

## Supplementary Files

### Materials and methods

#### Materials

Medial and lateral menisci originating from one young pig donor were collected from a local slaughterhouse. Tissues were rinsed in 5% (volume fraction) Betadine (Egis) in phosphate-buffered saline (PBS) solution, followed by two rinses in PBS. They were then transported in MACS<sup>®</sup> Tissue Storage Solution (Miltenyi Biotec, #130-100-008) on ice for up to 1 h and processed immediately after arrival.

#### Single-cell suspension preparation

The narrow pieces along the long axis on both sides (peripheral and inner) of each meniscus, in its middle part were dissected. Since the peripheral 10%-30% of the medial meniscus and the peripheral 10%–25% of the lateral meniscus are vascularized, we measured menisci dimensions and calculated the red areas to be dissected under direct vision, followed by discarding the very outer layer (about 1 mm), to avoid contamination with fatty and connective tissue. The samples representing the white zone were cut out from the inner side of the meniscus in the form of thin pieces, reaching no deeper than 20% of the meniscus width.

Samples were cut into small pieces, followed by enzymatic digestion and disaggregation steps. First, incubation was carried out with 4 mg/mL Pronase enzyme blend (Sigma-Aldrich, #10165921001) at 37 °C for 1 h, followed by a second incubation in 3 mg/mL Collagenase P (Sigma-Aldrich, #11213873001) at 37 °C for another 2 h. The mixture was then filtered through a 70 µm pluriStrainer<sup>®</sup> (pluriSelect, #43-57070-51) and centrifuged. The cell pellet was resuspended in 0.1% (volume fraction) bovine serum albumin (BSA, Sigma-Aldrich, #A9418-50G) in PBS and centrifuged to remove dead cells. Finally, the cells were filtered through a 30 µm pluriStrainer<sup>®</sup> (pluriSelect, #43-50030-03) and resuspended in 1% BSA in PBS to prevent cell re-aggregation.

#### Library generation

Subsequently, the cells isolated from meniscal zones were subjected to library construction using the Chromium Next GEM Single Cell 3' Reagent Kit v3.1 (10× Genomics, #PN-1000269). Samples were processed according to the manufacturer's protocol. Cell suspensions were first divided into the single-cell format with a Chromium X Series controller (10× Genomics), which encapsulates single cells within nanoliter-scale Gel Beads-in-emulsion (GEMs). During incubation, the content of GEMs produced barcoded full-length cDNA from polyadenylated messenger RNA (mRNA). After incubation, the GEMs were ruptured and the pooled fractions recovered. Silane magnetic beads were used to purify the first-strand complementary DNA (cDNA) from the reaction mixture. Barcoded, full-length cDNA was then amplified via polymerase chain reaction (PCR) to generate sufficient cDNA for library construction. Enzymatic fragmentation and size selection were used to optimize the size of the cDNA amplicons. Indexes and TruSeq Read 2 were added to the samples via End Repair, A-tailing, Adaptor Ligation, and PCR. The final libraries contained the paired-end (P5) and P7 primers used in Illumina bridge amplification. The prepared libraries were sequenced on the NextSeq500 (Illumina) device with the Mid Output Kit v2.5 (300 cycles).

#### scRNA-seq data pre-processing and quality control

Following sequencing, the output fastq files were used as input for the 10× Genomics Cell Ranger Software (cellranger-6.1.2) and mapped to the custom-built pig reference genome (RefSeq Sscrofa11.1) using the cellranger mkref pipeline provided by 10× Genomics. Downstream analysis was performed using R (v4.3.2) and the Seurat package (v5.0.1). Cell doublets (nFeature\_RNA>2000), cells containing more than 8% of mitochondrial genes (“ATP6”, “ATP8”, “COX1”, “COX2”, “CYTB”, “ND2”, “ND3”, “ND4”, “ND4L”, “ND5”, “ND6”), and cells with fewer than 200 genes (nFeature\_RNA) per cell were excluded from downstream analysis.

## Identification and analysis of cell clusters

Clustering of cells was performed using the Seurat package (v5.0.1). The data were log-normalized and integrated using the `IntegrateData` function. The results are shown as uniform manifold approximation and projection (UMAP) plots. The annotation of the main cell populations, such as chondrocytes, endothelial cells, proliferating endothelial cells, smooth muscle cells, monocytes, T-cells, and macrophages, was based on a research of the top 30 marker genes with the highest expression within each cluster, calculated using the `FindAllMarkers` function. Genes with differential expression were identified using the `FindMarkers` function, which is based on the non-parametric Wilcoxon rank sum test. *P*-values were adjusted using the Bonferroni correction with all features in the dataset.

## Pathway analysis

Pathway analysis was carried out with GO term enrichment using the GOnet software (<https://tools.dice-database.org/GOnet/>). Differentially expressed genes with an adjusted *P*-value of <0.05 were used as input.

## Monocle

To distinguish and visualize different cellular fates, we performed pseudotrajectory analysis using the Monocle 3 package (v1.3.5) in R (Cao et al., 2019; Qiu et al., 2017; Trapnell et al., 2014). The cellular trajectory ordered in pseudo-time was presented with multiple branches, and the CH3 subset of chondrocytes was annotated as a root-noodle. Several genes were visualized in the pseudo-trajectory pathway analysis.

## CellChat

To identify and visualize the cellular cross-talk within pig menisci, we applied the R package CellChat v1.0.6 with default parameters (Jin et al., 2021). Due to the unavailability of a dedicated pig interaction database, we used CellChatDB.human by selecting pig genes based on their homology with human genes (Jin et al., 2021). The interactions were identified and quantified based on the differentially expressed ligands, receptors and cofactors for each cell group ( $P < 0.05$ ). The “netVisual\_diffInteraction” function was applied to visualise differences in the strength of intercellular communication. For visualising cell-cell communication mediated by ligand-receptor pairs or signalling patterns, we used the ‘netVisual\_chord\_gene’ and ‘netAnalysis\_signalingRole\_heatmap’ functions, respectively. To identify differences in communication probabilities mediated by ligand-receptor pairs from certain cell groups to others, the “netVisual\_bubble” function was applied.

## Statistical analysis

For all statistical analyses, GraphPad Prism 8.0.1 was used. The statistical significance of cell cluster proportion between menisci and zones was calculated using a two-way ANOVA test.

## References

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