Full Title: Combining segmental bulk- and single-cell RNA-sequencing to define the chondrocyte gene expression signature in the murine knee joint

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Running Title: Transcriptome of murine chondrocytes

Abstract

Objective: Due to the small size of the murine knee joint, extracting the chondrocyte transcriptome from articular cartilage (AC) is a major technical challenge. In this study, we demonstrate a new and pragmatic approach of combining bulk RNA-sequencing (RNA-seq) and single cell (sc)RNA-seq to address this problem.

Design: We propose a new cutting strategy of the murine femur which produces three segments with a predictable mixed cell populations, where one segment contains AC and growth plate (GP) chondrocytes, another contains GP chondrocytes, and the last segment contains only bone and bone marrow. We analysed the bulk RNA-seq of the different segments to find common and distinct genes between the segments. Then, the segment containing AC chondrocytes was digested and analysed via scRNA-seq.

Results: Differential expression analysis using bulk RNA-seq identified 350 candidate chondrocyte gene in the AC segment. Gene set enrichment analysis of these genes revealed biological processes related- and non-related to chondrocytes, including, cartilage development (adj. p-value: 3.45E-17) and endochondral bone growth (adj. p-value 1.22E-4), respectively. ScRNA-seq of the AC segment found a cluster of 131 cells containing mainly chondrocytes. This cluster had 759 differentially expressed genes which enriched for extracellular matrix organisation (adj. p-value 7.76E-40) and other joint development processes. The intersection of the gene sets of bulk- and scRNA-seq contained 75 genes, where all but ten genes were previously implicated in cartilage homeostasis or osteoarthritis (OA) progression.

Conclusions: Our approach has the potential to detect the scarce disease phenotypes of chondrocytes in murine OA models.

Key words: articular cartilage; mouse models; chondrocytes; RNA-seq; scRNA-seq; cellular phenotyping

Introduction

Knee osteoarthritis (KOA) is one of the top five disabling conditions that affects more than one-third of people aged over 65 years and over 100 million individuals globally [1]. KOA is characterized by an erosion of the articular cartilage, which leads to inflammation and pain accompanied by joint stiffness and immobility. Despite its prevalence, the molecular mechanisms triggering KOA are poorly characterized and current therapies cannot halt or revert KOA. While animal models of KOA exist, the causative mechanisms leading to cartilage erosion are poorly understood. It is therefore of utmost importance to identify the key molecular factors and the associated signalling pathways responsible for changes in chondrocyte function and the pathological transformation of healthy cartilage during the establishment of KOA [2-4]. Such molecular factors can serve as predictive biomarkers, and also represent targets for the therapy of KOA.

Preclinical models of KOA in mice are widely used to study the underlying molecular mechanisms of the disease [5, 6]. However, the dissection of articular cartilage (AC) from mice knee joints is challenging due to the small size, low thickness, and the huge potential for contamination with e.g. subchondral bone or surrounding connective tissue [7]. These technical challenges create obstacles for studying the gene expression signatures of AC chondrocytes. In addition, chondrocytes are isolated within a voluminous extracellular matrix (ECM) that is neither vascularised nor innervated and constitute a very small fraction of the whole knee joint [8]. Thus, attempts to identify the chondrocyte specific gene expression signatures hidden within the mixed transcriptome of various cell types using RNA-sequencing on population level (bulk RNA-seq) is almost impossible. A solution for this problem would be single cell (sc) RNA-seq of AC chondrocytes. However, the method requires the preparation of a single cell suspension from the tissue of interest, during which the potential influence of enzymatic, mechanical or chemical pre-treatment's effects on the chondrocytes gene expression signature *in vivo* cannot be fully excluded [9, 10]. As chondrocytes are embedded in an ECM, an enzymatic digestion step is required. Hence, scRNA-seq analysis of musculoskeletal tissue such as bone and cartilage is a major challenge that has not been sufficiently resolved.

Here we present a new and pragmatic approach for extracting the gene expression signatures of chondrocytes from the articular cartilage (AC) of the murine knee joint using a combination of bulk RNA- and scRNA-seq. First, we propose a new cutting strategy for the joint which produces several control segments to help discriminate the chondrocyte-gene expression signature from the remaining joint cells' transcriptome using bulk RNA-seq. Second, we describe for the first time a fast protocol for the digestion of AC and the isolation of chondrocytes followed by scRNA-seq. This protocol allows for an unaltered gene expression signature of individual chondrocytes *ex vivo* by using the transcriptional inhibitor actinomycin-D (Act-D) during digestion

of the ECM. The combination of scRNA-seq and segmental bulk RNAseq enables a complete determination of the gene expression signature of murine AC chondrocytes, which will be key to understand underlying mechanisms leading to the establishment of KOA.

Methods

Some sections of the methods, including histology, bulk RNA library preparation and sequencing, bulk RNA-seq alignment and normalization, scRNA-seq alignment, normalization, clustering and differential expression analysis as well as gene set enrichment analysis are further described in the Supplementary methods.

Animals and tissue collection

Male C57BL/6J mice aged 10 weeks ($n = 6$) were purchased from Charles River Laboratories and were euthanized at an age of 12 weeks by cervical dislocation. The knee joints were collected, and the femur was carefully dissected. The femur was cut in 3 defined segments – segment A included the articular surface, subchondral bone and a small growth plate area of the femur (lateral and medial condyles). Segment B contained growth plate, bone marrow, non-loaded articular cartilage (*Facies patellaris femoris*) and bone while segment comprised bone marrow and bone. The cutting procedure was verified via histology and weight analysis.

Bulk RNA isolation

For bulk RNA isolation, segments were transferred to RNeasy Lysis Buffer (Qiagen) for 1 h at 4°C before removing the RNeasy Lysis Buffer and freezing samples at -80°C. Segment samples were cryopulverized (59012N, Biospec) and gently resuspended in TriFast™ (VWR), mixed with 1-bromo-3-chloropropane (Sigma Aldrich) and incubated for 10 min. Centrifugation was performed for 10 min at $10.000 \times g$ and the top aqueous phase was further collected for RNA isolation using the RNeasy Mini Kit (Qiagen) according to the manufactures' instructions. Purity of the RNA was analyzed via Nanodrop; RNA integrity and quality were verified via Agilent 2100 Bioanalyzer or Fragment Analyzer (RNA-sequencing).

Tissue digestion and cell preparation for single-cell RNA-sequencing

Femur preparation was done on ice with PBS supplemented with 2 µg/mL Actinomycin D (Sigma Aldrich). Segments were digested in 10 mg/ml Collagenase II (Nordmark) supplemented with 2 µg/mL Actinomycin D for 4 h at 37°C and constant rotation. The cell suspension was transferred to a cell strainer and washed with PBS/BSA. FACS analysis (MacQuant, Miltenyi

Biotec) was performed to determine cell concentration and vitality (DAPI staining; > 80 % cell vitality as cut off).

Single cell RNA-sequencing

For single cell library preparation, single cells were applied to the 10X Genomics platform using the Chromium Single Cell 5' Library & Gel Bead Kit (10x Genomics) and following the manufacturer's instructions for capturing ~3,000 cells. The amplified cDNA was used for 5' gene expression (GEX) library preparation by fragmentation, adapter ligation and index PCR. The quality of final single cell 5' GEX libraries was assessed by Qubit quantification and fragment analysis (DNF-474 High Sensitivity NGS Fragment Analyzer Kit, Agilent). The sequencing was performed on a NextSeq500 device (Illumina) using High Output v2 Kits (150 cycles) with the recommended sequencing conditions for 5' GEX libraries (read1: 26nt, read2: 98nt, index1: 8nt, index2: n.a.).

Results

Cutting of the murine femur provides sufficient RNA per segment and predictable mixed cell populations in each segment.

Chondrocytes can be found in the articular cartilage (AC) and the growth plate (GP) of the knee joint (Fig. 1a). We isolated the femur from mice and dissected it into three segments, labelled A, B and C. Segment A included the medial and lateral femoral condyle up to the GP; while segment B comprised the GP and metaphyseal area; and lastly, segment C contained half of the diaphysis (Fig. 1b). Given the small size of the murine joint, it was not possible to cut the femur condyles without including small parts of the GP. For this reason, the diaphyseal segment C served as a control for the signal interference from the GP in segment A and B. The cutting protocol was verified using H&E and Safranin-O staining, which clearly outlined the AC and GP in segment A, and the GP in B. There was no cartilage present in segment C (Fig. 1c). The cutting protocol also produced comparable samples for each segment, giving an average signal to noise ratio in their wet weight of approximately 4.6 (N=6), meaning there was no more than 21% variation in the cutting within each segment (Fig. 1d). Upon isolation of the RNA from each segment, we found that all samples yielded more than 1,000 ng of total RNA (Fig. 1e), with segment A yielding the lowest amount of RNA among the segments in the range of 2,772 ng to 6,135 ng. The RNA integrity number (RIN) values of the segment samples were found to be of sufficient quality for sequencing, with all segments showing a RIN value above 8 (Fig. 1f). In summary, each mouse joint was cut into three consistent segments with a predictable mixed cell population, and each segment contained adequate RNA quantity and quality for RNA-seq analysis.

Figure 1: The proposed segments of the mouse joint contain sufficient RNA per segment and predictable mixed cell populations. (a) H&E staining of sagittal cross-section of the murine knee joint. (b) Schematic indicating the approximate cutting region of the three segments of the femur. (c) Staining of sagittal cross-sections of representative segments: A, B, and C. H&E staining (top), Safranin-O staining (bottom): cartilage is red coloured. Scale bars indicate 200 µm. (d) Weight of samples of the segments represented as a bar graph (mean ± STD) and individual data points (n= 8). (e) Total RNA amount per segment represented as a bar graph (mean ± STD) and individual data points (n= 6). (f) RNA integrity number per segment represented as a bar graph (mean ± STD) and individual data points (n= 6).

Bulk RNA-seq analysis of the segments reveal AC chondrocyte candidate genes but cannot guarantee their chondrocyte specificity.

Since we aimed at delineating the transcriptome of AC chondrocytes and to reduce the signal interference from GP chondrocytes, we performed differential gene expression analysis between segment A and C, segment B and C, and segment A and B (Fig. 2a–c). To identify the differentially expressed genes, a Mann-Whitney U-test with a 3-fold change cut-off and a p-value of 0.05 was used. We found 350 genes which were upregulated in segment A with respect to segment C. Gene set enrichment analysis (GSEA) found these genes enriched in biological processes involved in cartilage development (GO:0051216, adj. p-value: 3.54E-17, genes: 28) and chondrocyte development (GO:0002063, p-value: 1.56E-4, genes: 8) (Fig. 2e). A comparable amount—274 genes—was upregulated in segment B when compared to segment C. These genes are involved in similar biological processes as those found in the comparison between segment A and C, such as cartilage development. However, there were also genes enriched in growth plate cartilage development (GO:0003417, adj. p-value: 8.81E-4, genes: 6) and endochondral bone growth (GO:0003416, adj. p-value: 1.22E-4, genes: 7) (Fig. 2f). As a next step, we studied the genes differentially expressed between segment A (containing AC and GP chondrocytes) and segment B (containing only GP chondrocytes) and found 71 genes to be upregulated in segment A. These we assumed to be potentially AC chondrocyte-specific. However, GSEA of these genes did not show enrichment in any biological process (Fig. 2d).

Other than chondrocyte-related processes, we also found that genes upregulated in segment A with respect to segment C were involved in the biological processes of peripheral nervous system neuron development (GO:0048935, adj. p-value: 1.11E-2, genes: 4) and ossification (GO:0001503, adj. p-value: 3.91E-8, genes: 25) (Fig. 2e). Similarly, among genes upregulated in segment B compared to segment C, we found genes enriched in growth plate cartilage development (GO:0003417, adj. p-value: 8.81E-4, genes: 6) and multinuclear osteoclast differentiation (GO:0072674, adj. p-value: 2.54E-3, genes: 4). This suggests that the segments contain genes from cells other than chondrocytes, for example, pre-osteoblasts, nerve cells and osteoclasts.

In summary, by juxtaposing the transcriptomes of the segments A, B, (containing chondrocytes) and C (containing no chondrocytes), we found a high variety of genes that may be AC chondrocyte-specific. However, we were not able to discern the transcriptomes of the different cell populations contained in the different segments.

Figure 2: Bulk RNA-seq analysis of the segments reveal potential AC-specific genes. (a–c) Volcano plots (top) highlighting the top ten genes differentially expressed between the two segments of interest. Venn diagram (bottom) stating the total number of genes which showed a larger than 3-fold change difference in expression between the two segments of interest. (n= 3) **(a)** Comparison between segment A and segment B. **(b)** Comparison between segment A and segment C. **(c)** Comparison between segment B and segment C. **(d–e)** Biological processes, molecular functions, and cellular components found in the gene set enrichment analysis (GSEA) of the gene set of interest. The associated GO terms are given in a bar graph with the length of the bar indicating the number of genes found in that term. **(d)** GSEA of genes more than 3-fold upregulated in segment A compared to B. **(e)** GSEA of genes more than 3-fold upregulated in segment A compared to C. **(f)** GSEA of genes more than 3-fold upregulated in segment B compared to C.

scRNA-seq analysis of the condylar segment A reveals that chondrocyte-like cells are scarce but cluster as a distinct population.

Since we assumed both chondrocyte phenotypes—from AC and GP—to be present in segment A, three samples were separately digested and analysed via scRNA-seq. Because we could not see any sequencing batch effects (Fig. 3a), the data from the three different experiments were combined for analysis. The aggregation of the three batches led to a total of 7,133 cells to be analysed. The Seurat-3 analysis pipeline deduced six clusters of different cell subpopulations (Fig. 3b). Using one versus all differential gene expression analysis with a cut-off of 3-fold change and GSEA, we discerned the cell subpopulations per cluster (see Supplementary Results A). Cluster 1 to 5 were enriched for hematopoietic stem cells. Therefore, cluster 1, 3, 4, and 5 comprised myeloid cells while cluster 2 included the lymphoid cells (Fig. 3). In more detail, cluster 1 was enriched for granulocytes (N=2,073) while cluster 4 comprised mainly neutrophils (N=789) which was also supported by the fact that both clusters showed overlapping gene expression (Supplementary Results A). Cluster 5 contained monocytes and macrophages (N=317) and showed expression of genes unique for antigen presenting cells which was comparable with cluster 2 (B-cells and precursors; N=1,336) (Fig. 3c; Supplementary Results A). Cells from the erythroid lineage and precursors were found in cluster 3 (N=1,055) which was supported by the identification of the corresponding biological processes (Fig. 3c). In cluster 6 mainly chondrocytes were found, even though the presence of osteoblast-specific genes indicated the presence of other musculoskeletal cells and GP chondrocytes (N=131).

In order to identify the chondrocytes in cluster 6, we searched for cells expressing cartilage-specific genes such as *Sox9* (89 cells; 68%), *Col2a1* (94 cells; 72%), and both genes together (78 cells; 60%) (Fig 3d, 3e). We concluded that the 78 cells expressing both *Sox9* and *Col2a1* were chondrocytes from AC or GP. Next, we asked the question whether the transcriptome of these cells could be affected by the digestion protocol in order to exclude false positive results. Since the digestion bias has not yet been addressed in detail in the sequencing field, we specifically looked for well-known effects of digestion. As a results, we did not observe an upregulation of *Mmp1*, *Mmp13*, *Tnfrsf1*, *ColX*, and a downregulation of *Col2A1*, *Col1A2*, *Acan* (see Supplementary Results B). Hence, the addition of ActD during tissue digestion seemed to preserve the phenotypic identity of the chondrocytes and appeared to inhibit potential digestion-induced alterations in the transcriptome.

GSEA of the 759 genes differentially expressed in cluster 6 (containing 60% chondrocytes) found some of these genes to be enriched in processes involving cartilage development (GO:0051216, adj. p-value: 1.65E-27, genes: 57), bone development (GO:0060348, adj. p-value: 7.3E-17, genes: 42), nervous system development (GEO:0007399, adj. p-value: 3.21E-10, genes: 143), and angiogenesis (GEO:0001525, adj. p-value: 6.83E-12,

genes: 57). Thus, we could deduce that the cells in cluster 6 are involved in production and upkeep of the ECM and bone in the joint (Fig. 4), and that they are a mixed population of chondrocytes, osteoblasts, and other cells.

Using scRNA-seq, we were able to extract a large set of genes belonging to the chondrocyte transcriptome. However, due to the small population size of the AC chondrocytes, they could not be discriminated from bone and other synovium-associated cells.

Figure 3: scRNA-seq analysis of the condylar segment A show chondrocyte-like cells cluster. (a) tSNE plot of the scRNA-seq analysis from segment A, with the cell colour highlighting the batch to which the cell belongs. **(b)** tSNE plot highlighting the cluster to which cells have been allocated and a list of the potential cell phenotypes in the clusters, the number of cells belonging to each cluster, and the number of differentially expressing genes (3-fold change cut-off) in comparison to all other clusters. **(c)** Columns of the heatmap contain selected differentially expressed genes in each of the clusters, rows contain their expression of in each cluster. Dendrogram showing the hierarchy of the clusters over the selected genes. **(d)** Box plot showing the expression of *Mmp13*, *Tnfrsf1*, *Col2a1*, and *Sox9* of cells within and outside cluster 6. **(e)** tSNE plots highlighting the magnitude of expression of the genes of interest in the cells from the scRNA-seq assay of segment A.

Figure 4: Gene set enrichment analysis of the 788 genes found to be differentially expressed in cluster 6 compared to all other clusters. Results originate from enrichment searches in the KEGG pathway, Reactome pathway, and Gene Ontology (Biological Processes, Molecular Function, and Cellular component) databases. The associated terms are given in a bar graph with the length of the bar indicating the number of genes found associated with that term. The name of the term and the adjusted p-value are stated adjacent to the bar on the left side.

Both approaches yield complementary data sets.

To date, there is no complete transcriptomic atlas of the cells in the murine joint. The well accepted assumption is that the transcriptome of the AC chondrocytes and GP chondrocytes have a larger overlap. If we use binary classification nomenclature, and say the genes resulting from the analysis of the respective methods found AC chondrocyte “positive” genes, and the remaining genes to be AC chondrocyte “negative” genes, then we can see that both approaches identify different sets of genes to be AC chondrocyte positive (see Fig. 5a for illustration). From our GSEA, we saw that the AC chondrocyte positive gene-set from bulk RNA-seq’s also contained genes well-known to be expressed in nerve cells. These genes, in binary classification nomenclature, are “false positive” AC chondrocyte genes. In contrast, in scRNA-seq, we observed the “false positive” genes to be genes associated with GP chondrocytes. Interestingly, the two approaches identified different, mutually exclusive, “false positive” genes. Thus, we hypothesised that using the intersection of the genes detected by both approaches would eliminate the “false positive” genes and enable us to delineate the AC chondrocyte transcriptome.

Using the intersection of bulk RNA-seq and scRNA-seq yields the AC chondrocyte-specific transcriptome.

To build a robust gene set of AC chondrocytes from the femoral cartilage, we combined all genes upregulated in segment A with respect to segment C and the genes upregulated in segment A with respect to segment B from the bulk RNA-seq. This gene set presumably contained chondrocyte-related genes. Then, we further reduced this gene set to chondrocyte-specific genes by taking the intersection of the genes in this set with genes upregulated in cluster

6 from the scRNA-seq data of segment A. This intersection gene set contained 75 genes (Fig. 5b, see Supplementary Results C: Table 1). In depth literature search revealed that a considerable number of these genes were AC cartilage-specific and had already been studied in the context of cartilage homeostasis or OA pathogenesis. The majority of genes encode for matrix proteins such as *Matn1* and *3*, *Lect1*, *Chadl*, *Epyc*, *Chad*, *Col9*, *Col2a1*, *Acan*, *Comp*, *Fmod*, *Ucma*, *Cilp2*, *Hapln*, *Clec3a* and *3b*, *Tnbs3* and *4*, *Fndc1*, *Prg4*, *Dpt*, *Cspg4* or factors and enzymes contributing to matrix formation or degradation: *Serpina 1a*, *1b*, *1d* and *1e*, *Prkg2*, *Car12*, *Tnxb*, *Htra4*, *Pcolce2*, *Gfpt2*, *Colgalt2*, *Has1* and *Ppp1r1b*. Out of the 75 genes, there were only 10 genes which could not be related to cartilage or a specific function, these were *Slurp1*, *Calml2*, *Ssc5d*, *Crispld1*, *Bpifb1*, *Mfap5*, *Medag*, *Gm11547*, *1500015O10Rik* and *3110079O15Rik*. Additionally, genes that are related to cellular functions were detected. For example, *Sod3* is abundant in the cartilage ECM maintaining the homeostasis by controlling reactive oxygen species (ROS). *Nov/Ccn3* encodes a matricellular protein with relevance in chondrocyte metabolism that represses endochondral ossification. *Mfi2*, *Syt8*, *Panx3*, *Anxa8*, *Fxyd 2* and *3*, *Itgbl1* and *Ntn1* are related to the active transport, cell membrane fusion, integrin interaction, or encode for transmembrane proteins. Some of these genes have been described to act anabolic (*Mfi2*, *Syt8*, *Fxyd2* and *3*, *Itgbl1*) while the rest is pro-catabolically supporting the progression of OA (*Panx3*, *Anxa8*, *Ntn1*). Moreover, inflammation-associated genes such as *Il17b*, *Cyt11*, *C1qtnf3*, *Rbp4* seemed to be expressed even under homeostatic conditions maintaining the balance between anabolic and catabolic factors. Interestingly, several genes were identified that are related to specific signalling pathways, for example, Wnt signalling (*Notum*, *Tnfrsf11b*, *Frzb*, *Rspo4*), indian hedgehog signalling (*Hhip*), Sox9 signalling (*Mia*), Smad signalling (*Meg3*), support cartilage differentiation, and maturation as growth factors (inhibitors) such as, *Sox9*, *Ctgf*, *Tgfb2*, and *Igfbp6*. *Sema 3c* and *3e* and *Ncmmap* were ranked as genes with association to the nervous system relevant for axonal guidance and myelin formation, respectively.

Discussion

The only cell type that can be found in fully developed mature articular cartilage is the chondrocyte, with a low species-specific volume density of 2 % (human) and 15–40 % (mouse) [11, 12]. The overall cartilage thickness on the femoral ends in the knee joint is reported to be approximately 2.35 mm in humans and 0.1 mm in mice [11, 13, 14]. It was found by Gardiner et al., that cartilage only contributes less than 0.2 % of RNA to the total RNA in the murine knee joint [7]. Due to this low resolution, they proposed to compare pathways rather than genes when performing meta-analysis of RNA studies on joint tissues. Though this is a step in the right direction, the approach still cannot exclude that many genes and pathways that have been described so far are attributed to potential other cell and tissue contamination within the samples. To overcome these unresolved technical issues, we proposed the novel and pragmatic approach of using multiple tissue segments—each segment with at least one mutually exclusive phenotype in its population—to be used as controls to delineate phenotypes of mixed cells in the joint. In particular, to delineate the AC chondrocyte transcriptome in the knee joint, we cut three segments, one segment containing AC and GP chondrocytes, one segment with only GP chondrocytes, and the last segment containing no chondrocytes. Using these three segments, we were able to delineate genes upregulated in AC chondrocytes and GP chondrocytes. The cutting strategy gave a robust gene-set list for chondrocytes; however, the approach could only capture the upregulated genes and not the whole chondrocyte transcriptome.

To date, insights into the single cell transcriptome of murine chondrocytes can only be gathered from limb developmental studies, where different stages in chondrocyte phenotype progression/development are described, for example, the formation of the growth plate [15, 16] or different bone marrow niches [17]. Hence, the literature on the chondrocyte single cell transcriptome in the murine joint is vacuous. In our study, scRNA-seq analysis of the condylar segment A revealed that chondrocyte-like cell cluster distinctly in the population but suffer from scarce cell presence. Nevertheless, we found that these chondrocyte-like cells had hundreds of genes which are significantly upregulated with respect to other cells in the joint, hence, giving them a distinct phenotypic signature. Due to lack of surface markers for cell sorting, we could only extract approx. 78 chondrocytes (1.1 % of all cells analysed) out of three femoral condyles. Even though the proportion of chondrocytes extracted seems low, it is in fact consistent with the proportion of chondrocytes which are found in the joint [7]. Taken together, the chondrocyte transcriptome is distinct enough to be detected in single cell assays, however, due to the small sampling frequency, heterogeneity in chondrocytes could not be detected with confidence.

Due to the nature of the problem of isolating chondrocytes in murine joints, we found that both bulk RNA-seq and scRNA-seq do suffer from poor specificity. Hence, we suggest performing both methods and using the intersecting genes as the true chondrocyte candidate genes. In this

work, the intersection of bulk RNA-seq and scRNA-seq yielded 75 genes which were AC chondrocyte-specific. Interestingly, beside the classical chondrocyte markers which are mainly related to ECM formation, degradation and integrity, we found new genes which were previously not associated with chondrocytes. For example, the gene *Sema3*, which has been described in context of axon promotion and repulsion [18] and was also found to be transactivated by *Sox9* [19]. Similarly, *Ncmip* plays a role in myelin formation and has also been listed in other transcriptome studies [7, 20]. Presumably, these genes are mainly expressed in deep layer chondrocytes of the osteochondral unit and mediate peripheral nerve growth. However, their implication on cartilage homeostasis, OA progression or even pain sensitization is unknown. In addition, *Clec3a* and *b* encode a cartilage-specific lectin which has been shown to be upregulated in GP chondrocytes but has not been described in detail in articular cartilage [21]. Vice versa, *Htra4*, a oligomeric chaperone protease which degrades misfolded secretory proteins has only been described in the context of arthritis and its involvement in cartilage degradation [22, 23]. Further genes such as *Slurp1*, *Calml3*, *Ssc5d*, *Crispld1*, *Bpifb1*, *Mfap5* and *Medag* were not related to cartilage or surrounding tissue at all, providing directions for further research studies. For examples, *Slurp1* regulates cell proliferation and exerts anti-inflammatory effects [24], while *Ssc5d* has been reported to be expressed in monocytes/macrophages and T-lymphocytes [25] and *Mfap5* can be found in synovial fibroblasts [26].

To isolate chondrocytes from cartilage, the treatment with collagenases is indispensable. Dissociation-induced alterations of the transcriptome are widely discussed in the community, although not very often studied or included in publications [10]. Notably, there is a fine line between the concentration of the collagenase, the digestion time, and the cell output, which need to be adjusted precisely to keep negative influences on the chondrocyte transcriptome as low as possible [9, 27, 28]. It was suggested that an additional treatment with transcription inhibitors might resolve this problem [29, 30]. Hence, in our study, we supplemented Actinomycin D (ActD), a chemotherapeutic medication and transcription inhibitor which has been shown to freeze the transcriptome in immune cells [31]. We did not find collagenase-induced upregulation of genes, suggesting that the ActD treatment paused the digestion-induced transcriptional changes. It must be noted that the chondrocytes in this experiment were healthy. In an OA (disease) setting, the chondrocytes are known to be under stress and undergoing differentiation or apoptosis. Future research is needed to investigate if ActD can preserve chondrocytes which are in a transient phase during disease progression.

Regarding the technical aspects, the natural next step is to achieve a higher yield of chondrocytes from the murine joint for scRNA-seq analysis. Due to the lack of surface markers, this has been proven to be a challenge. However, in our experimental workflow we were able to highlight the different cell populations appearing in scRNA-seq of the murine joint. Hence, we can use our results to tag all cells which are not chondrocytes, resulting in an up-sampling of

chondrocyte and chondrocyte-like cells. For example, we can use CD14 to exclude monocytes/macrophages, Ter119 to exclude erythrocytes/erythroblasts, and CD34 to exclude bone marrow cells.

We believe that our approach of combining the two RNA-seq methods will be effective in studying the disease phenotypes of chondrocytes in murine OA model in the future. Thus, target genes could be identified supporting the development of chondrocyte phenotype-specific treatment strategies.

Acknowledgement

We like to thank Prof. Frank Buttgereit, Dr. Timo Gaber and Dr. Max von Kleist for scientific discussions and support.

Contributions

V.S., M.F.M. and A.L. designed the study. V.S., A.L. and G.A.H. collected and analyzed data. V.S., F.H. and P.D. performed analysis of bulk RNA-seq and scRNAseq data. V.S. and A.L. prepared the main manuscript text. M.F.M., G.A.H. and A.M. revised manuscript. All authors finally approved the manuscript.

Role of the funding source

Preliminary experiments were financially supported by the Einstein Centre for Regenerative Therapies (Kickbox grant 2018). This work was supported by the state of Berlin and the “European Regional Development Fund” (ERDF 2014–2020, EFRE 1.8/11, Deutsches Rheuma-Forschungszentrum to G.A.H., F.H. and M.F.M.).

Conflict of interest

The authors declare no conflict of interests.

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Supplementary Methods

Histology

For histology femur segment samples were fixed with 4% paraformaldehyde at 4°C for 6 h and ran through a sucrose gradient (10 %, 20 %, 30 %) for 12-24h each. Cryo-embedding was performed using SCEM medium (Sectionlab) and cryo-slices of 7 µm were produced using cryo-films (Sectionlab) and stored at -80°C.

Hematoxylin and Eosin (H&E) staining: air drying, fixation with 4% PFA (10 min), washing with Aqua dest., first staining step in Harris's haematoxylin solution (7 min) (Merck, Germany), washing twice with Aqua dest., differentiation step in 0.25% HCl-solution, washing twice with tap water, second staining step in 0.2% Eosin (2 min) (Chroma Waldeck, Germany), differentiation and washing in 96% ethanol and 100% ethanol, fixation with xylol, covering of stained slices with Vitro-Clud® (R. Langenbrinck GmbH).

Safranin-O & light green staining: air drying, fixation with 4% PFA (10 min), washing with Aqua dest., first staining step in 1% Safranin-O solution (8 min) (Sigma Aldrich), washing twice with Aqua dest., second staining step in 0.1 % Light green (5 min) (Sigma Aldrich, Germany), washing in 1 % acetic acid and 100% ethanol, fixation with xylol, covering of stained slices with Vitro-Clud® (R. Langenbrinck GmbH).

Bulk RNA library preparation and sequencing

The mRNA-specific library preparation was performed using 10 ng of total RNA for each sample. For poly-A dependent cDNA synthesis and a first amplification step the Smart-Seq v4 mRNA Ultra Low Input RNA Kit (Clontech) was used according to the manufacturer's instructions. After quality control (DNF-474 High Sensitivity NGS Fragment Analyzer Kit, Agilent) and concentration measurement (Qubit dsDNA HS Assay Kit, Invitrogen), 1 ng of the purified cDNA was used for tagmentation and library completion with the Nextera XT library preparation kit (Illumina). Paired-end sequencing (2x75 nucleotides) was performed on a Illumina NextSeq500/550 using a Mid Output v2 Kit (150 cycles, Illumina).

Bulk RNA-Seq alignment and normalization

NextSeq500 (Illumina) raw sequence reads were mapped to the mouse GRCm38/mm10 genome with TopHat [1] in very-sensitive settings for Bowtie2 [2]. Gene expression was quantified by featureCounts [3].

Bulk RNA-Seq differential expression analysis

For the analysis of the mRNA and differentially expressed genes, DESeq2 [4] within R 3.5.1 in default settings was used.

Single cell RNA-seq alignment and normalization

Illumina output was demultiplexed and mapped to the mm10 reference genome by cellranger-3.0.2 (10x Genomics Inc.) using refdata-cellranger-mm10-1.2.0 in default parameter setting and 3000 expected cells.

Single cell RNA-seq clustering and differential expression analysis

Raw UMI-counts were further analyzed using R 3.6.1 with Seurat package [5], as proposed by Butler and colleagues, including log-normalization of UMI- counts, detection of variable genes and scaling. T-distributed Stochastic Neighbour Embedding and the underlying Principle Component Analysis was performed based on 30 components using variable genes and a perplexity of 30 as set by default.

Gene Set Enrichment Analysis

Gene set enrichment analysis was performed using g:profiler (version e99_eg46_p14_f929183). The default g:GOST method was used for calculating significance scores with correction for multiple tests [6]. We choose our adjusted p-value cut-off to be 0.05. The databases: GO molecular function, GO cellular components, GO biological process, KEGG, and Reactome were used for the enrichment.

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Supplementary Results

Supplementary Results A: Classification of the subpopulations identified with scRNA-seq in segment A.

Subpopulations were clustered based on the expression of specific genes and the enrichment analysis of certain biological processes. Cluster 1 enriched for granulocytes as supported by the expression of i) *CD33*, a myeloid-specific marker, ii) *Cxcr2*, which is known to be prominent on neutrophils, iii) *CD300lf*, an inhibitory receptor for myeloid cells and mast cells and iv) *Csf3r*, the granulocyte colony-stimulating factor (see supplementary Figure A). This is in line the found biological processes such as cytokine-mediated pathway (GO:0019221) and neutrophil activation (GO:0043312). In addition, gene expression overlaps were found with cluster 4 (neutrophils) for e.g. *CD177*, the neutrophil antigen B, *Camp*, found in polymorphonuclear leukocytes and *Ly6g*, a prominent marker for neutrophils. The biological processes such as protein targeting to ER (GO:0045047) and cotranslational protein targeting to membrane (GO:0006613) are not exclusively know for a specific cell type.

Cluster 2 shows high gene expression of *CD79a* and *b* as well as *Ms4a1* (CD20) specific markers for B-cells and B-cell precursors supported by the expression of CD69 an activation or retention marker. Biological processes enriched for respiratory electron transport (GO:0022904) and B-cell activation (GO:0042113).

Cells of the erythroid lineage can be clearly found in cluster 3 indicating the pronounced expression of genes encoding for enzymes involved in heme synthesis e.g. *Alas2* or protein unique for e.g. erythrocytes or erythroblasts e.g. *Hba-a1*, *Abcg* and *Hemgn*. Moreover, biological processes such as heme biosynthesis (GO:006783) and porphyrin-containing compound biosynthetic process (GO:0006779) are well known from erythroblasts and erythrocyte recycling [1].

In addition, cluster 5 enriched for monocytes and macrophages showing classical marker such as *CD14*, *CD74* and *Ccl2*. Furthermore, *Tnf* was expressed which is known to be upregulated during monopoiesis. With respect to biological processes cytokine-mediated pathways (GO:0019221) and cellular response to lipopolysaccharide (GO:0071222) were identified.

Finally, cluster 6 enriches for mainly chondrocytes (*Prg4*, *Chad*) which is stringently supported by the identified biological processes: extracellular matrix organization (GO:0030198) and skeletal system development (GO:0001501). However, the presence of osteoblast-specific markers (*Col1*, *Sparc*, *Sp7*) indicated the presence of GP chondrocytes and other musculoskeletal cells.

Supplementary Figure A: Classification of subpopulations. tSNE plots highlighting the cluster to which cells have been allocated and selected differentially expressing genes which support the allocation of distinct cell populations to the clusters.

Supplementary Results B: Effects of Actinomycin D on the single cell transcriptome

We know from literature that *Sox9* is not modulated during the ECM digestion [2]. Hence, we isolated the transcriptome of the cells in cluster 6 which had a non-zero *Sox9* expression. Then, we normalized the transcriptome of the cells by the *Sox9* expression and did the same with the transcriptome of the segments in bulk RNA-seq. Comparing the *Sox9* normalized expression levels of the cells in cluster 9 and the segments, we did not see an upregulation of genes *Mmp13*, *Tnfaip1*, *ColX* in the scRNA-seq compared to bulk (supp. Fig. B a–c). Similarly, we did not see the down regulation of *Col2a*, *Acan*, and *Comp* in the scRNA-seq compared to bulk (supp. Fig. B d–f). This suggests that the Act-D was successful in inhibiting at-least the previously establish digestion effects on chondrocytes.

Furthermore, we did find the upregulation of FAM46C encoding an active non-canonical poly(A) polymerase which has been described to support mRNA stability and gene expression [3]. Hu et al. reported the upregulation of FAM46C expression after ActD treatment [4]. The upregulation of this genes in our single cell samples indicates the effectiveness and functionality of the ActD treatment [3].

Supplementary Figure B: Normalized, specific gene expressions indicate no changes in transcriptome by digestions. (a–f) Boxplots of the *Sox9*-normalised expressions of the selected gene in cells of cluster 6. The mean expression in all cells is marked with a green triangle (scRNA-seq). The raw values are overlayed on the boxplot and coloured with a grey circle. The raw values of segments' *Sox9*-normalised expression of the gene of interest (bulk RNA-seq). Segment A is shown in red; segment B is coloured in green, and segment C is coloured in blue. (a) *Mmp13* (b) *Tnfaip1* (c) *Col10a1* (d) *Col2a1* (e) *Acan* (f) *Comp*.

Supplementary Results C: AC chondrocyte-specific genes identified by the intersection of bulk RNA-seq and scRNA-seq

Table 1: Selected differentially expressed genes which are AC chondrocyte-specific - well-known genes such as *Col2a1*, *Acan*, *Sox9*, *Tgfb2*, *Comp*, *Fmod*, *Prg4* are not additionally listed

Gene abbreviation	Gene name	Corresponding literature with respect to cartilage	References
Matn1, 3	<i>Matrilin1, 3</i>	<ul style="list-style-type: none"> cartilage matrix protein which interacts with proteoglycans – e.g. aggrecan in growth plate of developing bone, it is only expressed in the zone of maturation mice lacking Matn1 show alterations in type II collagen fibrillogenesis and fibril organization mice lacking Matn1/Matn3 show structural and biochemical abnormalities in articular cartilage 	[5, 6]
Sema3c, 3e	<i>Semaphorin 3c, 3e</i>	<ul style="list-style-type: none"> axonal guidance molecules which are postulated to have bi-functional properties involved in promotion and repulsion of axons mice lacking Sox9 show downregulated expression of Sema genes which strengthen the actin cytoskeleton studies most often name Sema3a to be anti-inflammatory 	[7-9]
Lect1	<i>Chondromodulin 1</i>	<ul style="list-style-type: none"> promotes chondrocyte growth and inhibits angiogenesis regulated by e.g. Sox9 during onset of OA it seems to be downregulated while it can attenuate OA progression 	[10]
Sod3	<i>Superoxide dismutase 3</i>	<ul style="list-style-type: none"> is expressed in cartilage and markedly reduced after OA development functions as a major scavenger of superoxide anions in extracellular spaces may have a vital role in maintaining cartilage ECM homeostasis through reactive oxygen species (ROS) control 	[11-13]
Calml3	<i>Calmodulin like 3</i>	<ul style="list-style-type: none"> is a calcium-sensing protein no specific literature on cartilage 	[14]
Serpina1e, 1a, 1d, 1b	<i>Serpin peptidase inhibitor, Clade A (antitrypsin)</i>	<ul style="list-style-type: none"> SerpinaA3-1/3 was expressed in a porcine impact model as acute-phase response and reduced levels may contribute to matrix degradation mechanical forces upregulated Serpina1 as inhibitors of proteolytic enzymes/ECM degradation 	[15, 16]
Col9a1, Col9a2, Col9a3	<i>Collagen 9</i>	<ul style="list-style-type: none"> stabilization of the fibrillar collagen network in the cartilage matrix anchorage of Matn3 and proteoglycans 	[17, 18]
Mfi2	<i>Melanotransferrin</i>	<ul style="list-style-type: none"> is expressed during development and in human cartilage antisense sequence (MFI2-AS) was reported to be upregulated in OA – knockout of MFI2-AS ameliorated LPS-induced OA 	[13, 19, 20]
Hhip	<i>Hedgehog interacting protein</i>	<ul style="list-style-type: none"> cell-associated regulator of hedgehog signaling - hedgehog target gene 	[21, 22]

		<ul style="list-style-type: none"> indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation 	
Syt8	<i>Synaptotagmin 8</i>	<ul style="list-style-type: none"> transmembrane protein involved in calcium-induced membrane fusion and exocytosis is expressed in rat articular cartilage with higher expression in deeper zone when compared to the superficial zone described as marker for aging in growth plate senescence 	[23-25]
Ucma	<i>Unique cartilage matrix-associated protein</i>	<ul style="list-style-type: none"> cartilage matrix associated protein which protects cartilage during inflammatory arthritis expression in mice is confined to cartilage zebrafish lacking Ucma have defects mice lacking lcma develop normally but develop more severe OA 	[26]
IL17b	<i>Interleukin 17b</i>	<ul style="list-style-type: none"> pro-inflammatory cytokine secreted by activated T-cells the IL-17 family of ligands and receptors may play an important role in the homeostasis of beyond the immune system Th17 cells stimulate production of MCSF and RANKL in osteoblasts and MSCs 	[27, 28]
Cilp, Cilp2	<i>Cartilage intermediate layer protein</i>	<ul style="list-style-type: none"> ECM protein abundant in articular cartilage may play a role in several diseases affecting cartilage is higher expressed in cartilage during early OA in chondrocytes it was shown that TGFβ induces <i>Cilp1</i> expression <i>Cilp1</i> was shown to inhibit TGFβ-mediated induction of ECM genes in nucleus pulposus cells 	[29-31]
Ncmap	<i>Noncompact myelin-associated protein</i>	<ul style="list-style-type: none"> plays a role in myelin formation listed in two other studies using transcriptomics (ATDC5; mice) 	[32, 33]
Tnfrsf11b	<i>Tumor necrosis factor receptor superfamily member 11B</i>	<ul style="list-style-type: none"> upregulation drives OA pathology by affecting the chondrocytes to engage an unbeneficial hypertrophic state an osteoblast-secreted decoy receptor that functions as a negative regulator of bone resorption provides instructions for making a protein called osteoprotegerin 	[34, 35]
Ctgf	<i>Connective tissue growth factor</i>	<ul style="list-style-type: none"> central mediator of tissue remodeling and fibrosis involved cartilage development expression of <i>Ctgf/Hcs24</i> was concomitant with development of OA lesions 	[36, 37]
Panx3	<i>Pannexin 3</i>	<ul style="list-style-type: none"> an important pro-catabolic mediator in OA deletion of Panx3 prevented the development of surgically induced OA <i>in vitro</i> studies demonstrated that Panx3-mediated ATP release accelerated hypertrophic differentiation 	[38, 39]
Frzb	<i>Frizzled-related protein</i>	<ul style="list-style-type: none"> secreted Wnt antagonist critical modulator of canonical Wnt signaling in cartilage biology 	[40, 41]

		<ul style="list-style-type: none"> mice lacking <i>Frzb</i> showed higher severity in cartilage degeneration 	
Anxa8	<i>Annexin A8</i>	<ul style="list-style-type: none"> expressed in human OA cartilage but not in normal cartilage marker of late-stage chondrocyte differentiation mainly unknown component of hypertrophic cartilage 	[42]
Rspo4	<i>R-spondin 4</i>	<ul style="list-style-type: none"> Wnt pathway modulator involved in the Wnt/β-catenin signaling cascade is downregulated when Sox9 is knocked-out protected bone and cartilage in a mouse model of arthritis 	[43-45]
Hapln1	<i>Hyaluronan and proteoglycan link protein 1</i>	<ul style="list-style-type: none"> stabilizes the aggregates of proteoglycan monomers with hyaluronic acid in the extracellular cartilage matrix disruption resulted in a severe chondrodysplasia 	[46]
Crispld1	<i>Cysteine-rich secretory protein LCCL domain-containing 1</i>	<ul style="list-style-type: none"> no significant change during OA function not clear 	[34]
Tnxb	<i>Tenascin XB</i>	<ul style="list-style-type: none"> involved in collagen deposition and fibril formation deficiency leads to Ehlers–Danlos syndrome (joint hypermobility) 	[47]
Chadl	<i>Chondroadherin-like protein</i>	<ul style="list-style-type: none"> small leucine-rich protein is expressed in cartilage and modulates chondrocyte differentiation appears to have a negative regulatory role, possibly ensuring the formation of a stable ECM 	[48]
Igfbp6	<i>Insulin-like growth factor binding protein</i>	<ul style="list-style-type: none"> can function as IGF carriers, protects IGFs from degradation during transport contribute to the maintenance of tissue homeostasis is a cross-species tendon marker act as growth inhibitors on growth plate chondrocytes 	[49-51]
Dpt	<i>Dermatopontin</i>	<ul style="list-style-type: none"> structural ECM component overexpressed in hip OA chondrocytes 	[52]
Meg3	<i>Maternally expressed gene 3</i>	<ul style="list-style-type: none"> may play a role in angiogenesis and promotes cell proliferation interacts with Smad7 significantly downregulated in OA cartilage 	[53-55]
Bpifb1	<i>BPI fold containing family B, member 1</i>	<ul style="list-style-type: none"> autoantibodies against the lung specific protein unknown role in cartilage 	-
Cspg4	<i>Chondroitin sulfate proteoglycan</i>	<ul style="list-style-type: none"> transmembrane protein, glycoprotein and proteoglycan expression is restricted to a limited number of cell types e.g. articular chondrocytes induced changes in expression of aggrecanases and MMPs may be important in the pathogenesis of OA by regulating proteolytic activity or apoptosis related pathways 	[56, 57]
Clec3a, Clec3b	<i>C-type lectin domain family 3 member A</i>	<ul style="list-style-type: none"> cartilage-specific lectin is present in resting, proliferating, and hypertrophic growth plate cartilage 	[58]

Fxyd2, 3	<i>FXYD domain-containing ion transport regulator 2, 3</i>	<ul style="list-style-type: none"> Fxyd2 is the gamma subunit of the Na, K-ATPase shown to be expressed in chondrocytes involved in sodium and potassium transport and homeostasis 	[59, 60]
Htra4	<i>HtrA serine peptidase 4</i>	<ul style="list-style-type: none"> predicted to be an oligomeric chaperone protease which degrades misfolded secretory proteins was reported to be upregulated in mouse model of arthritis and contributed to cartilage degradation 	[61, 62]
Epyc	<i>Epiphycan</i>	<ul style="list-style-type: none"> is a dermatan sulfate proteoglycan plays an important role in maintaining joint integrity Epn/Bgn double-deficient mice show high severity of OA during aging 	[63, 64]
Pcolce2	<i>Procollagen C-endopeptidase enhancer 2</i>	<ul style="list-style-type: none"> glycoprotein that binds the COOH-terminal propeptide of type I procollagen and potentiates its cleavage by procollagen C-proteinases 	[65]
Itgbl1	<i>Integrin-β-like 1</i>	<ul style="list-style-type: none"> inhibits integrin signaling and promotes chondrogenesis physically interacts with integrins to down-regulate activity 	[66]
Rbp4	<i>Retinol binding protein 4</i>	<ul style="list-style-type: none"> adipokine produced by cartilage and is correlated with MMPs in OA is expressed in chondrocytes during mouse development involved in bone growth (secondary ossification center) 	[67, 68]
Ntn1	<i>Netrin 1</i>	<ul style="list-style-type: none"> involved in chondroprogenitor mobility highly expressed in OA chondrocytes 	[69, 70]
Thbs3, Thbs4	<i>Thrombospondin-3, 4</i>	<ul style="list-style-type: none"> is structurally similar to cartilage oligomeric matrix protein plays a role in the regulation of skeletal maturation mice lacking Thbs4 showed transient reduction of articular cartilage thickness correlates with disease severity in OA knee cartilage 	[71-73]
Notum	<i>Notum</i>	<ul style="list-style-type: none"> carboxylesterase that acts as a key negative regulator of the Wnt signaling attenuates Wnt/β-catenin signaling to promote tracheal cartilage patterning 	[74]
Fbln7	<i>Fibrulin-7</i>	<ul style="list-style-type: none"> cell adhesion molecule expressed in teeth, cartilage, blood vessel walls, and placentae 	[75]
Nov	<i>CCN3 - nephroblastoma overexpressed</i>	<ul style="list-style-type: none"> matricellular proteins with a remarkable relevance within the context of cartilage metabolism mice lacking CCN3 have been shown to display osteoarthritic changes in their knee articular cartilage reported to activate the expression of genes related to articular chondrocytes and to repress genes related to endochondral ossification in epiphyseal chondrocytes 	[76]
Mfap5	<i>Microfibril associated protein 5</i>	<ul style="list-style-type: none"> known to be expressed in synovial fibroblasts 	[77]
Gfpt2	<i>Glutamine-fructose-6-phosphate aminotransferase</i>	<ul style="list-style-type: none"> involved in matrix biosynthesis 	-
Colgalt2	<i>Collagen Beta Galactosyltransferase 2</i>	<ul style="list-style-type: none"> implicated in bone and cartilage involved in collagen formation 	[78]

Ssc5d	<i>Soluble scavenger receptor cysteine-rich domain-containing protein</i>	<ul style="list-style-type: none"> a gene primarily expressed in monocytes/macrophages and T-lymphocytes a soluble scavenger protein which was previously identified to be elevated in synovial fluid of OA patients 	[79]
Medag	<i>Mesenteric estrogen dependent adipogenesis</i>	<ul style="list-style-type: none"> expressed in human cartilage (more pronounced in females) seems to be a regulator in OA progression 	[79, 80]
Cyt11	<i>Cytokine-like 1</i>	<ul style="list-style-type: none"> mice lacking <i>Cyt11</i> showed normal cartilage and bone development but exhibit augmented osteoarthritic cartilage destruction increased during chondrogenesis, and decreased during hypertrophic maturation inducer of chondrogenic differentiation of MSCs appears to have strong pro-angiogenic effects 	[81, 82]
C1qtnf3	<i>Complement C1q tumor necrosis factor-related protein 3</i>	<ul style="list-style-type: none"> identified as a growth factor, and promotes proliferation of chondrogenic precursors and chondrocytes mice lacking C1qtnf3 exhibited higher severity arthritis scores than WT controls 	[83, 84]
Ppp1r1b	<i>Protein phosphatase1 regulatory subunit 1b</i>	<ul style="list-style-type: none"> encodes for Darpp-32 expressed in neurons and regulated by dopamine expressed in growth plate chondrocytes expression is decreased when Sox9 is downregulated in intervertebral disks 	[44, 85, 86]
Slurp1	<i>Secreted Ly6/uPAR-related protein-1</i>	<ul style="list-style-type: none"> regulates cell proliferation maybe regulated by hypoxia exerts anti-inflammatory effects 	[87]
Car12	<i>Carbonic anhydrase 12</i>	<ul style="list-style-type: none"> catalyze the reversible reaction of carbonate hydration, are important for cartilage homeostasis Car12 expressed in full-thickness cartilage 	[88]
Mia	<i>Melanoma inhibitory activity/cartilage-derived retinoic acid-sensitive protein</i>	<ul style="list-style-type: none"> promote chondrogenesis upstream of the transcription factor Sox9 MIA/CD-RAP-deficient mice revealed an enhanced calcified cartilage layer in knee articular cartilage and a disordered arrangement of chondrocytes 	[69, 89, 90]
Fndc1	<i>Fibronectin type III domain containing 1</i>	<ul style="list-style-type: none"> classical matrix protein 	-
Prkg2	<i>cGMP-dependent protein kinase 2</i>	<ul style="list-style-type: none"> is required for the proliferative to hypertrophic transition of growth plate chondrocytes during endochondral ossification loss of PRKG2 function in rodent and bovine models results in dwarfism 	[91]
Chad	<i>Chondroadherin</i>	<ul style="list-style-type: none"> a leucine rich repeat extracellular matrix protein with functions in cell to matrix interactions the protein acts as an anchor to the matrix by binding tightly to collagens type I and II as well as type VI mice lacking <i>Chad</i> showed disturbed bone and cartilage homeostasis but no altered longitudinal growth 	[92]
Susd5	<i>Sushi domain containing 5</i>	<ul style="list-style-type: none"> impact cartilages ECM organization and cartilage development influences the hyaluronic acid binding 	[93]

<i>Has1</i>	<i>Hyaluron synthase 1</i>	<ul style="list-style-type: none"> responsible for cellular hyaluronan synthesis expression and activity of HAS1 are induced by pro-inflammatory factors like interleukins and cytokines, suggesting its involvement in inflammatory conditions mice lacking <i>Has1</i> exhibited chronic joint inflammation 	[94, 95]
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