation. At designated time points explants were fixed and embedded in paraffin. Five micrometer sections were stained with Alcian blue and Alizarin red S for histological evaluation of hypertrophic differentiation and endochondral ossification. Image analysis was performed using the Image J software package. Cartilaginous ends of the tibiae were isolated and subjected to gene expression analysis using RT-qPCR and protein expression analysis using ELISA.

**Results:** Explanted tibiae cultured under normoxic conditions became significantly more elongated compared to tibiae cultured under hypoxic conditions. Remarkably, macroscopic and histological analysis showed that the cartilaginous area of the primary physis became significantly larger in hypoxia. Strikingly, tibiae cultured under normoxic conditions progressively increased the width of the hypertrophic zone and decreased their resting zone, while under hypoxic conditions the opposite phenomenon was observed. Moreover, the calcified area of hypertrophic cartilage was significantly larger under normoxic conditions. Although the length of calcified tissue was significantly shorter under hypoxic conditions, it was more extensively calcified. Gene expression analysis showed that under normoxic conditions hypertrophic marker genes such as Col10A1, MMP13, RUNX2, COL6A1 and ALPL were significantly higher expressed compared to hypoxic conditions. In contrast, under hypoxic conditions markers for hyaline cartilage such as Acan, Col2a1 and Sox9 and secreted articular cartilage markers Grem1, Frzb and Dkk1 were significantly higher expressed. Indeed, addition of Grem1, Frzb and Dkk1 to explanted tibiae cultured under normoxia mitigated longitudinal growth, by inhibiting hypertrophic differentiation and endochondral ossification, in a concentration dependent manner.

**Conclusions:** Collectively our data suggests that oxygen levels play a powerful role in the terminal differentiation of hypertrophic chondrocytes and formation of endochondral bone. Where normoxia induces up regulation of hypertrophic related genes, hypoxia triggers tibiae to produce chondrogenic markers and suppressed hypertrophic differentiation at least partially by stimulating the expression of the secreted antagonists Grem1, Frzb and Dkk1. Together this suggests that the high oxygen levels, as found in the hypertrophic zone, actively contribute to the hypertrophic differentiation of cartilage and its subsequent endochondral ossification.

### 207 INHIBITION OF MAMMALIAN TARGET OF RAPAMYCIN (mTOR) INDUCES AUTOXYTHESIS IN ARTICULAR CARTILAGE AND REDUCES SEVERITY OF EXPERIMENTAL OSTEOARTHRITIS

**B. Caramelo1, A. Hasegawa1, M. Tajimuchi1, E. Bianco2, R. Terkeltaub1, M. Lotz1. 1The Scripps Res. Inst., La Jolla, CA, USA; 2Ostearticular and Aging Res. Lab, INIBIC-Complejo Hosp.ario Univirrio A Coruña, A Coruña, Spain; 3Dept. of Med., Univ. of California, San Diego, La Jolla, CA, USA**

**Purpose:** Osteoarthritis (OA) is characterized by insufficient extracellular matrix synthesis and articular cartilage degradation. Autophagy is an essential cellular homeostasis mechanism for the removal of dysfunctional intracellular macromolecules and organelles. Previous findings indicated deficient autophagy in aging and OA cartilage. The Mammalian Target of Rapamycin (mTOR) is a key inhibitor of autophagy. In this study we determined whether inhibition of the mTOR pathway is associated with disease-modifying activity in experimental OA.

**Methods:** Experimental OA was induced by transection of the medial meniscotibial ligament and the medial collateral ligament (MML+MCL) in 2-month old C57Bl/6 mice (n = 36). Rapamycin (1 mg/kg weight/day) (n = 18 mice) or DMSO vehicle control (n = 18 mice) were administered intraperitoneally for 10 weeks. The morphological changes in the articular cartilage and synovium were examined by histology using semiquantitative scoring systems. Immunohistochemical and immunofluorescence staining were employed to analyze the effect of rapamycin on mTOR signaling pathway, autophagy and cartilage homeostasis.

**Results:** Intraperitoneal rapamycin treatment affected the mTOR signaling pathway in mouse knee joints as indicated by inhibition of p856, a direct target of mTOR and activation of LC3, a main marker of autophagy. The rate of progressive cartilage degeneration in mouse knee joints after surgical destabilization of the medial meniscus was significantly reduced (P < 0.01) in the rapamycin treated group compared to the vehicle treated group. In addition, the histological evaluation showed a significant decrease in synovitis (P < 0.05) after rapamycin treatment. The effect of rapamycin was directly correlated with maintenance of cartilage cellularity and a decrease in ADAMTS-5 expression in mouse knee joints.

**Conclusion:** These results suggest that inhibition of mTOR reduces cartilage degeneration and OA progression in experimental OA. Pharmacological inhibition of mTOR by rapamycin or other agents may be a potentially effective therapeutic approach for the treatment of OA.

### 208 REDUCED IGF SIGNALING MAY BE RESPONSIBLE FOR THE DECLINE IN CARTILAGE MATRIX GENE EXPRESSION IN DEGENERATED AREAS WITHIN OSTEOARTHRITIC CARTILAGE

**N. Fukui1,2, N. Tanaka2, Y. Ikeda2, T. Yamaguchi2, M. Miyamoto3, T. Tashiro4, Y. Katsuragawa5. 1The Scripps Res. Inst., La Jolla, CA, USA; 2Ostearticular and Aging Res. Lab, INIBIC-Complejo Hosp.ario Univirrio A Coruña, A Coruña, Spain; 3Dept. of Med., Univ. of California, San Diego, La Jolla, CA, USA**

**Purpose:** In osteoarthritis (OA), cartilage undergoes degenerative changes primarily in the weight bearing areas. Previous studies have shown that the expression of type II collagen and aggrecan is obviously elevated in preserved areas, but it is considerably attenuated in degenerated areas. Although this decline in matrix gene expression could be involved in the loss of cartilage in OA, a mechanism for the decline has not yet been elucidated. The purpose of this study was to clarify this mechanism through the analyses of human cells and cartilage samples.

**Methods:** This study was performed under the approval of an institutional review board. RNA was obtained from 10 end-stage OA cartilages at degenerated and preserved areas, respectively, and gene expression profiles were determined in respective RNA samples by cDNA microarray. The result of microarray analysis was confirmed by qPCR analysis combined with laser capture microdissection (LCM; Pixell II, Arcturus) for accurate acquisition of cartilage tissues from specific regions. The result of gene expression analysis was confirmed by immunohistochemistry and protein quantification of tissue extracts by BioPlex (BioRad). A signal pathway involved in the decline in matrix gene expression in degenerated areas was specified by explant culture experiments. RNAi and adenoviral transduction experiments were performed to elucidate the underlying mechanism for the decline.