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Research Article

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Pig meniscus single-cell sequencing reveals highly active red zone chondrocyte populations involved in stemness maintenance and vascularization development

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Abstract: Meniscus injuries are widespread and the available treatments do not offer enough healing potential. Here we provide critical support for the use of pigs as a biological model for meniscal degeneration and the development of cutting-edge therapies in orthopaedics. We present a single-cell transcriptome atlas of the meniscus, consisting of cell clusters corresponding to four major cell types: chondrocytes, endothelial cells, smooth muscle cells, and immune cells. Five distinct chondrocyte subclusters (Ch0-Ch4) were annotated, of which only one was widespread in both the red and white zones, indicating a major difference in the cellular makeup of the zones. Subclusters distinctive to the white zone appear to be responsible for cartilage-specific matrix deposition and protection against adverse microenvironmental factors while those in the red zone exhibit characteristics of mesenchymal stem cells and are more likely to proliferate and migrate. Additionally, they induce remodelling actions in other chondrocyte subclusters and promote the proliferation and maturation of endothelial cells, inducing healing and vascularization processes. Considering they have substantial remodelling capabilities, these subclusters should be of great interest for tissue engineering studies. We also show that the cellular makeup of the pig meniscus is comparable to that of humans, which supports the use of pigs as a model in orthopaedic therapy development.

Key words: Single-cell sequencing; Transcriptome atlas; Meniscus; Chondrocyte; Pig model; Orthopaedic

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1 Introduction

The meniscus is a fibrocartilaginous tissue essential for knee joint stress distribution and stability. The structure of the meniscus is divided into three zones based on their extracellular matrix composition and degree of microvascularization: the outermost red zone, which is rich in blood vessels; the innermost white zone with a low blood supply; and the intermediate red-white zone. The meniscus has a minimal ability to repair and is prone to damage due to the transfer of heavy loads and shear forces in the knee (Rai and McNulty, 2017). Meniscus injuries in the outer zone are more likely to heal, but those in the internal white zone often result in irreversible damage (Makris et al., 2011). Although suturing and partial or complete meniscectomy are still the most prevalent methods of treating meniscus tears, most patients develop osteoarthritis (OA) between ten and twenty years after an injury. The long-term success rate of meniscal repair is only 23.1%, as shown by a meta-analysis published in 2012 (Andersson-Molina et al., 2002; Beaufils et al., 2017; Doral et al., 2018; Nepple et al., 2012). These data suggest that novel therapies should focus on avoiding surgery while maintaining the mechanical characteristics of the meniscus. Therefore, it is hoped that developing treatment strategies like tissue engineering (TE) and xenotransplantation will considerably benefit meniscus repair.

Domestic pigs (*Sus scrofa domesticus*), being physiologically and anatomically similar to humans, have already proven to be a valuable model in studying human diseases and testing new treatments and medical devices (Lunney et al., 2021). The field of orthopaedic research is no exception, as pig models have been widely used for health conditions such as juvenile osteochondritis dissecans, meniscal degeneration, and post-traumatic osteoarthritis (Fang et al., 2020; Goetz et al., 2015; Tóth et al., 2019). Xenotransplantation between pigs and humans, particularly involving the transplantation of bones and cartilage-like structures, has already emerged as a promising area of research (Bracey et al., 2019; Sommaggio et al., 2016). Also, TE uses a range of cell types and organic scaffolds originating from pigs for facilitating tissue repair, regeneration in 3D and recapitulation of meniscus functions after severe trauma (Kwon et al., 2019; Semba et al., 2020). This means that pigs could become a solution for the constant shortage of organ donors and lack of efficient treatment methods, by offering high tissue availability and genetic engineering capabilities. Although both pig-based TE and xenotransplantation offer the chance to restore an organ's physiological function, an in-depth understanding of its genetics is necessary for the highest level of performance. While there are challenges that must be addressed, ongoing exploration of pig omics data aims to improve our understanding of the degree of biological similarity and difference between humans and pigs. Using single-cell sequencing of porcine menisci, we attempted to fill a knowledge gap thereby opening new possibilities for advanced orthopaedic treatments.

For this purpose, we chose single-cell RNA sequencing (scRNAseq) technology, which has emerged as the cutting-edge method for exposing the diversity and complexity of individual cellular transcriptomes. Moreover, it reveals the hierarchical architecture of many cell types and their roles within highly organized tissues and organs (Jovic et al., 2022). The 10x Genomics Gene Expression microfluidic approach, which involves the production of microdroplets, each comprising a single cell, reverse transcription mixture, and a gel bead coupled with oligo sequences, enables deep investigation of single cells transcriptomes. The oligos are designed to catch poliA transcripts, tag them with a cell-identifying barcode and a unique mRNA ID (for further quantitative investigation), and ultimately allow the production of libraries compatible with long and short-read sequencing platforms. In contrast to bulk NGS approaches, this cutting-edge technology not only allows cell type identification but also enables the uncovering of regulatory connections between genes and monitoring of developmental trajectories of various cellular lineages.

Only one publication using a single-cell transcriptome sequencing approach focused on zonal characterization has presented the entire spectrum of cell populations that constitute the human meniscus (Fu et al., 2022). To the best of our knowledge, no data on pig meniscus cell composition are currently available. In this study, single-cell transcriptome sequencing (scRNA-seq) was performed to define and juxtapose cell populations that occur in specific zones of the pig meniscus. Comparisons of annotated cell types with those reported in human studies are discussed.

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2 Results

2.1 scRNA-seq of pig menisci reveals the presence of seven cell clusters and high similarity between the medial and lateral meniscus

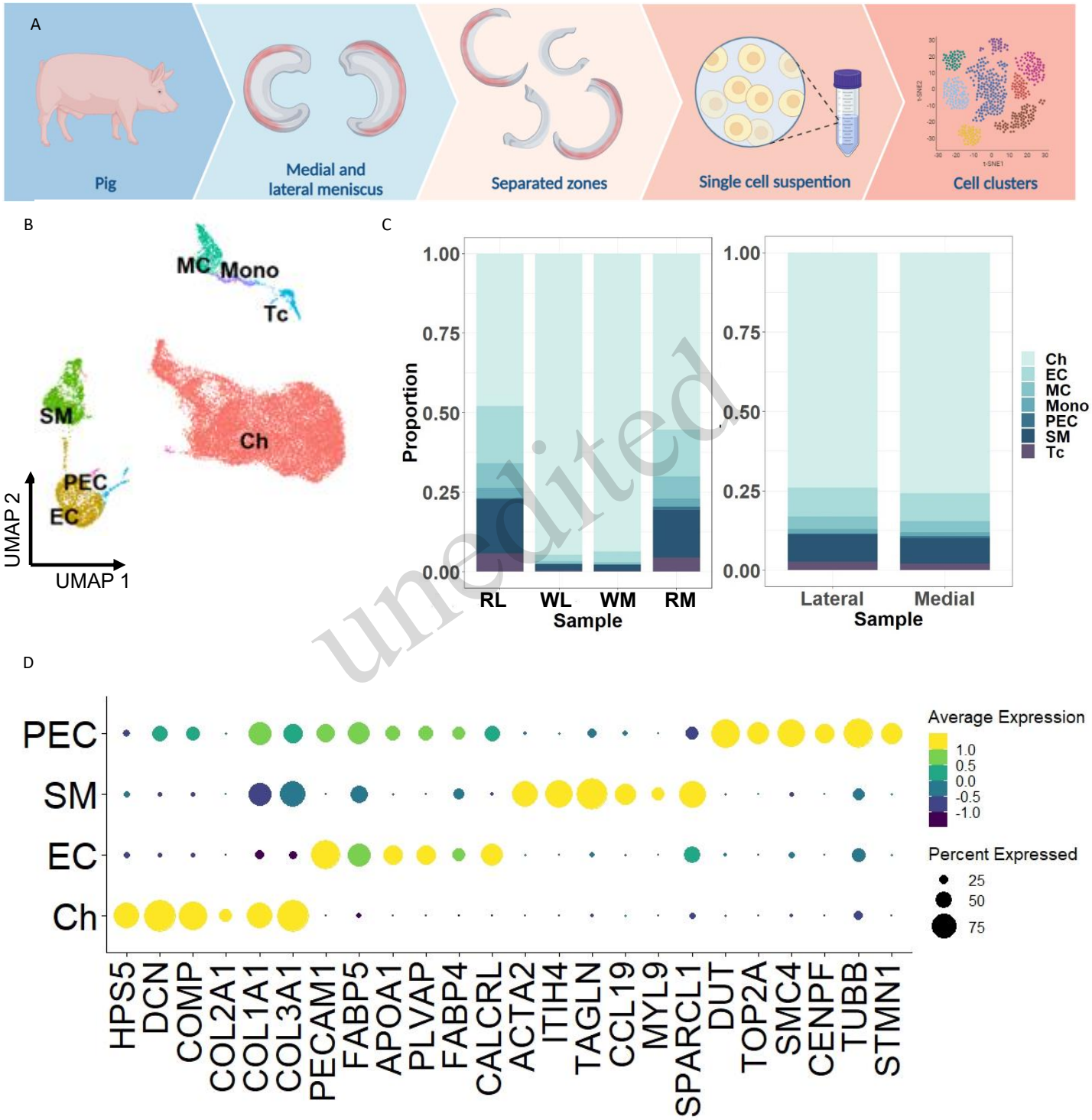

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Fig. 1. scRNA-seq analysis of pig menisci reveals the presence of distinct cell types and their diverse distribution within the zones. **A**. Sample preparation scheme (Created with BioRender.com). **B.** UMAP plot of identified clusters corresponding to cell types: **Ch** – chondrocytes, **EC** – endothelial cells, **PEC** – proliferating endothelial cells, **SM** – smooth muscle cells, **Mono** – monocytes, **Tc-** T-cells, **MC** - macrophages **C.** Cluster proportion in each analysed sample. **RL** – red zone, lateral meniscus, **WL** – white zone, lateral meniscus, **WM** – white zone, medial meniscus, **RM** – red zone, medial meniscus. Clusters proportion in the medial and lateral meniscus. **D.** The expression of six top marker genes per identified non-immune cell clusters.

Porcine menisci - lateral and medial, each divided into red and white zones, were retrieved from a single pig for single-cell sequencing, resulting in the construction of the very first cell transcriptome atlas for this tissue in pigs (Fig. 1A). The cell type composition was compared across these two anatomically divergent menisci and between zones with variable degrees of vascularity. All samples achieved a satisfactory percentage of living cells (ranging from 85 to 100%) enabling the generation of a reliable dataset. The sequencing data underwent a quality control procedure, excluding cells with more than 8% of mitochondrial transcripts and less than 200 genes detected in each cell (nFeatures_RNA), as they indicated apoptotic, or lysed cells and empty droplets. Eventually, a dataset of 26 928 high-quality cells was used for cluster identification and cell type annotation (Table S1). Of these cells, 14 233 originated from the medial meniscus, and 12 695 from the lateral meniscus. When divided based on zonal distribution, 12 168 cells came from the red regions and 14 760 from the white regions.

Seven cell clusters corresponding to four major cell types were identified and visualized on a UMAP plot (Fig. 1B). Cell clusters were annotated based on the highly expressed marker genes (Kozhemyakina et al., 2015) (Table S2). We distinguished a cluster of chondrocyte cells (Ch) expressing collagens (*COL1A1*, *COL2A1, COL11A1*), aggrecan (*ACAN*), decorin (*DCN*) and *SOX9*, a transcription factor involved in chondrocyte differentiation and maintenance of the chondrocytic phenotype (Akiyama et al., 2002; Gelse, 2003; Lefebvre & Dvir-Ginzberg, 2017; Li et al., 2018) (Fig. 1D). Chondrocyte clusters constituted almost 75% of the meniscus cells, making them the majority in each analysed sample (Fig. 1C). The characteristics of specific chondrocytic subclusters are presented below. The next most abundant cell type (9.3%) comprised two clusters of endothelial cells (ECs), identified based on the expression of canonical markers *PECAM1* and *PLVAP* (Guo et al., 2016; Privratsky & Newman, 2014) (Fig. 1D). Within this group, a small subpopulation of proliferating ECs (PECs) was identified. Besides markers typical for ECs, this cluster expressed genes involved in cell proliferation (*TOP2A*) and mitosis such as *SMC4* and KIF11 responsible for chromatin condensation and spindle formation during mitosis (Ali & Abd Hamid, 2016; Rapley et al., 2008; Thadani et al., 2018) (Table S2). EC populations, along with another identified cell type, smooth muscle cells (SMs accounting for 8.3%) expressing *ACTA2* and *MYL9*, are derived from blood vessels (Sun et al., 2020; Xu et al., 2022). The last identified group of three clusters belonged to immune cells, representing about 7.5% of the meniscus population, and most likely comprised macrophages (MC, *CD68+*), T-cells (Tc, *CCL5+*) and monocytes (mono, *HLA-DRA+*) (Cajander et al., 2016; Chistiakov et al., 2017; Korbecki et al., 2020) (Table S2).

We then compared the distribution of cell clusters in the medial and the lateral menisci. This comparison was conducted due to previously documented variability between these two menisci in terms of susceptibility and prevalence of injuries, as well as clinical outcomes after treatment. All the identified clusters were observed in both menisci (Fig. 1C), and the cells within them exhibited similar expression profiles and cell-type specific markers (Krych et al., 2020). The high similarity of the two types of menisci was confirmed through analysis of variance, which showed no statistically significant difference in cluster frequencies at $p=0.05$. The analysis showed no significant divergences in either cell type composition or in frequencies between the medial and lateral menisci. Therefore, we chose to combine data from the scRNA-seq of the same zones originating from the medial and lateral menisci in the following integrative analysis of cells within zones.

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2.2 Zonal comparison of cell type composition confirmed a divergent cellular makeup

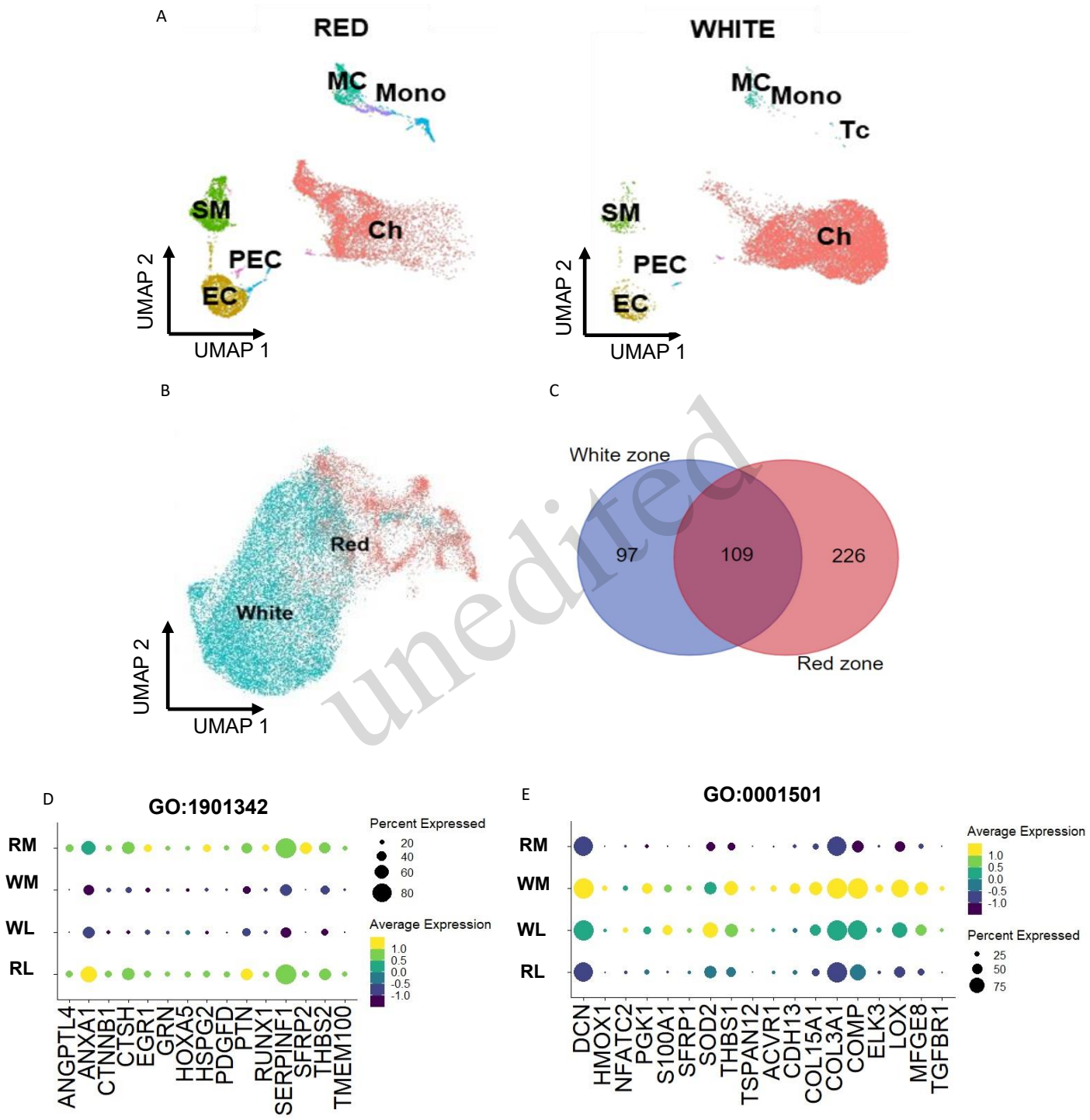

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Fig. 2. Menisci zones differential transcriptional signature reflects their regenerative potential **A**. UMAP plot comparing clusters in menisci zones **Ch** – chondrocytes, **EC** – endothelial cells, **PEC** – proliferating endothelial cells, **SM** – smooth muscle cells, **Mono** – monocytes, **Tc-** T-cells, **MC** - macrophages **B.** UMAP plot of chondrocyte cluster showing different contribution of zonal chondrocytes. **C.** Venn diagram showing overlap between GO terms upregulated in chondrocytes originating from white and red zones **D.** Dot-plot showing genes involved in GO:1901342 – regulation of vasculature system **E.** Dot-plot showing genes involved in GO:0001501 – skeletal system development.

To further explore the intricate nature of the porcine meniscus, we compared two distinct zones, the red zone and the white zone, that are known to be anatomically different. This difference is largely due to the level of vascularization and divergent extracellular matrix (ECM) composition resulting in different healing capacities (Heckmann et al., 2006; Makris et al., 2011). Firstly, we visualised the distribution of cells using UMAP plots (Fig. 2A) and examined the percentage of clusters in each zone, followed by statistical analysis. All the annotated clusters were present in both zones, yet their distribution differed significantly. The main factor contributing to this significant discrepancy was the degree of vascularization. In the red zone, about 33% of cells were annotated to clusters forming blood vessels, with 17% being endothelial cells (EC+PEC) and another 16% marked as SM cells. In contrast, of cells originating from the white zone, only 2.8% were identified as ECs and 1.9% as SM cells. This indicates the presence of only trace vascularity in the white zone, but not its complete absence, which is in accordance with results from GO analysis showing pathways related to vasculature development. Consequently, the distribution of immune cells also varied greatly, ranging from 1.06% in the white zone to 15.4% in the red zone.

The remaining cell types predominantly consisted of chondrocyte clusters, which made up 51.5% in the red zone and over 94% in the white zone. Furthermore, the expression patterns of certain genes encoding ECM components showed distinct differences. Of particular relevance to the functional biology of cartilage-like tissue is the production of collagens. As anticipated, chondrocytes in the white zone exhibited elevated levels of *COL2A1* expression, while *COL1A1* was more abundant in chondrocytes from the red zone. Additionally, genes encoding proteoglycans characteristic of meniscus tissue showed zone-specific expression patterns. Among them, *COMP* and *VCAN* were strongly expressed in the red zone, while *ACAN* appeared to be more characteristic of the white zone (Fig. 3C). Furthermore, a differential analysis of cells forming this chondrocyte cluster (Ch) revealed striking variation in gene expression patterns, indicating their involvement in distinct metabolic pathways (Fig. 2B, Table S3 and S4). A pathway enrichment Venn diagram constructed for differentially expressed genes (p_val_adj ≤ 0.02) showed that the zones shared as few as 109 pathways (Fig. 2C).

In chondrocytes from the red zone significant gene enrichment was observed in metabolic pathways associated with developmental processes, cell motility and adhesion, and ECM organisation. Proliferation, immune system control, and the formation of blood vessels also appeared to be critical aspects of red zone chondrocyte biology (Fig 2D). Hence, we consider that these cells exhibit a high rate of metabolism, significant proliferative capacity, and tissue healing ability. In contrast, chondrocytes specific to the white zone showed gene enrichment related to chondrocyte differentiation, ECM organisation and bone development (Fig. 2E). Of particular importance are SOX5 and SOX6, which control the late stages of chondrocyte differentiation and have a positive effect on their proliferation and cartilage matrix formation, and SOX9 activated in the early stages of pre-chondrocyte formation (Smits et al., 2001). The abundance of transcripts of collagen IX, which plays a crucial role in connecting collagen fibres and proteoglycans to provide mechanical strength, further indicates the structural role of white chondrocytes and their propensity to develop cartilage tissue (Yan et al., 2009). Additionally, these cells are closer to their final developmental stage and showed signs of declining metabolic activity, as expected in meniscus tissue.

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2.3 Heterogeneity of chondrocyte cells points toward divergent function and healing capabilities of the zones

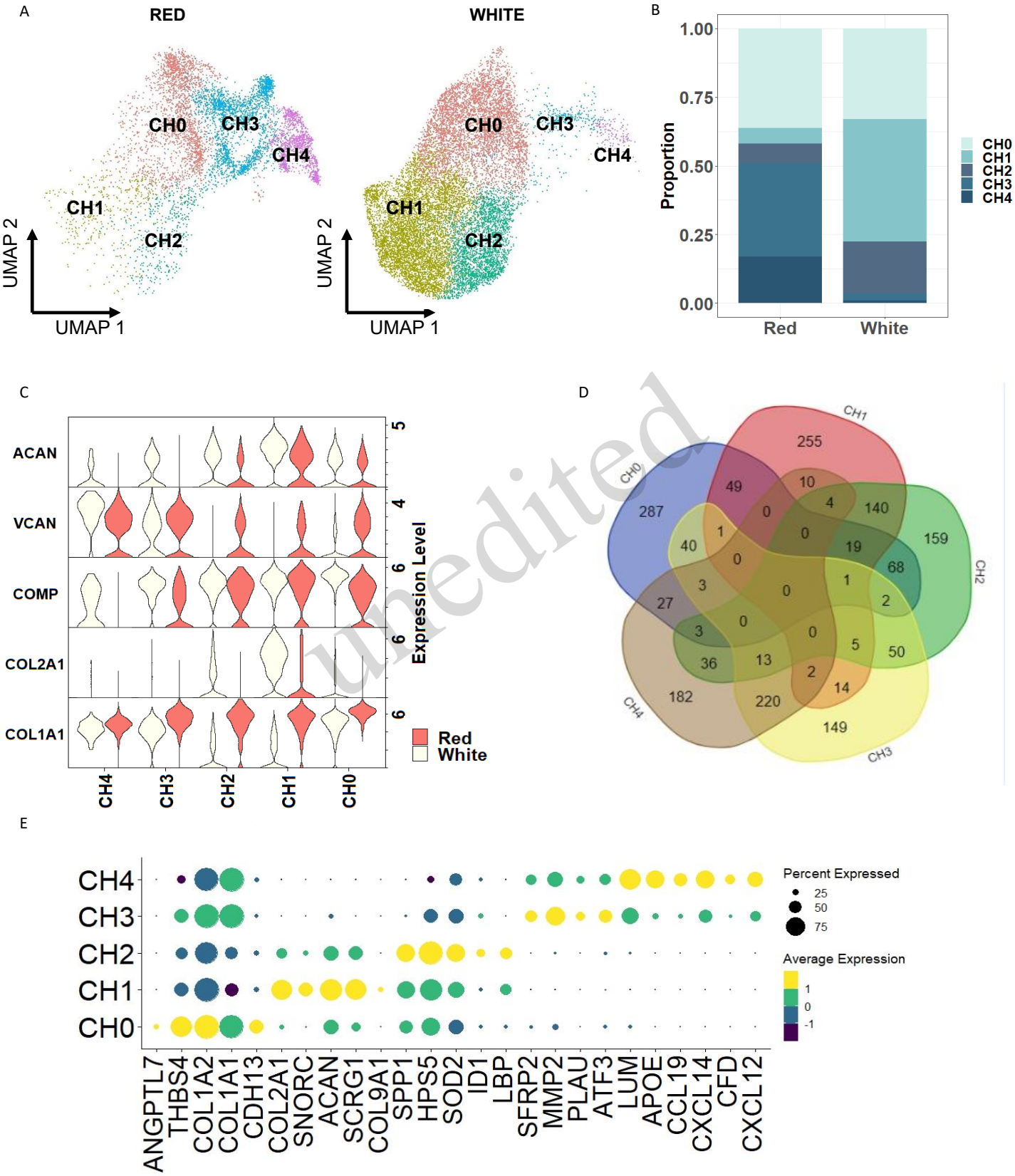

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Fig. 3. Menisci chondrocytes subclusters differ between distinct zones.
A. UMAP plot comparing chondrocytes subsets in menisci zones – red and white. **B.** Chondrocyte subset proportion in red and white zones. **C.** Chondrocyte subclusters marker genes expression in the white and red zones. **D.** Venn diagram showing the overlap of 500 genes with highest expression in chondrocyte subsets **E.** Dot plot showing selected genes with the highest expression within chondrocyte subset.

Due to the above-mentioned discrepancies, an additional investigation of the large cell cluster expressing chondrocyte-specific molecular markers was conducted to better discriminate among cell populations. As a result, five subclusters were identified (Ch0-Ch4, Fig. 3A) with distinct frequencies in the white and red zone samples (Fig. 3B). The subclusters were analysed based on the identification of highly expressed genes (Fig. 3C, Table S5). Remarkably, according to the Venn diagram showing the TOP500 highly expressed genes for each subcluster, not many genes were shared by subclusters (Fig. 3D). Of those highly expressed, none are present in all subclusters, and the total percentage of genes shared by three or four clusters is about 3%. This suggests significant functional differentiation of subclusters.

Subcluster Ch0 exhibited expression of genes involved in osteogenesis and chondrocyte ossification. Among those exclusively expressed were: OMD, which positively regulates osteogenesis; OGN, which enhances bone formation; and ASPN, which is a negative regulator of chondrogenesis involved in collagen biomineralization (Eyre & Weis, 2013; Lin et al., 2021; Nakamura et al., 2017; Rajagopal et al., 2016; Tagliaferri et al., 2015; Xu et al., 2015). Additionally, genes involved in the negative regulation of vasculature development pathways were enriched, including SERPINF1, SPARC, and THBS4. This expression profile suggests that the detected subcluster has an osteophytic phenotype. However, osteophytes are not only osteo-cartilaginous outgrowths in osteoarthritic joints: they are also considered transient repair tissue derived from precursor cells (Gelse et al., 2003) . The top differentiating gene for this subcluster, ANGPTL7, has been linked to chondrocyte differentiation, endochondral ossification, and tissue remodelling (Nishiyama et al., 2021; Takano et al., 2021). Therefore, based on pathway analysis, we expect that this subcluster plays an important role in the meniscus remodelling process, which likely results from mechanical stimuli (Hashimoto et al., 2002). Consequently, we believe that referring to the subcluster "0" as osteophytic is inadequate since it implies that the cells are a step toward bone formation. Instead, we believe that this subcluster reflects the cells' ability to respond to increasing mechanical pressure. Another distinctive gene expressed in this cell group is the proapoptotic gene PEDF, suggesting a transient phenotype of chondrocyte-like cells vulnerable to terminal differentiation and cell death. The presence of these cells in both the red and white zones indicates that this may represent the last stage of chondrocyte differentiation in the meniscus tissue.

In the white zone, subcluster Ch1, the second most frequent subcluster, predominantly expressed genes encoding ECM proteins. Among them, collagens such as COL2A1, COL9A1, and COL11A1, proteoglycans such as ACAN and SNORC, and the ECM stabilizing protein HAPLN1 were highly expressed. Due to the elevated expression of genes associated with maintaining stemness (SCRG1) and differentiation status (S100A1), as well as activation of multiple pathways associated with development and morphogenesis, this population appears to have recently undergone differentiation. The elevated expression of EFEMP1, a gene responsible for ECM integrity maintenance and trafficking, and VIT, involved in matrix assembly and cell proliferation, indicates that this subcluster is undergoing constant remodelling (de Vega et al., 2009; Wilson et al., 2012; Zhang & Marmorstein, 2010) . Analysis also showed clear enrichment in chondrocyte differentiation and cartilage development pathways as well as in genes responsible for collagen synthesis, modification degradation, and assembly of collagen fibrils and other multimeric structures. Since this profile closely resembles what is believed to be the canonical cartilage chondrocyte transcriptome, the most likely function of subcluster 1 is matrix deposition.

The third most common subcluster of cells, Ch2, was rich in gene transcripts connected to stress response, inflammation, and tissue remodelling. Overexpression of the GADD45 gene family, implicated in response to cell injury and stress, suggests stressful environmental growth conditions and the presence of DNA-damaging agents (Salvador et al., 2013) . The presence of several cytoprotective gene transcripts such as CLU (anti-apoptotic, anti-inflammatory, and antioxidant properties), SOD2 (coding for superoxide dismutase), CP (a metalloprotein involved in the peroxidation of transferrin), and MT1A (an oxidative stress-response gene protecting against hydroxyl free radicals), as well as the abundance of gene transcripts connected to inflammatory response (NFKBIA), post-traumatic cartilage destruction (LBP), or inflammation processes and tissue remodelling (CHI3L1), suggests a suboptimal meniscal microenvironment for cells (Drecourt et al., 2018; Duprez et al., 2012; Koike et al., 2015; Tarquini et al., 2020; Ungsudechachai et al., 2021; Won et al., 2021; Zhang et al., 2021; Zhao et al., 2020) . However, the overexpression of SPP1 (a matrix remodelling marker) noted in the meniscus model of post-traumatic osteoarthritis, HPS5 (a regulator of lysosome synthesis and vesicular trafficking), CCDC80 (a promoter of cell adhesion and matrix assembly), THBS (an adhesive glycoprotein induced in sites of tissue damage or active remodelling that mediates cell-to-cell and cell-to-matrix interactions as an

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adaptive stress response), and MGP (a powerful inhibitor of cartilage mineralization) indicates that the cells are remodelling in response to these stressful conditions (Gautam et al., 2004; Lynch et al., 2012; Manabe et al., 2008; Salazar-Noratto et al., 2019; Yagami et al., 1999). Additionally, the abundance of gene transcripts (like FOS and CCN2) and pathways involved in cell proliferation and differentiation, suggests that these cells can handle stressful growth conditions quite well and are unlikely to follow apoptotic pathways (Güller et al., 2008; Takigawa, 2013). In summary, these cells exhibit anti-oxidative and anti-inflammatory properties against stressful growth conditions within the meniscus, and in response, they remodel the ECM and clear the micro-environment to ensure the survival of neighbouring cells. Therefore, they may serve as protectors to other chondrocytes.

Cells forming subcluster Ch3, which constitute most of the red zone, express genes with divergent functions, involved in stem cell modulation, matrix remodelling, and inflammatory responses simultaneously. Several gene transcripts connected to stem cell regulation, including CRABP1 and IGFBP2, known to modulate stem cell behaviour by suppressing cell proliferation and sensitizing them to growth factors, were highly expressed (Fisher et al., 2005; Y.-L. Lin et al., 2017). Similarly, SFRP2 and TNC, proteins characteristic of mesenchymal stem cells (MSCs), exhibited elevated expression levels in this subcluster. Both proteins enhance MSC survival rates, regulate differentiation, and enhance therapeutic efficacy when exposed to unfavourable factors (Alfaro et al., 2008; Rodrigues et al., 2013). Other intensively transcribed genes identified in this subcluster are important for matrix organization. For example, MMP2, typical of the red zone, is involved in vascular remodelling and angiogenesis within the meniscus due to its degrading potential toward aggrecan and collagens (Fuller et al., 2012). ADAMTS1, interacting with MMP2 in several pathways, is a hypoxia-induced disintegrin and metalloproteinase, promoting cell proliferation and migration. Lumican is a collagen-binding proteoglycan responsible for biomechanical strength in connective tissues (Chakravarti, 2002). Taken together, these genes suggest a predicted pattern characteristic of vascularized, mechanically durable tissue with the ability to differentiate. Overexpression of anti-inflammatory genes is a well-known phenomenon specific to MSCs. On one hand, the remodelling and healing process is facilitated by the production of anti-inflammatory agents that enable the recruitment of other MSCs to the site of injury. On the other hand, MSCs, by modifying immune cells, foster a microenvironment favourable to the healing process (Liu et al., 2018). Genes identified in a highly expressed set specific to cluster Ch3 included antiviral ISG12 and IGS15, antimicrobial chemokines CXCL2 and CXCL14, involved in allergic inflammation POSTN, and genes responsible for immune cell infiltration ABL2 (Hara & Tanegashima, 2012; Izuhara et al., 2014; Li et al., 2022; Wang et al., 2021). Considering the aforementioned, we predict that the Ch3 population would closely resemble the phenotype of MSCs while also displaying certain chondrocytic characteristics, allowing us to refer to them as chondrocyte progenitor cells. Due to their immunomodulatory and matrix remodelling properties, these cells are of great interest in tissue engineering and the management of meniscus injuries.

The smallest subcluster, Ch4, expresses many genes involved in the breakdown of the ECM, as well as those implicated in cell migration and proliferation. Genes such as apolipoprotein E (APOE), responsible for clearing lipoproteins, lumican (LUM), which induces matrix degradation, and serpin family E member 2 (SERPINE2), which regulates metalloproteinases, are implicated in catabolic pathways (Barreto et al., 2020; Santoro et al., 2015; Zhou et al., 2022). Versican, a key ECM component involved in cell migration and proliferation due to its matrix-loosening and hydrating capabilities, is encoded by the highly expressed VCAN gene. Versican is a hygroscopic proteoglycan that interacts with other ECM components, affecting their assembly and remodelling. In the meniscus, it has an exceptional ability to resist compressive and tensile stresses caused by loading, thanks to its binding with hyaluronan (Rahmani et al., 2006). SDC2 and GSN, two additional abundant gene transcripts in this subcluster, are linked to cell migration and proliferation. Additionally, this subcluster has high quantities of various collagen transcripts that code for collagen IV, which forms a basement membrane, collagen V, which plays a key role in the healing process, and collagen VI, an ECM organiser (Theocharidis & Connelly, 2019). Based on this transcriptional profile, the cells forming this subcluster are expected to be remodelling cells. This remodelling behaviour might be connected to vascular system development, since multiple pathways involved in angiogenesis have been identified for this subcluster (ex. GO:0001525; GO:0045765; GO:0045766).

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2.4 Single-cell trajectory analysis of chondrocyte subclusters reveals two distinct cell fates associated with zonal composition.

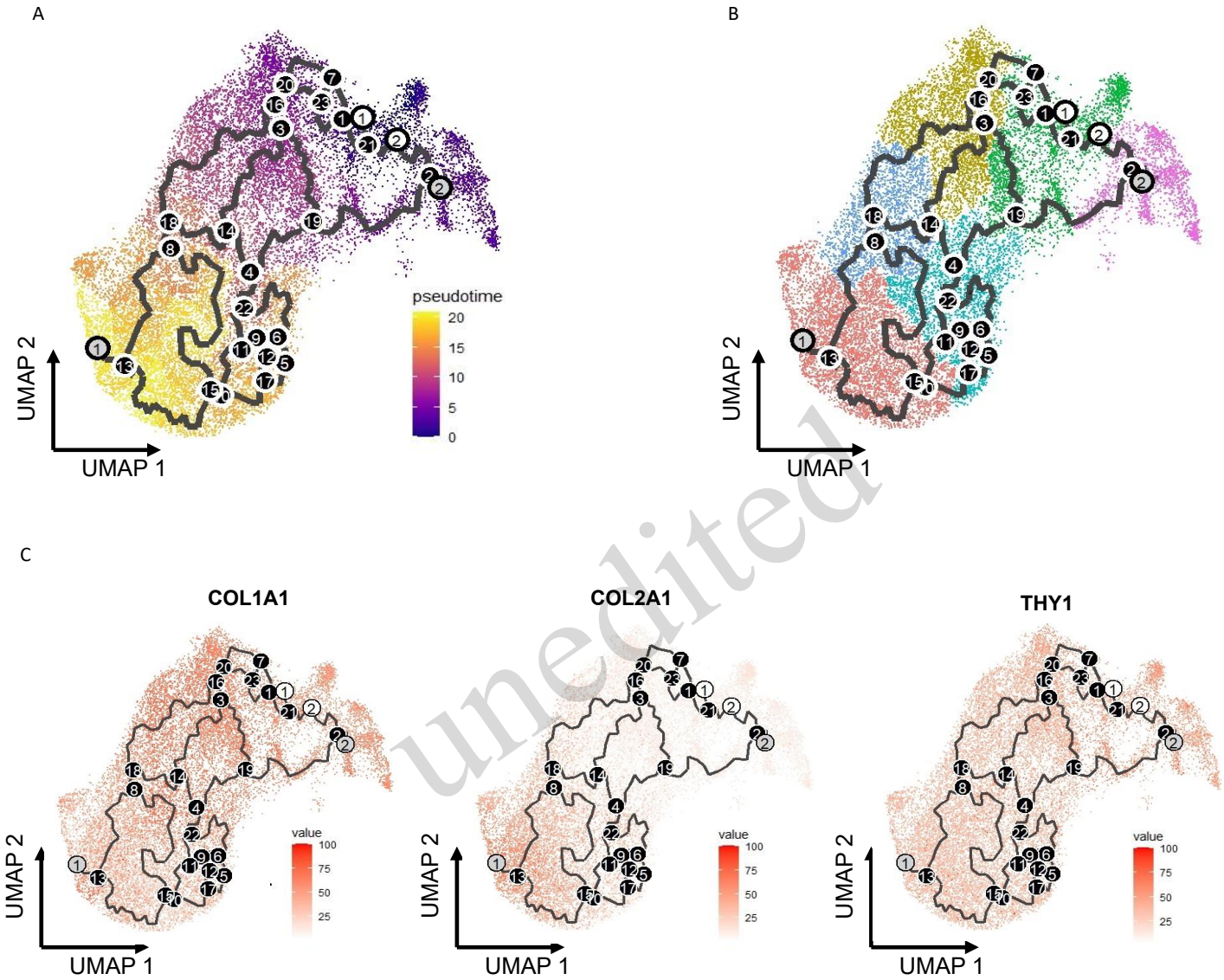

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Fig. 4. Single-cell trajectory analysis of chondrocyte subclusters reveals two distinct cell fates compliant with zonal composition.
A. UMAP plot showing possible cell fates of CH3 subset (defined as a root-noodle). **B.** UMAP plot showing defined six subsets based on the pseudotrajectory analysis with defined cell fates **C.** UMAP plots showing the expression of selected genes (COL1A1, COL2A1, THY1) on pseudo-trajectory plot.

To study the differentiation of chondrocytes, we used the computational approach of trajectory analysis using the Monocle3 R package. We chose a chondrocyte subcluster Ch3 as the starting point for our trajectory, based on the numerous proliferation-associated GO terms (ex. GO:0008284, GO:0042127) allocated for this subcluster and expression of a group of genes identified as MSC/progenitor cell markers including: CD34 – a marker gene present in many progenitor cell types indicative for stem cell activity; CD248 – an MSC marker and negative regulator of bone formation; THY1 – a surface marker for synovial MSCs exhibiting increased differentiation potential for chondrocytes (Naylor et al., 2012; Sidney et al., 2014) (Fig. 4C). A trajectory representing the two distinct cell fates (1 and 2), matching to the zonal distribution of chondrocyte subclusters, was established (Fig. 4A and 4B). Cell fate 1 is in line with the white zone's usual development into classical, matrix-depositing chondrocytes (Ch1) expressing COL2 and increased levels of ACAN (Fig. 4C). The Ch4 cells, found only in the red zone and intimately associated with the development of vasculature, occupy fate 2, which denotes the opposing termini of the pseudotime trajectory (Fig. 4B). These findings align with the meniscus's zonality, emphasising the need to consider diverse composition when conducting studies on meniscus regeneration and treatment strategies.

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2.5 Analysis of cellular interactions identifies the significant stimulatory activity of chondrocytes toward other cells and the proangiogenic and remodelling cha

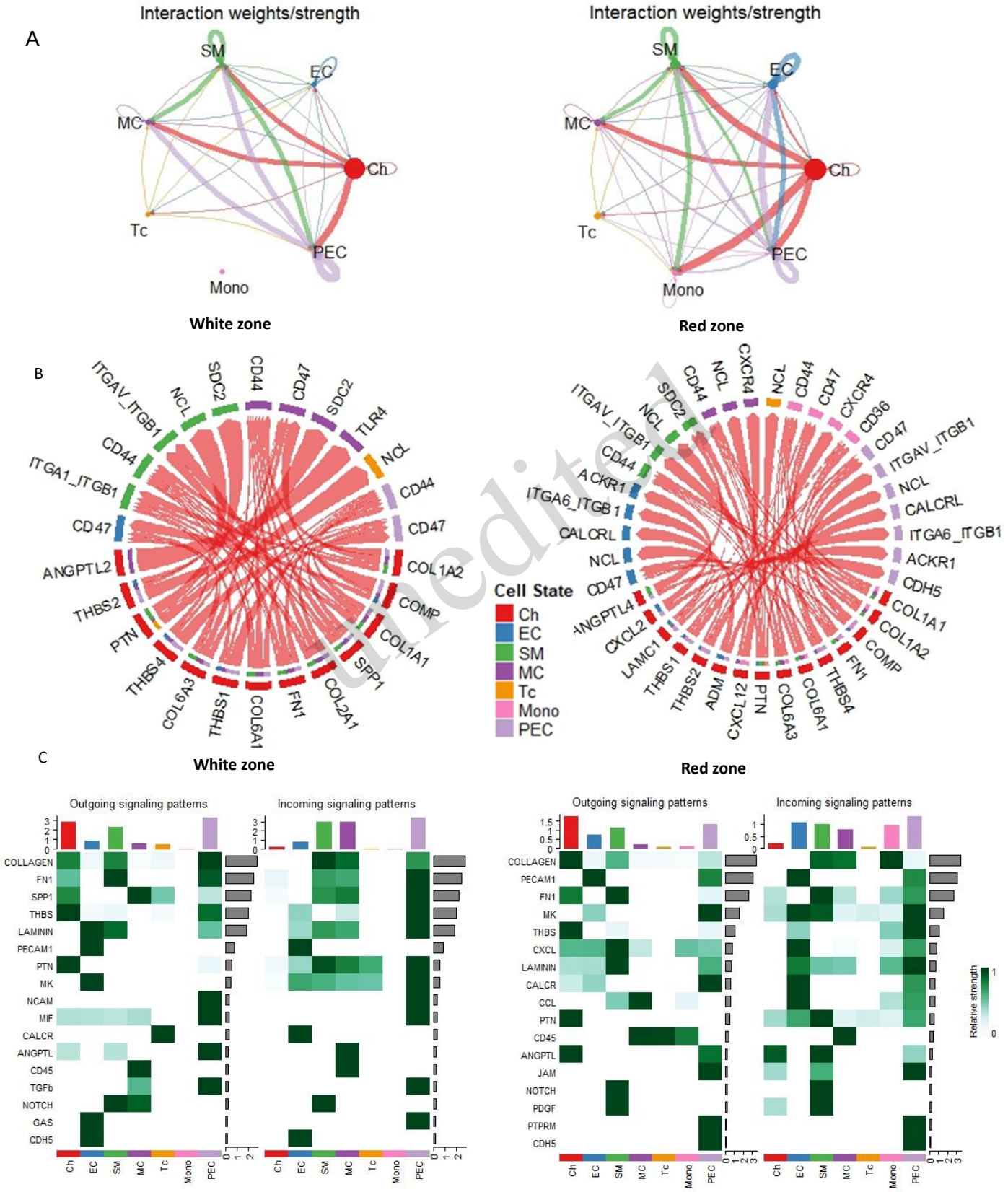

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Fig. 5. Cell–cell interactions of the cell clusters explored based on the ligand–receptor expression.
A Circle plot of the ligand-receptor pairs interaction weights/strengths of all cell types in white (left panel) and red (right panel) zones. String thickness represents the weight. **B** Chord plot of ligand-receptor pairs among chondrocytes and other cell types in white (left panel) and red (right panel) zones. **C** Heatmap depicting outgoing and incoming signalling patterns in regard to zones. Colour range represents relative strength of interaction.

Cell–cell interactions of the cell clusters and subclusters were explored based on ligand–receptor expression. We adapted human ligand–receptor database CellChatDB to match all pig ligand–receptor pairs for each cell type based on gene homology. Overall signalling pattern analysis indicated higher activity in red zone Ch compared to white zone Ch (Fig. 5A). Chondrocytes residing in both zones were interacting with cells forming blood vessels (SMs and PECs and weakly with ECs) and with cells identified as macrophages (MCs). Interestingly, monocytes (mono) are intensively stimulated by red zone chondrocytes but not white zone ones. Most contributing was the CD44 receptor, responsible for induction of M2-like polarity of macrophages involved in wound healing and tissue repair (Fig. 5B) (Kim et al., 2019).

Another interesting observation was that meniscus chondrocytes are prone to send signals and affect other cell types, while receiving hardly any (Fig. 5C). The most abundant networks are collagen, THBS and FN1 (Fig. 6). Collagens are most likely inducing anti-apoptotic effects and enhancing proliferation via CD44 receptor expressed on immune and vasculature-associated cells (Wu et al., 2019). The THBS signalling network is associated with proliferation, vascularization and healing processes. Particularly interesting is the THBS4-CD47 pattern observed between chondrocyte-PEC in both zones (Liao et al., 2022). Thbs4 is known to be expressed by cells in response to increased mechanical load and CD47 activation induces proliferation in stem cells (Kaneshige et al., 2022). Other highly active signalling patterns pointing toward chondrocyte stimulation of PECs are COMP-CD47 and FN1-(ITGAV+ITGB1). COMP-CD47 has already been reported in bone marrow as an important hematopoietic progenitor of cell expansion, while fibronectin 1 action on integrins has been reported as inducing epithelial to mesenchymal cell transition (Ding et al., 2017; Wen et al., 2022). The presence of these patterns indicates chondrocyte involvement in stemness maintenance and PEC proliferation stimulation, induced by mechanical stimuli.

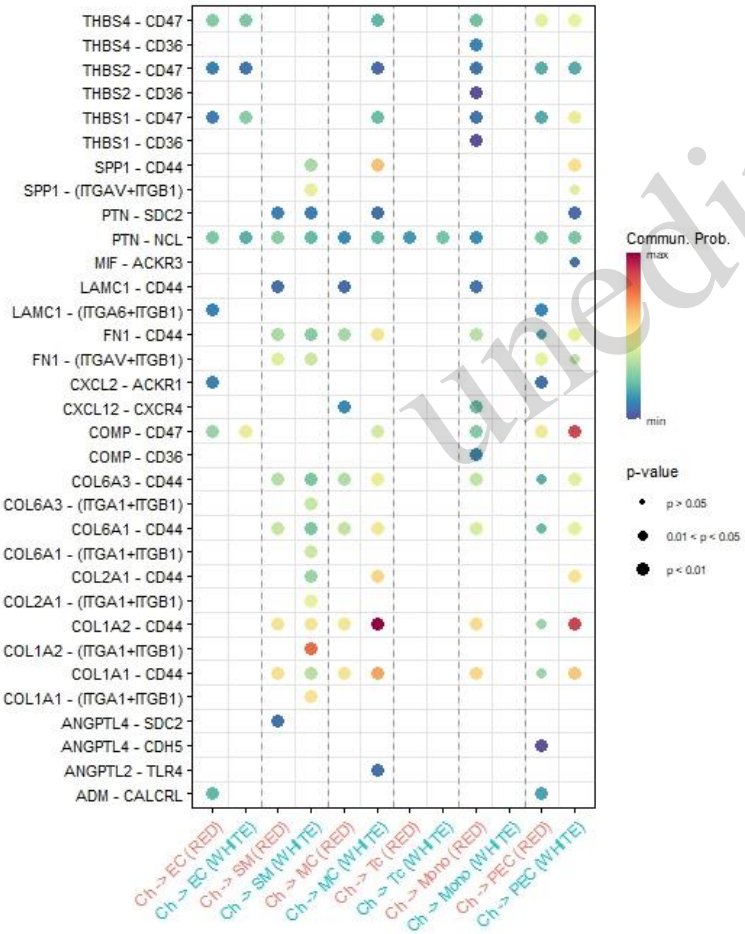

Fig 6. Upregulated and downregulated signaling ligand-receptor pairs in communication of chondrocytes with other cells in red and white zones. Colour range represents the communication probability and dot size represents p-value.

When chondrocyte subclusters are considered, incoming and outgoing signalling patterns confirm the dual functional characteristics and pseudotime fate of chondrocytes forming red (Ch3 and Ch4) and white (Ch1, Ch2) zones (Fig. 7A, 7B). Subcluster Ch0, widespread in both zones, and recognised as showing signs of tissue remodelling toward ossification, expresses collagen signalling networks, stimulating SMs, MCs, Mono, PECs and Ch4 via CD44 (Fig. 7). Additionally, two patterns between Ch0 and PEC, described above, were observed: THBS4-CD47 and COMP-CD47. Conversely, Ch0 seems to be activated by Ch3 and Ch4 through periostin, which is recognized for delivering signals through interactions with integrins, crucial for tissue development and remodelling during wound healing (Ontsuka et al., 2012). Subclusters Ch1 and Ch2, residing mostly in the white zone, show profiles similar to those of outgoing signals of Ch0, but with weaker interaction strength, while incoming signalling profiles show Ch1 stimulation with several ligands (mostly NCAM, JAM and MK from PEC, and PTN from other chondrocytes) and close to zero incoming signals for Ch2 (Fig. 7). The most pronounced outgoing signalling

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patterns in Ch1 and Ch2 concern the SPP1 ligand, encoding osteopontin, highly expressed in osteoarthritic cartilage and chondrocytes exhibiting ageing characteristics (Qu et al., 2023). Chondrocytes identified as progenitory (Ch3) appear to interact strongly with Ch4 but only weakly with other chondrocyte subclusters (Fig. 7). Numerous networks of Ch3 and Ch4 were observed including collagen, fibronectin, pleiotrophin and angiopoietin-like (Fig 7). *PTN* is expressed in sites of early vascular development in healing wounds and is associated with an angiogenic phenotype (Christman et al., 2005) while ANGPTL4 promotes cartilage matrix remodelling by inhibiting expression of collagen type II and aggrecan and by up-regulating the level of certain MMPs (Mathieu et al., 2014). Adding enhancement of proliferation induced by collagens and fibronectin activatory action in wound repair, a clear picture of Ch4 activation by Ch3 toward meniscus remodelling, vascularization and healing can be drawn (Tao et al., 2018). Ch3 appears also to enhance proliferation of endothelial cells (PEC) via THBS, COL and FN1 ligands. Interestingly, PECs are sending NCAM signals to Ch3, most likely preventing hypertrophic differentiation during chondrogenic differentiation of these MSC-like cells and preventing OA pathogenesis. Ch3 cells are also stimulated by platelet derived growth factor (*PDGF*) expressed by SM cells, which is known to be a potent stimulator of cell proliferation as well as cartilage formation (Schmidt et al., 2006).

Despite being the smallest subcluster, Ch4 remodelling chondrocytes appear to have the highest number and strength of interactions (Fig. 7). They display a pattern of incoming and outgoing signalling that is comparable to Ch3, but with certain advancements, such as the stimulation of monocytes and the expression of laminines that interact with other cell types (Fig. 7). Recent findings suggest that laminins expressed by cartilage progenitor cells promote chondrogenesis and provide a favourable microenvironment for its regeneration (Sun et al., 2017). Patterns identified in our dataset showed Ch4 chondrocytes signalling toward PEC and EC via laminin ligands, which are known to direct differentiation of endothelial progenitor cells toward mature ECs (Yap et al., 2019). The pattern responsible for Ch4-Mono interaction – CXCL12/CXCR4 – is also involved in vascularization and regeneration processes, namely monocyte polarisation toward M2 macrophages, vascular formation, cell proliferation and migration (Fang et al., 2022). Additionally, activation of CXCR4 is known to increase expression of matrix metalloproteinases, resulting in collagen destruction and tissue remodelling (Mousavi, 2020).

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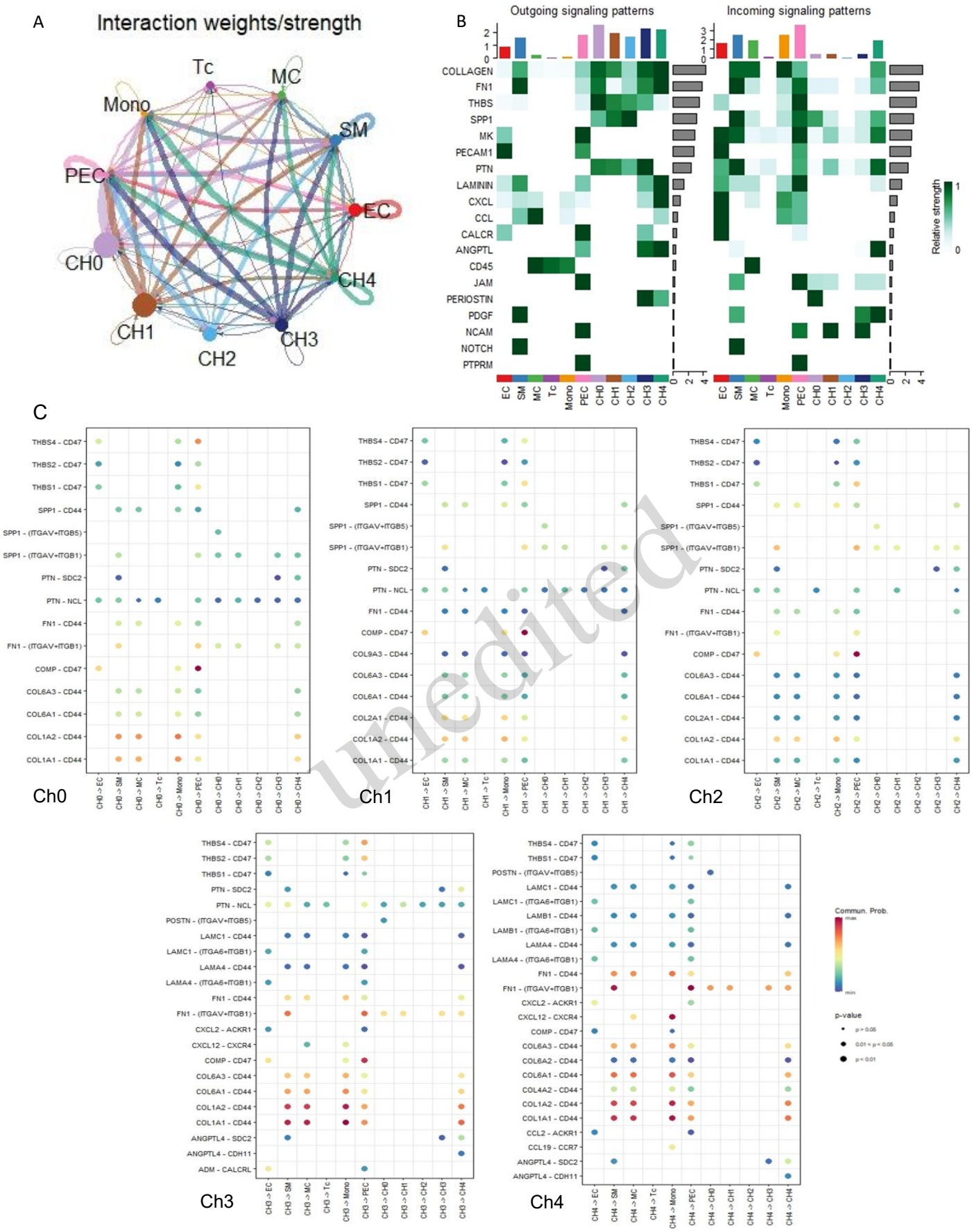

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Fig. 7. Cell–cell interactions of the chondrocyte subclusters explored based on the ligand–receptor expression **A** Circle plot of the ligand-receptor pairs interaction weights/strengths of all cell types. String thickness represents the weight. **B** Heatmap depicting outgoing and incoming signaling patterns for chondrocyte subclusters. Colour range represents relative strenght. **C** Signaling from each chondrocyte subcluster. Colour range represents the communication probability and dot size represents p-value

3 Discussion

Seven cell clusters corresponding to four major cell types were identified based on the expression of marker genes: chondrocyte-like cells (Ch) expressing collagens, aggrecan, and positive for SOX9; endothelial cells (ECs) expressing *PECAM+* and *PLVAP+*; proliferating endothelial cells (PECs) expressing *TOP2A+, SMC4+,* and *KIF11+*; smooth muscle cells (SMs) expressing *ACTA2+* and *MYL9+*; and immune cells, most likely macrophages (MC) expressing *CD68+*, T-cells (Tc) expressing *CCL5+*, and monocytes (mono) expressing *HLA-DRA+*.

While the frequencies of these clusters differed significantly when comparing zones, the analysis of medial and lateral menisci revealed no qualitative or quantitative differences in cell type distribution and cell transcriptional profiles. This led to the conclusion that the variable prevalence and treatment outcomes observed between medial and lateral meniscus injuries are most likely due to the higher forces acting on the medial side of the knee (Halder et al., 2012). Epidemiological studies support this hypothesis, indicating that medial meniscus tears are more common in older patients with elevated BMI and suffering from a range of degenerative knee conditions, whereas lateral meniscus injuries more frequently concern young, male patients with no history of osteoarthritis or similar conditions and are often connected to ligament injury, suggesting that those tears are sport-related (Mitchell et al., 2016; Thein et al., 2017). It is not surprising, therefore, that lateral meniscus tears heal more efficiently and require fewer total or partial meniscectomy surgeries, even though there is no specific genetic component that can be added (Krych et al., 2020).

It was discovered that the chondrocyte cluster interacts quite actively with other types of meniscal cells. It is surprising how many outgoing signalling patterns have been discovered, but very few incoming signalling patterns have been observed, leading to the conclusion that chondrocytes act as stimulators rather than stimulated cells. All of the cell types identified in the meniscus, and PECs in particular, are impacted by chondrocytes. They promote proliferation, stemness maintenance and matrix remodelling, which results in increased vascularization and further tissue repair. Thorough investigation of the chondrocyte-like cluster revealed the presence of five subclusters (Ch0-Ch4). Only Ch0 was abundant in both of the regions under study. Subclusters Ch1 and Ch2, which are most likely responsible for cartilage-specific matrix deposition and protection against adverse microenvironment factors, respectively, representing one branch of the pseudotime trajectory, were specific to the white zone. Those subclusters send signals via the osteopontin pathway – characteristic of differentiated, ageing chondrocytes – not noticed in Ch3 and Ch4 chondrocytes. In addition to being metabolically active and expressing chondrocyte-specific genes encoding collagens and proteoglycans, Ch3 and Ch4, which make up most of the red zone, exhibit characteristics of MSCs and are prone to proliferation and migration. They also induce remodelling actions on other chondrocyte subclusters, potentially inducing healing and vascularization processes. What is more, Ch3 cells are most likely enhancing PEC proliferation, while Ch4 direct differentiation of PECs toward mature endothelial cells. Therefore, those two subclusters have greater remodelling properties and capacity for usage in tissue engineering.

These findings are consistent with the work of Fu et al. (2022) who performed single-cell sequencing on healthy human menisci and detected chondrocytes, ECs, and immune cells. In contrast to our research, they additionally annotated a group of cells known as "pericytes." However, this cell population expresses significant quantities of *ACTA2*, *MYL9*, and other muscle contractile genes and is found mainly in the red zone of the meniscus, resembling the "smooth muscle cells" discovered in our investigations. Furthermore, Fu et al. noted that using only genetic markers makes it extremely difficult to differentiate between pericytes and smooth muscle cells. We assume that the functional characteristics of these two populations are identical, and any differences exist only in terms of nomenclature.

Regarding chondrocyte populations, most of our results agree with those of Fu et al. (2022). However, comparing cell subtypes is challenging due to the abundance of data and the subtle variations between distinct cell clusters. Except that we identified three clusters for white zone chondrocytes instead of two, the gene markers reported by Fu et al. as being specific to the white zone are present in our data labelled as white zone chondrocytes. Our Ch1 shares *APOD*, *FN1*, and *ECRG* markers with Fu's chondrocyte-1 population and exhibits anti-angiogenic characteristics and remodelling actions. The subcluster Ch1 in our study displays similar genetic markers and ECM remodelling capabilities as Fu's chondrocyte-1 population (both clusters are positive for *CHI3L1*, *HTRA1*, *FN1*, and the *TIMP* superfamily). The Ch2 cells in our study, which correspond to the white zone, express markers common to Fu's chondrocyte-3 cell type (*CHI3L1, CDON*). Similar to our study,

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Fu et al. found two red zone clusters expressing genes such as *POSTN, MMP2, ADAMTS2, CFD, FNDC, VCAN*, and genes from the *CXCL* superfamily, which suggests remodelling and immunomodulatory effects. The main discrepancy concerns the last chondrocyte cluster identified by Fu et al. as expressing genes linked to proliferation; these markers were not found in any of our chondrocyte clusters but were prevalent in the *PECAM/PLVAP+* proliferating endothelial cells (PECs). Interestingly, in our dataset, the cells referred to as progenitory chondrocytes (Ch3) associated with the Ch-2 and Ch-4 clusters were found to express MSC markers and display early signals of differentiation, which were not reported by Fu et al. Although more thorough research on the integration of data from different species is needed for a detailed examination of cell type similarities, it is clear that the cell types found in porcine-origin menisci are consistent with data from humans. Due to the lack of automated software for functional annotation of detected clusters, single-cell data analysis studies are currently focused on subjective approaches and addressing specific hypotheses.

Single-cell sequencing of the human meniscus has been covered in another study by Sun et al. (2019). However, we have chosen not to compare our data with their results due to several concerns, the main one being sample treatment prior to sequencing. Our and Fu's experiment settings used freshly extracted meniscus tissue cells that had been subjected to single-cell library preparation, whereas Sun et al. analysed in vitro cultured cells. The study by Sun et al. focused solely on adherent cell populations, and it is likely that the cells' expression patterns were dramatically changed during the culture process. Additionally, the single cell suspension and partitioning methods were different (cell sorting as opposed to droplet generation). Fu et al. discussed this in significant detail and claimed that these differences led to discrepancies in the generated data, resulting in significantly distinct cell clustering. The mismatch in results highlights the need to thoroughly review the methodology before attempting to compare the data.

Our study was conducted on one individual. Sequencing of additional samples, as well as adding spatial transcriptomic data to the picture, will help to further validate our results. However, analysing 26 928 cells seems adequate for creating a cell atlas for a single, specific tissue, especially since Wang et al.'s pig cell atlas included a comparably high number of analysed cells for only two tissue types (liver and lung), while some tissues were described based only on a few thousand cells (with a minimal number of 1 527 for subfornical organ) (Wang et al., 2022). Many cell atlases published in high impact journals are based on analyses carried out on single individuals and lack external validation. The reproducibility issue concerning single cell genomics is still under debate due to the complexity of single cell datasets and numerous analytical choices influencing final outcomes. This issue has been extensively discussed in an opinion piece recently published in PLOS GENETICS (Gibson, 2022).

In this paper, we present a single-cell transcriptome atlas of the medial and lateral menisci of the pig, demonstrating that their molecular landscapes are identical. By dividing the menisci into red and white zones, we identified their significantly diverse chondrocyte compositions. Detailed analysis of cell expression profiles, functional potential and signalling revealed high metabolic activity and stimulatory actions of the chondrocytes residing in the red zone of the meniscus. The results also pointed toward the exceptional role of two chondrocyte subclusters in meniscus remodelling, vascularization and healing as well as in stemness of progenitor epithelial cell maintenance. Additionally, we have shown that the cellular makeup of pig tissue is similar to that of human tissue, providing crucial support for the use of pigs as a biological model for meniscal degeneration and the development of cutting-edge therapies like tissue engineering and xenotransplantation.

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Data availability statement

Data are available in a public, open access repository Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE241228.

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Author contributions

Monika MANKOWSKA Conceptualization; Data curation; Investigation; Methodology; Project administration; Writing – original draft; Writing – review & editing

Monika STEFANSKA Data curation; Formal analysis; Investigation; Methodology; Software; Visualization; Writing – original draft; Writing – review & editing

Anna Maria MLECZKO Investigation; Resources; Validation
Katarzyna SARAD Software; Visualization; Writing – original draft
Witold KOT Investigation; Writing – original draft
Lukasz KRYCH Data curation; Formal analysis; Investigation; Software
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Eric Lars-Helge LINDBERG Writing – review & editing
Jakub Dalibor RYBKA Conceptualization; Funding acquisition; Supervision; Writing – review & editing

All authors read and approved the final manuscript and, therefore, had full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Monika Mankowska, Monika Stefanska, Anna Maria Mleczko, Katarzyna Sarad, Witold Kot, Lukasz Krych, Julia Anna Semba, Eric Lars-Helge Lindberg, Jakub Dalibor Rybka declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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Supplementary information
Table S1-S5; Materials and methods

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