were examined using the immunohistochemistry of the mouse limb cartilage. Cell proliferation was assessed by PCNA staining. For gain- and loss-of-function analyses, genes or the specific siRNAs were overexpressed in ATDC5 cells with stable retroviral transfection or in primary chondrocytes with adenoviral transfection. Transcriptional regulation was examined by luciferase assay using ATDC5 and non-chondrogenic HeLa cells transfected with a reporter construct containing a promoter fragment of the differentiation marker genes above. Transactivity of β-catenin was determined by the TOPFlash system. Computational predictions were performed using the online resource NetworKin for in vivo kinase-substrate relationships, and the GEO database for expressions in the growth plate cartilage.

**Results:** Both isoforms were largely unphosphorylated and active in the early differentiation stages of chondrocytes in cultured ATDC5 cells and in the mouse limb cartilage. Although Gsk3α−/− mice and Gsk3β−/− mice showed normal skeletal development, their compound Gsk3α−/−;Gsk3β−/− mice exhibited dwarfism with impairment of early chondrocyte differentiation without affecting the later differentiation or proliferation. Gain- and loss-of-function analyses using cultures of ATDC5 cells and primary chondrocytes from the knockout mice with overexpression of GSK-3 isoforms and their siRNAs revealed that GSK-3α and GSK-3β-induced early chondrocyte differentiation with functional relationship of GSK-3α and GSK-3β in a developmental stage. This was independent of the canonical Wnt signaling, since the transcriptional activity of β-catenin was not altered by the Gsk3 genotypes. Instead, computational predictions followed by SSO9 and COL2A1 promoter assays identified RelA (NF-κB p65) as a key phosphorylation target of GSK-3. Further analyses by mutagenesis in the 12 putative phosphorylation sites of RelA revealed that the Thr254-phosphorylation was essential for RelA in the RelA−/− chondrocyte culture caused suppression of the early differentiation markers, which was restored by the RelA overexpression, but not by the Thr254 mutant or the GSK-3 overexpression. Lastly, we created conditional knockout mice of Rela in undifferentiated limb mesenchyme (Pex1-Cre;Rela−/−) and in cartilage (Col2a1-Cre;Rela−/−), and confirmed that both mouse models exhibited dwarfism with impairment of early chondrocyte differentiation, similar to the Gsk3α−/−;Gsk3β−/− mice.

**Conclusions:** This study is the first to show in vivo evidence of the functional relationship of GSK-3α and GSK-3β through analysis of compound knockout mice. Redundant functions of the two isoforms through phosphorylation of RelA at Thr254 play a crucial role in early stages of chondrocyte differentiation.

53 THE TRANSCRIPTIONAL REGULATION OF MMP13 BY CPG METHYLATION IN HUMAN CHONDROCYTES: HIF2-ALPHA IS A POTENTIAL TARGET

K. Hashimoto1, M. Otero1, K. Imagawa2, M.C. de Andrade3, H.I. Roach4, R.O. Orefio5, M.B. Goldring1. 1 Hosp. for Special Surgery, New York, NY, USA; 2Univ. of Southampton, Southampton, United Kingdom

**Purpose:** In osteoarthritis (OA), articular chondrocytes undergo phenotypic change and acquire the ability to over-express matrix-degrading genes. We previously showed that DNA methylation at Cpg sites in the relevant promoters is correlated with the aberrant expression of several genes, including MMP13. However, how CpG methylation status intervenes to determine the promoter activity of genes such as MMP13 is still not known. The aims of the study were to determine (1) whether CpG methylation directly affects MMP13 promoter activities, (2) which CpG sites are responsible for changes in MMP13 promoter responses and (3) what transcription factors are involved in CpG methylation-related promoter regulation of MMP13.

**Methods:** The methylation status of the MMP13 proximal promoter and the MMP13 gene expression were quantified in healthy and OA human primary chondrocytes by pyrosequencing and RT-qPCR, respectively. Three MMP13 reporter constructs, spanning −372/+14, −214/+14 and −86/+14 bp of the proximal human MMP13 promoter were generated by PCR-amplification and cloned into a CpG-free luciferase reporter vector. CpG-mutant constructs were generated by substituting CG to TG using the wild-type −214/+14 MMP13 construct as template. Luciferase activities were quantified for each reporter construct, untreated or treated with DNA methyltransferase (M.SssI), and transfected in human immortalized C28/I2 chondrocytes, alone or in co-transfection with expression vectors encoding HIF1α, HIF2α, ESE1, Cfos, cJun, Runx2, USF1, CAT1 or Sp1. Chromatin immunoprecipitation (ChIP) assays were performed in C28/I2 cells in co-transfection experiments utilizing non-methylated or methylated MMP13 constructs, and HIF2α expression vectors, and analyzed by qPCR utilizing specific PCR primers for the transiently transfected MMP13 constructs.

**Results:** The aberrant MMP13 gene expression in human primary OA chondrocytes (25-fold higher compared to non-OA chondrocytes) correlated with increased DNA demethylation of the MMP13 proximal promoter, with the CpG site at −110 bp showing the greater percentage of demethylation from the seven CpG sites contained within the −330−/−14 bp proximal sequence. Luciferase assays revealed that methylation of the wild-type MMP13 reporter constructs lead to a significant decrease on their basal activities. Mutation of different proximal CpG sites indicated that only the mutation at the −110bp-CpG was able to repress the basal activity of the non-methylated MMP13 promoter. In agreement, the MMP13 promoter activity was preserved in a mutant construct in which all the proximal CpG sites except for −110bp were mutated, and methylation treatment repressed the activity of the same reporter construct. Over-expression of different transcription factors indicated that only the HIF2α-driven MMP13 transactivation was affected by CpG methylation at −110bp, correlating with the decreased HIF2α binding to the methylated MMP13 proximal promoter in ChIP assays.

**Conclusions:** We here show that CpG methylation directly represses the MMP13 promoter activity in vitro, and that the methylation status of the −110bp CpG site is critical for the promoter activity, leading to its decreased basal and HIF2α-driven activity, and reducing the binding of HIF2α to the proximal promoter. The latter indicates that HIF2α shows less affinity to its methylated DNA binding site, and suggests that HIF2α is the major to be a potential target of epigenetic transcriptional regulation of MMP13.

54 INTERACTION OF HUMAN MESENCHYMAL STROMAL CELLS AND REGULATORY T-CELLS IN OSTEOARTHRITIS


**Purpose:** There is an increasing body of evidence that inflammatory T cells from the synovium have a major role in the progression of osteoarthritis by overproduction of cytokines and growth factors. The balance between inflammatory and regulatory T cells is considered to play an important role in this process. Mesenchymal stromal cells have been extensively investigated for their tissue regeneration potential through the last two decades. More recently, interest has partially focused on immunogenic properties of MSC, and their abilities to regulate T-cell function. However, their role in regulation of immunity in arthritic diseases, most importantly in osteoarthritis, remains mostly unknown. The purpose of this study was to determine the influence of mesenchymal stromal cells (MSC) from osteoarthritis (OA) patients on regulation and recruitment of regulatory T-cells.

**Methods:** Bone marrow (BM) and synovial membrane (SM) was harvested from OA patients during total hip arthroplasty (n = 19). MSC were isolated from BM and SM and cultured in DMEM-LG until passage 2. Functional analysis and immunophenotyping confirmed the presence of typical MSC markers. Mixed lymphocyte cultures enriched in CD4+CD25+CD127− regulatory T-cells (Treg/MLC) were derived from whole blood taken from healthy donors. Treg/MLC and MSC were
cultivated for five days and after separation analysed for the presence of typical MSC markers and Treg markers by FACS. Single MSC and TregMLC cultures were used as controls. IL-2, 4, 6, 10, 17a, IFN-γ and TNF were assessed after 2 and 5 d of cocultivation by cytometric bead array (CBA) analysis.

In a second step, TregMLC cultures were analyzed in the presence of IL-6-complemented media with varying concentrations and media taken from MSC cultures (DMEM-LG). The abundance of IL-6 in these culture conditions was verified at different time points by CBA analysis.

Results: Both MSC from BM and SM were able to maintain the percentage of Tregs in coculture, while in TregMLC without addition of MSC, there was a significant decrease of the Treg fraction after 5 days. The effect of Treg maintenance was independent from Treg/MSC ratio. Compared to MSC cultures alone, T-cell-MSC cocultures showed a significant increase in IL-6 abundance after 2 and 5 days of culture. Cocultivation with MSC taken from synovial membrane showed significantly higher amounts of IL-6 production compared to BM-MSC-T-cell cocultures. Cultured without T-cells, SM-derived MSC showed a higher IL-6 production than BM-MSC. Overall IL-6 production was significantly reduced after 5 days of coculture, compared to the amounts observed at day 2. However, the differences between SM- and BM-MSC remained significant.

The effect of Treg maintenance could be replicated by IL-6 addition to culture media in a dose-dependent way. However, Treg maintenance was highest when IL-6 was given from MSC cultures, even if the abundance of IL-6 found in these media was surmounted in naive DMEM-LG. IL-2, 4, 10, 17a, IFN-γ and TNF-concentrations showed no significant differences between these groups.

Conclusions: The immunogenic potential of MSC has already induced certain therapeutic approaches in autoimmune diseases. Despite the knowledge that MSC are potent regulators of T-cell function in various diseases, the precise role in the modulation of osteoarthritis is mostly unknown. It is known that MSC can regulate and recruit Tregs in vitro.

Our data clearly demonstrate that maintenance of Treg phenotype in MSC-T-cell cocultures can be mediated by MSC derived from osteoarthritis patients. IL-6 seems to play an important role in mediating these processes. However, other soluble factors secreted by MSC can be supposed to regulate T-cell and Treg function more specifically. We postulate that synovial inflammation is responsible for the significant differences in IL-6 secretion by MSC derived from synovial membrane and bone marrow. However, this does not seem to affect MSC potency to maintain the Treg phenotype.

55 MORE INFLAMMATORY SIGNS ON ULTRASOUND IN INTERPHALANGEAL JOINTS IN EROSIVE HAND OSTEOARTHRITIS

M. Kortekaas, W.-Y. Kwok, M. Reijnierse, T. Huizinga, M. Kloppenburg,
Leiden Univ. Med. Ctr., Leiden, Netherlands

Purpose: Erosive hand osteoarthritis (HOA) is a subset of hand osteoarthritis (HOA) associated with a higher clinical burden than non-erosive disease. The processes that lead to erosive evolution are still unknown. The clinical course of HOA is characterised by episodes of inflammatory signs, as assessed during physical examination. Recent ultrasound (US) studies demonstrated a high incidence of inflammatory signs in both HOA and HOA.

In the present study the presence of inflammatory signs assessed by US in erosive and non-erosive interphalangeal joints in patients with HOA in comparison to interphalangeal joints from patients with non-erosive HOA was investigated.

Methods: Of 55 consecutive HOA patients, fulfilling the ACR criteria, at least 45 years of age, interphalangeal joints from both hands were used. Conventional radiographs of both hands were scored according to Verbruggen and Veyes; E(rosive) and R(emediated)-phases were defined as erosive. Using US all 18 interphalangeal hand joints were scored for synovial thickening, effusion, greyscale (GS) synovitis, and Power Doppler Signal (PDS) on a four-point scale.

Generalized estimated equation (GEE) analyses were performed to study the association between inflammatory signs and erosiveness in individual joints. GEE was also performed to study the association between the N(normal), S(tationary), Joint space loss, E and R-phases and US inflammatory signs. Relative risks were presented as odds ratios (OR) with 95% confidence intervals (95% CI). In multivariate analyses adjustments were made for confounders (age, sex and BMI).

Results: Of 55 HOA patients (mean age 61 years, 86% females) 51% showed at least one erosive joint. In 94 erosive joints GS synovitis, synovial thickening, effusion and PDS were found in 57%, 13% 50% and 15%, respectively; in 896 non-erosive joints in 29%, 10% 26% and 8%, respectively. Summated scores of PDS, GS synovitis and effusion were higher in EOA than in non-erosive HOA. GS synovitis was more frequent in S, J, E and R-phases compared to N-phases. PDS was only associated with E-phase (5.3 (1.3–20.5)) not with other phases. Non-erosive joints in EOA demonstrated more PDS (3.2 (1.6–6.4)), GS synovitis (2.2 (1.3–3.7)) and effusion (2.2 (1.2–3.8)) in comparison to joints in non-erosive HOA.

Conclusions: Inflammatory signs are more frequent in EOA than in non-erosive HOA, especially in the erosive joints. Remarkably, also interphalangeal joints without erosions in patients with EOA demonstrated more inflammatory US signs in comparison to interphalangeal joints of patients with non-erosive HOA. These results suggest an underlying systemic cause for erosive evolution.

56 THE ASSOCIATION OF PERIPHERAL AND CENTRAL SENSITIZATION WITH MUSCLE CO-ACTIVATION: A COMMON MECHANISM AFFECTING PAIN AND FUNCTION IN KNEE OA?

1BUSM, Boston, MA, USA; 2Columbia, New York, NY USA; 3Iowa City, IA, USA; 4Children’s Hosp., Boston, MA, USA; 5Aalborg Univ., Aalborg, Denmark; 6UCSF, San Francisco, CA, USA; 7UBA, Birmingham, AL, USA

Purpose: Deep somatic pain decreases agonist and increases antagonist activity during dynamic muscle activity (pain adaptation theory). Muscle co-activation (antagonistic hamstring muscle activation during quadriceps agonist activity) is of interest in knee OA because it may increase joint stability on the one hand, but may contribute to worsening structural lesions on the other. Experimental pain studies support central mechanisms affecting muscle function, but whether this occurs in OA is not known. This is relevant because knee OA is associated with peripheral and central sensitization (nervous system alterations in pain signalling), which in turn is associated with pain severity, but its association with motor function changes is not known. We therefore examined whether sensitization is associated with altered motor activity in knee OA as assessed by muscle co-activation.

Methods: The MOST Study is a NIH-funded cohort of persons with or at risk of knee OA. We obtained surface EMG of the medial and lateral quadriceps and hamstrings during knee extension and flexion maximum voluntary contractions (MVC) using an isokinetic dynamometer (Cybex) at 60°/s. Hamstring muscle co-activation was defined as the median of 4 trials in a single limb of hamstring muscle EMG activity during knee flexion MVC, standardized by MVC, during knee flexion MVC (% of max). Medial and lateral hamstring muscles were considered separately and combined as overall hamstring co-activation (using a root mean square approach) as our outcomes. Our two sensitization exposures were assessed in the same limb as hamstring co-activation: (1) Temporal summation (augmented pain response to repetitive mechanical stimuli; a marker of central sensitization) was defined as being present when, after touching the skin over the patella with a monofilament repeatedly at 1 Hz for 30 s, the subject reported new or increased pain at the patella. (2) Pressure pain threshold (PPT) is a marker of peripheral +/− central sensitization at sites of disease/inflammation, or of central sensitization when assessed at an otherwise normal area. PPT was assessed with an algometer (1 cm² tip) at the patella as the point at which the participants indicated that the pressure first changed to slight pain. The average of 3 trials was used to calculate the PPT. Because muscle co-activation was not normally distributed, we categorized it into age- and sex-specific tertiles.

We measured temporal summation and PPT pain sensitivity in 563 participants who had data from the surface EMG and sensitization assessments (mean age 67.3 ± 6.7, mean BMI 30.3 ± 5.6, 58% female, 39% with tibiofemoral OA). Temporal summation was present in 7% of the median (IQR) for PPT was 20.9 (20.8–21.0) for overall hamstring co-activation was 10.3% (5.7–15.1). Temporal summation was associated with >30% greater odds of having the highest tertile of medial and overall hamstring co-activation compared with the lowest tertile, but not with lateral hamstring co-activation (Table). Low PPT (lowest tertile)