S1: Chondrocyte isolation and culture, RNA extraction and sequencing, RT-PCR and data analysis

Cartilage samples were obtained from ten OA patients undergoing knee replacement surgery at Coxa Hospital for Joint Replacement, Tampere, Finland. The patients fulfilled the American College of Rheumatology classification criteria for OA [1], and patients with diabetes mellitus were excluded from the study to prevent potential diabetes-related metabolic changes from confounding gene expression results [2]. Patient characteristics were as follows: (mean age 67 years [SEM 3.8 years], eight females, Kellgren-Lawrence grade 3.7 [SEM 0.15]).The study was approved by the Ethics Committee of Tampere University Hospital, Tampere, Finland, and performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Cell culture

Full-thickness pieces of articular cartilage from femoral condyles, tibial plateaus and patellar surfaces were removed aseptically from subchondral bone with a scalpel, cut into small pieces and washed with phosphate buffered saline (PBS). Chondrocytes were then isolated by enzyme digestion for 16 hours at 37 °C in a shaker by using a collagenase enzyme blend (0.25 mg/ml Liberase^{TM} Research Grade medium; Roche, Mannheim, Germany). Isolated chondrocytes were plated on 24-well plates (2.0 x 10^5 cells/ml) in the culture medium DMEM (Dulbecco's Modified Eagle Medium; Sigma-Aldrich, St. Louis, MO, USA) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (250 ng/ml), (all three from Gibco/Life Technologies, Carlsbad, CA, USA), containing 10 % fetal bovine serum (Lonza, Verviers, Belgium). After 24 hours, the following stimuli were added: 1) none, 2) ibuprofen 10 µM (Sigma-Aldrich), 3) IL-1β 100 pg/ml (R&D Systems Europe Ltd, Abingdon, UK) or 4) bothIL-1β 100 pg/ml and ibuprofen 10 µM. The cells were then cultured for 24 hours.

RNA extraction and sequencing

Culture medium was removed after 24 hours, and total RNA of the chondrocytes was extracted with GenElute™ Mammalian Total RNA Miniprep kit (Sigma). Total RNA was treated with DNAse I (Qiagen, Hilden, Germany). RNA concentration and integrity were confirmed with the 2100 Bioanalyzer (Agilent Technologies).

Total mRNA of the samples was sequenced in the Biomedicum Functional Genomics Unit, University of Helsinki, Finland using the Illumina NextSeq 500 system. Sequencing depth was 15 million paired-end reads 100 bp in length. Read quality was first assessed using FastQC [3], and the reads were trimmed using Trimmomatic [4]. Trimmed reads were aligned to reference human genome with STAR [5]. Count matrices were prepared with the featureCounts program [6]. Differential expression was assessed with edgeR using patient number as an additional experimental factor for pairwise comparisons [7]. Gene expression levels are given as trimmed means of M-values (TMM normalization). For the purposes of further analysis, genes with a minimum of 1.5 fold change (FC) in abundance and FDR-corrected p-value < 0.05 were deemed biologically and statistically significant. Pseudogenes and known non-protein coding transcripts were excluded. Functional analysis was performed using Ingenuity Pathway Analysis (IPA) (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis) and protein interactions were studied with STRING [8]. Gene functions were obtained from the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene).

Quantitative reverse transcription/polymerase chain reaction

Chondrocytes from a different set of ten OA patients (mean age 66 years [SEM 3.6 years], six females, Kellgren-Lawrence grade 3.6 [SEM 0.16]) were isolated and cultured as described above. RNA (150 ng / sample) was reverse-transcribed to cDNA for RT-PCR using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). cDNA obtained from the reverse transcriptase reaction was diluted 1:20 with RNAse-free water and subjected to quantitative RT-PCR using TaqMan Universal PCR Master Mix and the ABI Prism 7500 Sequence detection system (Applied Biosystems).

Primers and probes for human interleukin 6 (IL-6) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Metabion International AG (Martinsried, Germany). The primer and probe sequences (IL6: forward: TACCCCCAGGAGAAGATTCCA, reverse: CCGTCGAGGATGTACCGAATT, probe: CGCCCCACACAGACAGCCACTC, GAPDH: forward: AAGGTCGGAGTCAACGGATTT, reverse: GCAACAATATCCACTTTACCAGAGTTAA, probe: CGCCTGGTCACCAGGGCTGC) and concentrations were optimized according to the manufacturer's instructions in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C. mRNA levels of other studied genes were determined with TagMan Gene Expression Assays (Applied Biosystems): peroxisome proliferator activated receptor gamma (PPARγ, assay number Hs01115513_m1), peroxisome proliferator-activated receptor gamma coactivator 1-beta (PPARGC1B, Hs00993805_m1), colony stimulating factor 2 (CSF2, Hs00929873_m1), interleukin 23 subunit alpha (IL23A, Hs00372324_m1), hyaluronan synthase 1 (HAS1, Hs00758053_m1), insulin-like growth factor-binding protein 4 (IGFBP4, Hs01057900_m1) and ADAM metallopeptidase with thrombospondin type 1 motif 6 (ADAMTS6, Hs01058097_m1).

PCR reaction parameters were as follows: incubation at 50 °C for 2 minutes, incubation at 95 °C for 10 minutes, and thereafter 40 cycles of denaturation at 95 °C for 15 s and annealing

and extension at 60 °C for 1 minute. Each experimental reaction was performed in duplicate. For IL6, the relative mRNA levels were quantified using the standard curve method as described in Applied Biosystems User Bulletin number 2. To calculate the relative expression of mRNAs determined with TaqMan assays, the $2^{(-\Delta\Delta CT)}$ method was used. According to the method, the cycle threshold (C_T) value for genes of each gene was normalized to the C_T value of GAPDH mRNA in the same sample.

ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of prostaglandin E2 (PGE2), prostaglandin F2 alpha (PGF2α), prostaglandin D2 -methoxime (PGD2-MOX, a stable form of PGD2) and 6-keto-prostaglandin F1 alpha (6-keto-PGF1α,a surrogate of prostacyclin synthesis) in the cell culture media of chondrocyte samples from nine separate donors. All ELISA kits were purchased from Cayman Chemical and the measurement protocols carried out according to the manufacturer's instructions.

Statistics

For the RNA-Seq data analysis, normalisation was performed and differential expression was studied using a generalised linear model implemented in edgeR [7] using patient number and treatment as experimental factors. For the PCR and ELISA measurements, ANOVA with Bonferroni's post-test was performed using GraphPad InStat version 3.06 for Windows.

References:

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