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Single-cell RNA sequencing identifies the expression of hemoglobin in chondrocyte cell subpopulations in osteoarthritis

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Abstract

In recent years, chondrocytes have been found to contain hemoglobin, which might be an alternative strategy for adapting to the hypoxic environment, while the potential mechanisms of that is still unclear. Here, we report the expression characteristics and potential associated pathways of hemoglobin in chondrocytes using single-cell RNA sequencing (scRNA-seq). We downloaded data of normal people and patients with osteoarthritis (OA) from the Gene Expression Omnibus (GEO) database and cells are unbiased clustered based on gene expression pattern. We determined the expression levels of hemoglobin in various chondrocyte subpopulations. Meanwhile, we further explored the difference in the enriched signaling pathways and the cell-cell interaction in chondrocytes of the hemoglobin high-expression and low-expression groups. Specifically, we found that SPP1 was closely associated with the expression of hemoglobin in OA progression. Our findings provide new insights into the distribution characteristics of hemoglobin in chondrocytes and provide potential clues to the underlying role of hemoglobin in OA and the mechanisms related to that, providing potential new ideas for the treatment of OA.

Keywords Single-cell sequencing, Hemoglobin, Osteoarthritis, Chondrocyte

Introduction

Mammals are able to meet their body's oxygen requirements through the continuous circulation of blood carrying large amounts of hemoglobin [1]. However, blood vessels are not present in cartilage tissue, and chondrocytes within it must obtain the required oxygen by diffusion through neighboring tissues [2]. Previous studies believe that the amount of oxygen supply within cartilage is limited and chondrocytes produce energy primarily through glycolysis controlled by the hypoxia-inducible factor (HIF) signaling pathway [3, 4].

The hemoglobin belongs to the family of Hb protein that are essential for O_2 transportation in mammals, and the physiological properties of hemoglobin depend on the ordered assembly of its subunits. Electrostatic attraction between the positively charged α-subunit and

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the negatively charged β-subunit encoded by the HBA and HBB genes in hemoglobin promotes the formation of α-β-dimers [5–10]. Myocytes and neurons are known could express large amount of myoglobin and neuroglobin that could bind and store oxygen, allowing for timely and prolonged oxygen delivery during times of high oxygen consumption such as exercise [11–17], the mobility and oxygen-carrying function of myoglobin in muscle was reported in previous studies [18, 19]. It is recently reported that chondrocytes also have a similar strategy to adapt the low-oxygen environment [20], chondrocytes could form a kind of hemoglobin which is membrane-free called "hedy" in their cytoplasm, hedy is similar to the hemoglobin of erythrocytes, allowing the chondrocytes to bind and store oxygen dispersed from the hypoxic environment, and to supply oxygen for a short period of time. Depletion of hemoglobin leads to a lack of hedy, resulting in massive chondrocyte death and delayed skeletal development. However, the expression pattern of hemoglobin in chondrocytes remains unclear.

The heterogeneity and species diversity of chondrocytes have received increasing attention in recent years [21]. Studies have shown that chondrocytes in articular cartilage include a variety of groups [22–26], including proliferative chondrocyte (ProCs), pre-hypertrophic chondrocyte (preHTC), fibrocartilage chondrocyte (FC), homeostatic chondrocyte (HomC), regulatory chondrocyte (RegC), hypertrophic chondrocyte (HTC), senescent cell (SNC), and chondrogenic progenitor cell (CPC). It is found different cell populations have different functions. PreHTCs have the ability to regulate hypertrophic differentiation, whereas HTCs regulate matrix mineralization in cartilage [22, 23]. FCs are associated with the integrity of cartilage extracellular matrix and the process of cartilage fibrosis [27, 28]. SNCs were found to be cell cycle arrested, selective removal of SNCs could attenuate osteoarthritis (OA) progression [25], meanwhile CPCs could self-renew and differentiate to multiple lineages, which helps to promote OA cartilage repair and maintain homeostasis [22]. The expression pattern of hemoglobin in different types of chondrocytes remains unclear.

OPN (osteopontin), a protein encoded by SPP1 gene, is widely distributed in various tissues, it is reported OPN is associated with a variety of biological processes including proliferation, migration and inflammation [29–31]. In previous studies, it is reported that the expression level of OPN is significantly elevated after OA [32, 33]. Recently, it is also observed that the number of chondrocytes in cell populations that highly expressed SPP1 was elevated in OA cartilage, cells with high expression level of SPP1 are more angiogenic and exhibit more features of senescent cells [34]. All of above studies suggest a close relationship between the expression of SPP1 and the happening of OA. However, the potential physiological and pathological effect of SPP1 in the process of OA might remain unknown, more study is needed to further elucidate the specific role of SPP high expressing cells in OA.

In this study, we investigated the expression of hemoglobin in different chondrocyte subpopulations by analyzing a single-cell sequencing database, and compared the differences of hemoglobin expression in OA tissue and normal tissue. We also observed the relationship between the expression level of hemoglobin and SPP1 in different chondrocyte subpopulations. This study tried to further explore the expression characteristics of hemoglobin in OA process and to provide new insights for the treatment of OA.

Methods

Raw data collection

Data from 10× genomic single-cell RNA sequencing (scRNA-seq) of 7 knee cartilage samples were obtained from the GEO (https://www.ncbi.nlm.nih.gov/geo/.) database (Series GSE169454), the sample set consisted of cartilage tissue from 3 control samples from normal knee cartilage tissue (GSM5203389, GSM5203390, GSM5203391) and 4 OA samples from OA knee cartilage tissue (GSM5203392, GSM5203393, GSM5203394, GSM5203395).

Screening and normalization of scRNA-seq data

Using R (v4.3.2, https://www.r-project.org/) and the "Seurat" package [35] (https://cran.r-project.org/web/packages/Seurat/.) to process the single-cell RNA data. We used the "PercentageFeatureSet" function to determine the percentage of mitochondrial genes in each cell, then we set the parameter pattern of the mitochondrial genes as "^MT-". Cells with mitochondrial genes < 15% were included in subsequent analyses and log-normalized, other cells with higher gene percent were excluded. Subsequently, we excluded the genes that expressed in fewer than 10 cells and excluded cells that expressed genes fewer than 200 with the "Subset" function. In addition, we excluded non-cellular and cellular aggregates. Principal Component Analysis (PCA) is used for unsupervised clustering, and the "JackStraw" function is used to determine the number of principal components and visualize them [36–38].

Determination of differential genes expression and cell grouping

After filtering, the gene expression matrix of three control samples and four OA samples were merged separately and the batch effect was excluded using harmony (v1.0, lambda = 1, max.iter.harmony = 20) in the R package. Clustering was made by inter-cluster differential nonlinear dimensionality reduction (t-distributed stochastic neighborhood embedding, t-SNE) and the

"FindAllMarkers" function (minimum % = 0.3, log function threshold=0.25) was used to find marker genes. Distinct cell types were labeled by canonical marker genes such as SOX9, COL2A1, ACAN (chondrocytes), CKS2, HMOX1 (RegC), P3H2, DDX21, UPP1, CDV3, NGF, C3orf52 (ProC), TGFBI, S100A4, THBS3, ADAMTS5, TPPP3 (preHTC), COL1A1, TMSB4X, ID3, PRSS23, OLFML3, LAMA4, HES1, SEPW1 (FC), WWP2, BHLHE41 (HTC), JUN, BRD2, RGS16, CCNL1, SNHG12, DDIT3, TRA2A, RGS2, KMT2E, H2AFX (HomC). The cell clusters were then annotated based on the reported cell-specific marker genes [39–41].

Bioinformatics analysis of HBB + and SPP1 + chondrocytes

Chondrocytes in the HBB high-expressing group were determined with the log2 (FC) expression value of HBB greater than 1, cells in this group were defined as HBB+chondrocytes, and chondrocytes with low HBB expression were defined as HBB- chondrocytes. Similarly, chondrocytes with high expression level of SPP1 were defined as SPP1+chondrocytes, and cells with low expression level of SPP1 were defined as SPP1- chondrocytes. Differential gene expressions (DEGs) of HBB+and HBB- chondrocytes and DEGs of SPP1+and SPP1- chondrocytes were calculated by "FindMarkers" function in Seurat R package, and volcano plots were drawn. Using "FindAllMarkers" function, the gene expression profile in each cell group was provided. "Doheatmap" function was used to plot a heatmap showing the expression level of differential genes between SPP1+and SPP1- chondrocytes and between HBB+and HBB- chondrocytes. The expression of HBA1, HBA2, HIST1H1E1, COCH, DIO3, DDX3Y, OXT, AMTN, DEFB1, FST between HBB+and HBB- chondrocyte groups, and the expression of IBSP, HBA2, HBA1, HBB, CXCL14, COL10A1, RP34Y1, EF1AY, ODX3Y between SPP1+and SPP1- chondrocyte groups were compared and showed in volcano plot, the expression level was also demonstrated by violin plot. DEGs in HBB+and HBB- chondrocytes with $\log_2$ fold change≥1, and DEGs in SPP1+and SPP1- chondrocytes with $\log_2$ fold change≥1 were selected, and were generated for KEGG enrichment analysis using the "clusterProfiler" R package (V.4.2.2) [42, 43].

Cell–cell communication

After identifying chondrocyte subtypes, we analyzed cell-cell communication using the CellChat (V.1.1.3) pipeline [44, 45]. CellChat objects were created from merged Seurat objects. Set the CellChatDB paracrine/autocrine signaling interaction dataset as the reference database. Calculate the probability of communication using a 20% truncated mean (computeCommunProb, set type as "truncatedMean", trim=0.2). Cell-cell communication was inferred by aggregating cell-cell communication networks with default parameters. Using above methods, we visualized the number and strength of cellular interactions among different cell subpopulations and aggregated cell-cell communication networks were shown. The signaling pathway networks in the high and low expression groups were displayed using heatmaps.

Gene set enrichment analysis (GSEA)

Based on the identified DEGs, GSEA analysis was used to compare the gene expression profile between the HBB high-expressing and HBB low-expressing groups respectively. Genes were aligned according to the different expression levels, with a view to seeing whether they were enriched at the top or bottom of the sequence. In this study, GSEA was used to compare the differences in cell signaling pathways between the HBB high-expressing and HBB low-expressing groups.

Animal model

8-week-old C57BL/6 male mice (n=12, specific-pathogen-free level, weighing approximately 20 g) were obtained from the Animal Experimentation Center at the General Hospital of Northern Theater Command. Animal procedures in this study were approved by the Northern Theater General Hospital Animal Medical Research Ethics Committee (approval NO. 2023-49) and conducted according to the Animal Research: Reporting of In Vivo Experiments Guideline [46]. Animals were divided into sham and OA groups. The animals were housed in a 12-h cycle bright/dark environment with adequate feeding and water. OA model was established using the destabilizing of medial meniscus (DMM) procedure [47]. Specifically, anesthesia was applied to the mice using 1% (W/V) pentobarbital (40 mg/kg), opened joint capsule, the medial meniscus tibial ligament was exposed and cut in mice of OA group, while the mice of sham group were only subjected to joint capsule opening. 8 weeks after DMM modeling, mice were anesthetized with a lethal dose of ketamine/xylazine mixture (ketamine: 120 mg/kg, xylazine: 12 mg/kg), the were then decapitated using gentle techniques.

Tissue processing

After mice scarifying, 0.1 M PBS and 4% paraformaldehyde were perfused transcranial. The obtained joint tissues were postfixed in 4% paraformaldehyde and soaked in sucrose solution until sinking, then the tissues were sliced using frozen sectioning machine (Leica, Wetzlar, Germany) along the coronal position, the frozen sections were obtained and placed in -20 °C refrigerator.

Immunostaining and hematoxylin & eosin (HE) staining

Frozen sections were punched with 0.1% Triton X-100 for 5 min, washed, and then incubated with immunostaining

blocking buffer (Beyotime, Shanghai, China) for 1 h. The sections were then incubated overnight with primary antibody SPP1 (1:200, Proteintech). After washing with PBS, the sections were incubated for 1 h at room temperature (37 °C) using anti-rabbit Alexa fluor coupled secondary antibody (Abcam). Finally, nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). HE staining was performed on frozen sections. Images were observed and captured using a fluorescence microscope (Zeiss, Jena, Germany).

Statistical analysis

In this analysis, we used R version 4.3.2. Data were expressed as mean ± standard deviations (mean ± SDs), t-test was used to determine the statistical difference in the standardized expression of genes between groups. Normality of data was tested using the Shapiro-Wilk test. In all two-sided tests, $p \leq 0.05$ was considered a statistically significant difference.

Results

scRNA-seq and cell grouping of samples in control and OA groups

After combining the scRNA-sequencing data from control group and OA group separately, we performed cell cycle effect removal, data normalization, and data downscaling, then we clustered on the data. UMAP plots showed the distribution of diverse undifferentiated clusters in the control and OA groups (Fig. 1A). An overview of cells from different samples in the control group and OA group and the consistency of data are shown in Fig. 1B and C. Among the 17 cell subcategories identified by clustering, clusters 13, 15 in the OA group were identified as hemoglobinocytes based on the marker gene HBA1, HBB and HBA2. Cells in above groups were removed to exclude the interference of erythrocytes in the results. Clusters 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 13 were identified as chondrocytes in control group and clusters 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 were identified as chondrocytes in OA group based on the chondrocyte marker. In control group cluster 12 and 15 were identified as fibroblasts, pericytes and osteoblasts, cluster 16 was identified as low-quality cells and was excluded in further analysis, clusters 14 was identified as immune cells. In OA group, clusters 16 was identified as osteoblast subset and cluster 14 was identified as immune cells subset. The indicated markers including SOX9, COMP, COL2A1, and ACAN for chondrocyte expressed in control and OA tissue are shown in Fig. 1D. Based on above cell annotations, we merged the cell subpopulations and got the chondrocyte group. In addition, to preliminarily observe the distribution of hemoglobin in the samples, we visualized the expression of hemoglobin genes including HBB, HBA1 and HBA2 in control samples and OA samples, which showed that hemoglobin-related genes accounted for a relatively larger proportion in OA samples (Fig. 1E).

Evaluation of hemoglobin genes in the chondrocyte subpopulations

Among the clustered cells, we isolated chondrocyte subpopulations in the control group and OA group, then we repeated the cell undifferentiated clustering analysis according to chondrocyte subsets markers. T-SNE plots showed the distribution of 7 chondrocyte subpopulations in control and OA chondrocytes (Fig. 2A-B). We initially calculated the proportion of each chondrocyte subpopulation in the control and OA samples, the results showed that the RegC subpopulation accounted for a relatively greater proportion in control samples, and FC and preHTC chondrocyte subpopulations accounted for a greater proportion in OA samples (Fig. 2C). Next, we found the expression level of hemoglobin indicated markers including HBB, HBA1, and HBA2 is much higher in OA chondrocytes compared with control chondrocytes. (Fig. 2D-E). We gathered the cells with HBB high expression in OA group and observed the proportion of HBB high-expression chondrocytes in different chondrocyte subpopulations (Fig. 2F-G). We also observed that HBB was highest expressed in ProC, FC and preHTC subgroups (0.44, 0.42, 0.36), the increased proC, FC and preHTC chondrocytes in HBB high-expressing cells suggested that these populations might be critical for hemoglobin expression in OA (Fig. 2H).

Difference in gene expression and biological process between HBB + and HBB- chondrocytes in OA

We performed gene differential analysis of HBB + and HBB- chondrocytes in OA group and identified 5236 differential expression genes (DEGs), of which 2694 genes were up-regulated and 2542 genes were down-regulated in HBB + cells (Fig. 3A). With the absolute value of log2(FC) as the cut-off value of 1.2, we found a total of 639 specifically up-regulated genes and 587 specifically down-regulated genes (Fig. 3B). We identified genes related to the composition of human hemoglobin, ribosome construction and protein synthesis, including HBA1, HBA2, HIST1H1E, which were significantly increased in HBB high-expressing chondrocytes, while genes related to apoptosis and patterns of programmed cell death, ubiquitination of proteins, construction of basement membrane and cell-cell junction, such as FST and AMTN genes, were significantly decreased (Fig. 3C). Afterwards, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the screened DEGs using R package "clusterProfiler", and observed that HIF-1, TNF, mTOR and apoptosis signaling pathways were enriched in HBB high-expressing

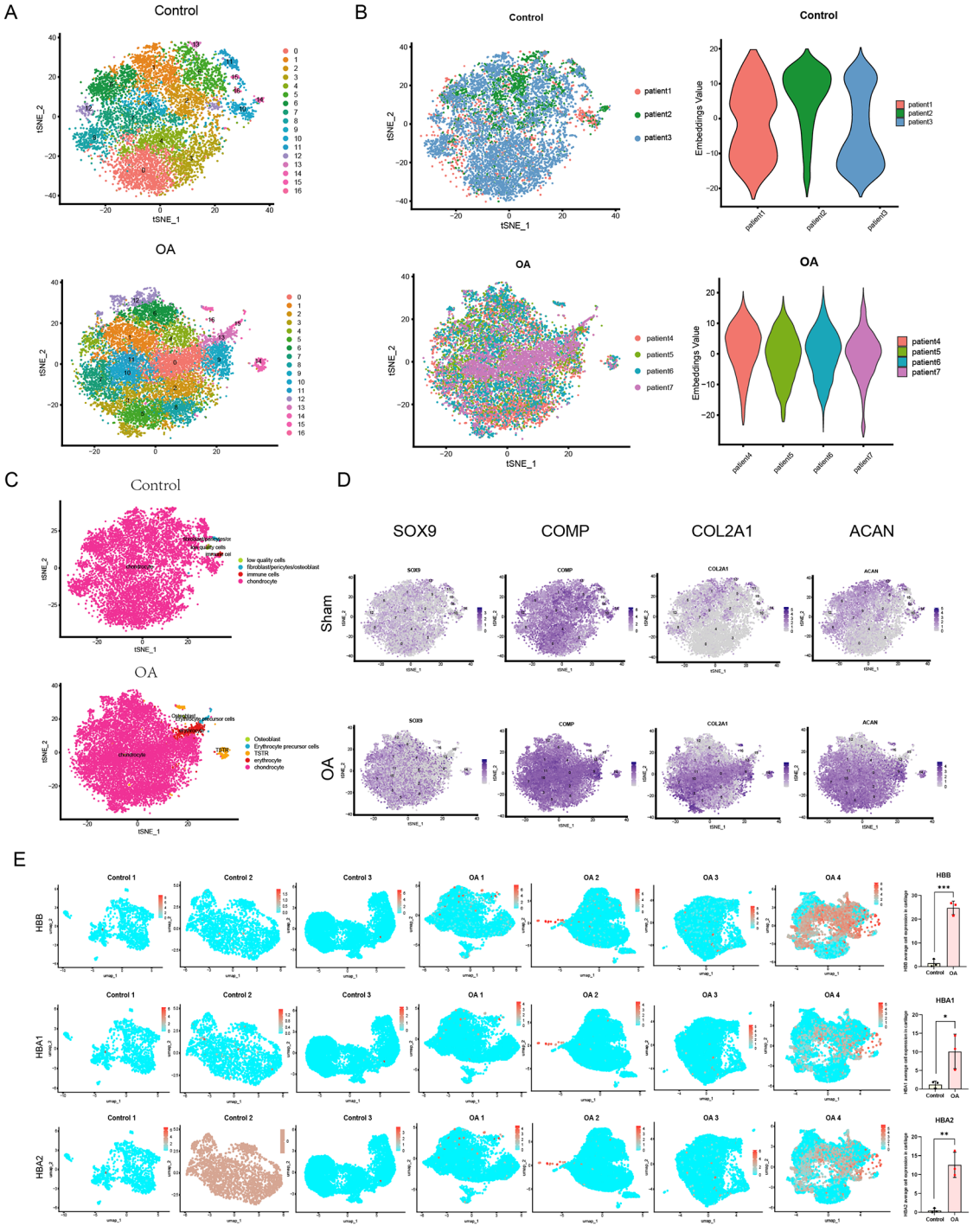

Fig. 1 Single-cell RNA sequencing and identification of cell populations and gene signatures. **A**. The umap of human control and OA group chondrocyte monocyte transcriptome were visualized and colored according to the grouping obtained by undifferentiated cluster analysis. **B**. Distribution of patient samples in chondrocytes in control and OA groups. **C**. Visualization of umap colored according to cell types for human control and OA articular tissue single-cell transcriptomes. **D**. Dot plots showing the expression of indicated markers including SOX9, COMP, COL2A1, and ACAN for chondrocyte in control and OA articular tissue single-cell transcriptomes on the umap map. **E**. Dot plots showing the expression of indicated markers including HBB, HBA1, and HBA2 for hemoglobin in control and OA chondrocytes on umap map. The average cellular expression level of HBB, HBA1, and HBA2 in chondrocytes samples from different group (control, OA) is shown. Data is expressed as mean ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

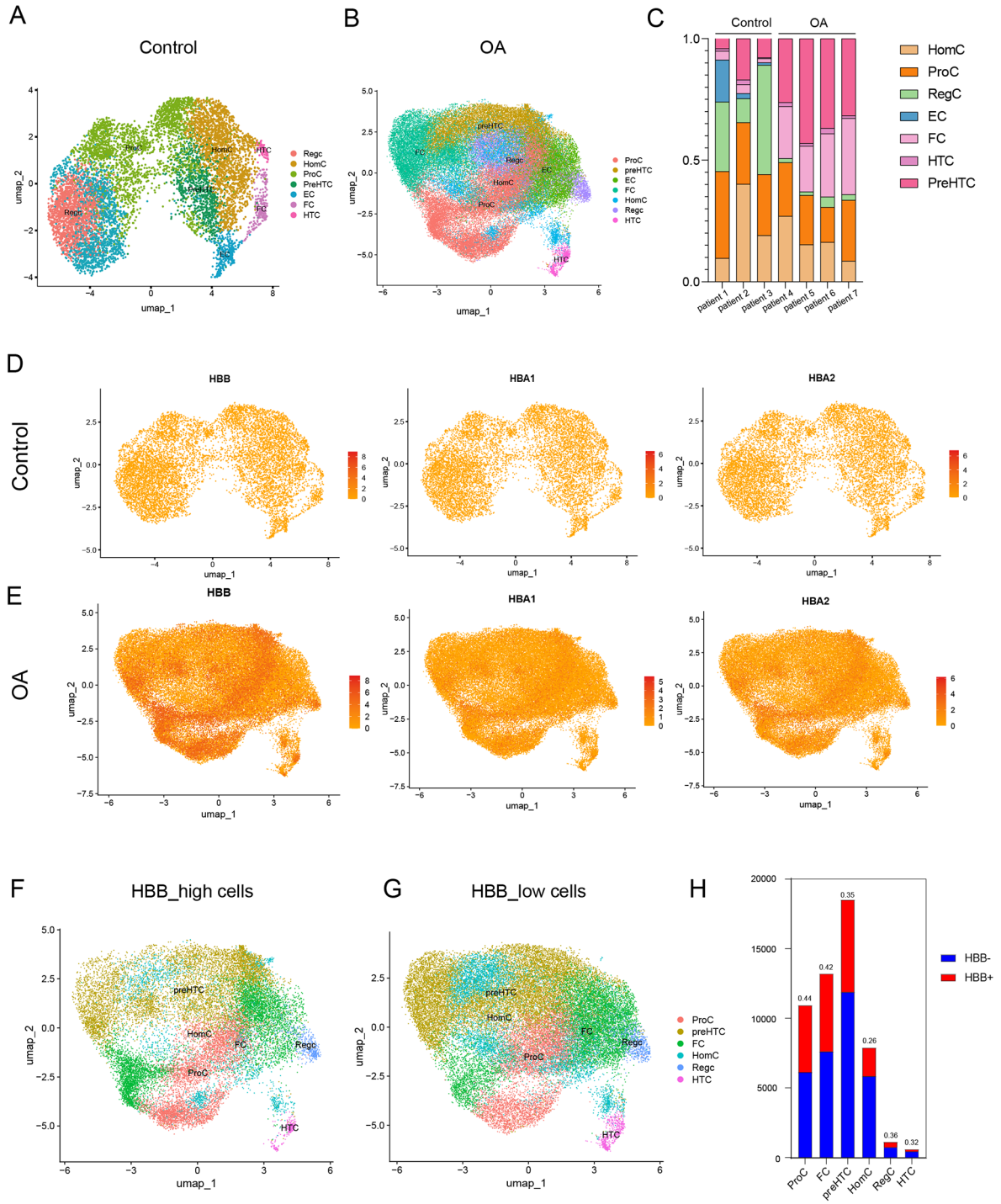

Fig. 2 Subclustering of control and osteoarthritis (OA) group chondrocytes and characteristics of HBB expression in chondrocytes. **A-B**. Subclustering of control and OA chondrocytes further identified 7 distinct subtypes. Color-coded tsne plot is shown and each chondrocyte subcluster is defined on the right. RegC: regulatory chondrocyte, HomC: homeostatic chondrocyte, ProC: proliferative chondrocyte, preHTC: prehypertrophic chondrocyte, EC: effector chondrocyte, FC: fibrocartilage chondrocyte, HTC: hypertrophic chondrocyte. **C**. Cell proportions of chondrocytes subclusters in control and OA tissue. RegC subpopulation accounted for a greater proportion in control samples, and FC and preHTC subpopulations accounted for greater proportion in OA samples. **D-E**. Dot plots showing the expression of indicated markers including HBB, HBA1, and HBA2 for hemoglobin in OA chondrocytes is higher than that in control chondrocytes on the umap map. **F-H.** The proportions of 7 chondrocytes subpopulations in HBB high-expressing and HBB low-expressing cells, and HBB is highest expressed in ProC, FC and preHTC chondrocyte subgroups

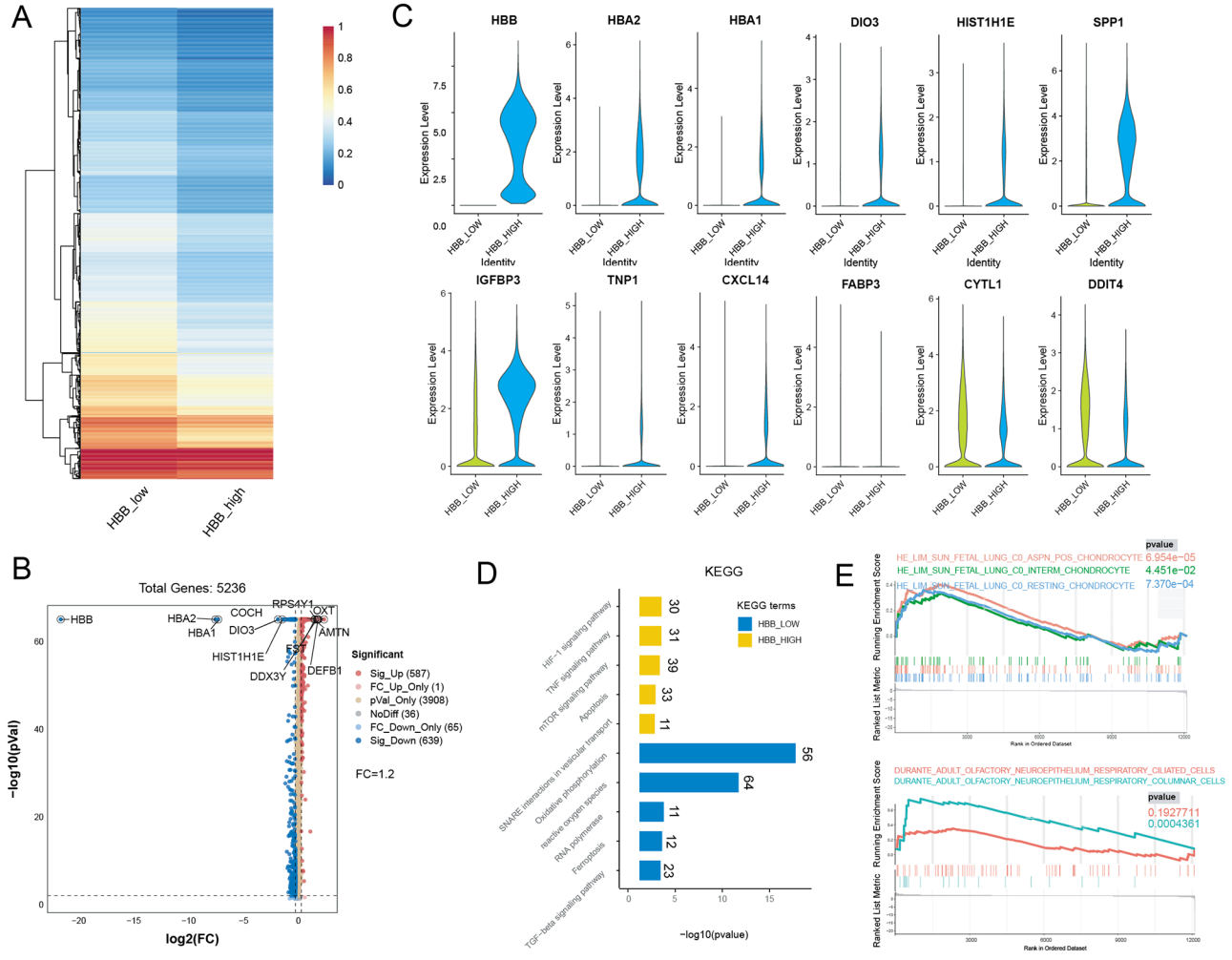

Fig. 3 Characteristics of HBB high-expressing chondrocytes in osteoarthritis (OA) tissue. **A**. Heatmap of differentially expressed genes between HBB low-expressing and HBB high-expressing chondrocytes. **B**. Volcano plot of differentially expressed genes in HBB low-expressing and high-expressing chondrocytes. **C**. Violin plots demonstrating the expression of representative genes in HBB low-expressing and HBB high-expressing chondrocytes chondrocytes. **D**. KEGG biological process enrichment analysis of differentially expressed genes between HBB low-expressing and HBB high-expressing chondrocytes. **E**. GSEA enrichment illustrating the difference of genes expression profile in HBB high-expressing and HBB low-expressing chondrocytes in pathways related with chondrocytes and respiration

cells, whereas pathways such as oxidative phosphorylation, reactive oxygen species, TGF-beta, and ferroptosis signaling pathways were enriched in HBB low-expressing cells, suggesting a significant difference between the physiological functions of HBB high-expressing chondrocytes and HBB low-expressing chondrocytes (Fig. 3D). We used GSEA to evaluate the difference of genes expression profile in HBB high-expressing cells and HBB low-expressing cells in pathways related with chondrocytes and respiration (Fig. 3E).

Potential ligand–receptor interactions analysis in HBB + and HBB- chondrocytes

"CellChat" was used to analyze the number of interactions and the interaction intensity of intercellular communication between chondrocyte subtypes in HBB high-expressing chondrocytes and HBB low-expressing chondrocytes (Fig. 4A, C). It could be observed that HBB high-expressing chondrocytes have a comparable quantity in cellular communication compared to HBB low-expressing chondrocytes, while with a slight decrease in cellular communication intensity. Also, we observed the major outgoing signaling pathways and major receptive signaling pathways of each chondrocyte subpopulation in the above two cell groups (Fig. 4B, D). Collagen was the highest cell-interacting pathway in both populations, however, the intensity of the expression of SPP1 pathway was notably higher in the HBB high-expressing cell population than in the HBB low-expressing group. Given that the ProC chondrocyte subpopulation and the FC chondrocyte subpopulation contain the largest proportion of HBB + cells, we analyzed the major interaction

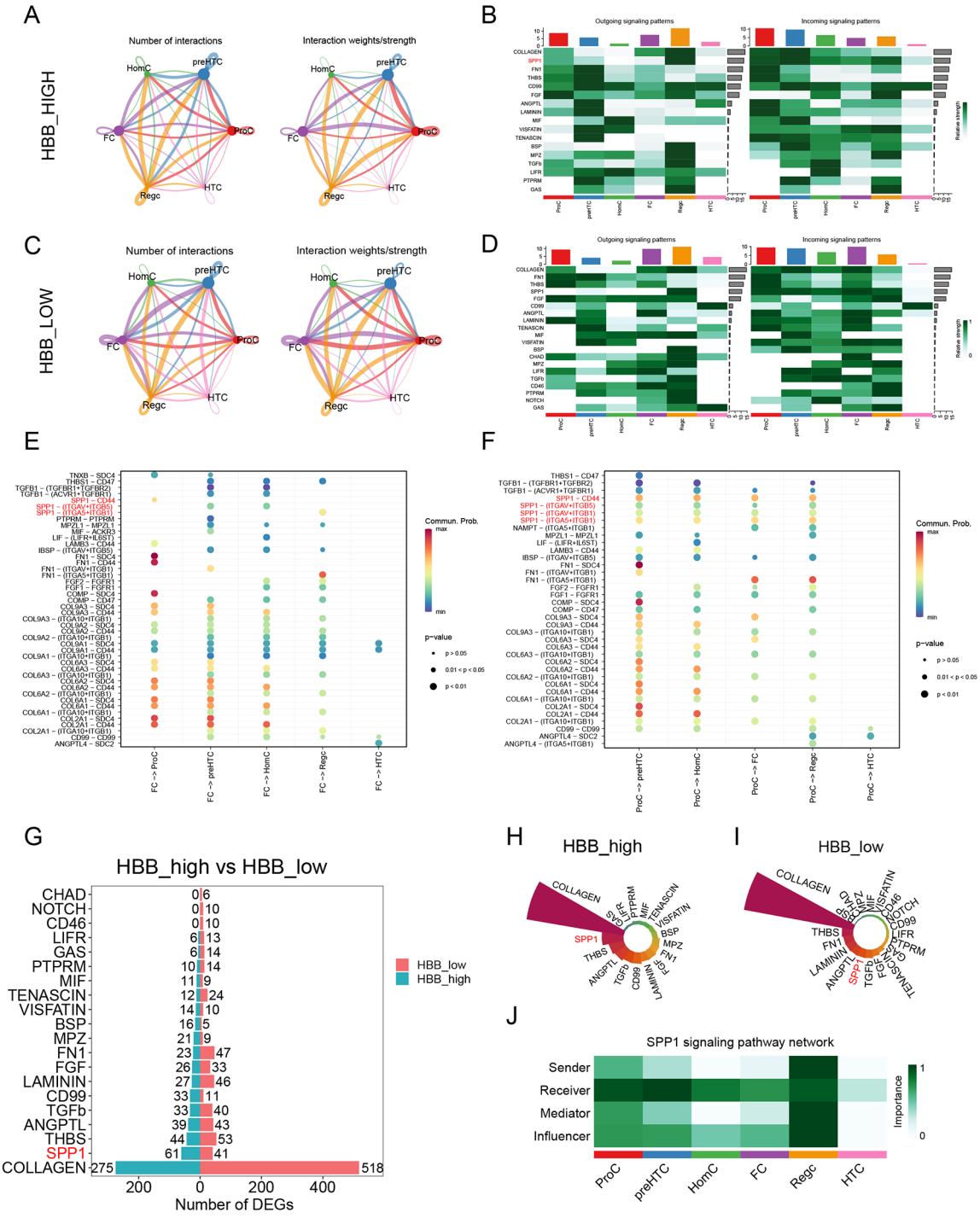

Fig. 4 Potential ligand–receptor interactions analysis in HBB high-expressing and HBB low-expressing chondrocytes. **A-D.** The quantity and intensity of interaction between different chondrocytes subpopulations and high outgoing and incoming signaling pattern in different chondrocytes subpopulations in HBB high-expressing chondrocytes (**A-B**) and HBB low-expressing (**C-D**) chondrocytes. **E-F.** The ligand–receptor pairs exhibit the major interaction pathways between FC (E) or ProC (F) chondrocyte subpopulation and any one of the chondrocyte subpopulations in HBB high-expressing chondrocytes. **G-I.** The expression of representative signaling pathways in HBB low-expressing and HBB high-expressing chondrocytes. The number of SPP1 associated differentially expressed genes (DEGs) is high HBB high-expressing chondrocytes (H) and HBB low-expressing chondrocytes (I), and in the comparison of HBB high-expressing and HBB low-expressing chondrocytes (G). **J.** Heatmap illustrating the roles of different cell subpopulations in the expression of the SPP1 associated pathway in HBB high-expressing chondrocytes, and SPP1 associated signals were mainly generated by RegC and received by all other kind of cell subpopulation

pathways between them and the other chondrocyte subtypes in HBB+cell population. We observed that the SPP1-associated pathway showed high expression intensity and significance in both two cell groups (Fig. 4E-F). In addition, we compared the differences in the major expressed signaling pathways in the HBB high expression chondrocyte and HBB low expression chondrocyte groups and observed that the SPP1 related pathway was highly expressed and much more abundant in the HBB high-expressing cells compared to the HBB low-expressing cells (Fig. 4G-I). Finally, we further explored the role played by different cell subpopulations in the expression of the SPP1 associated pathway, and observed that SPP1 associated signals were mainly generated by RegC and received by all other kind of cell subpopulations, including preHTC and ProC. (Fig. 4J)

Distribution and potential role of SPP1 + cells in OA

After observed that the high SPP1 expressing cells account for a large part in the HBB+cell population, we further investigated the characteristics of the SPP1 high-expressing cell population to find if this cell population has special effect in OA process. First, we observed the distribution of SPP1 high-expressing cells, it is showed that SPP1 high-expressing cells were mainly distributed in the OA samples (Fig. 5A). In addition, we observed the proportion of SPP1 high-expressing cells in different chondrocyte subpopulations, we observed that the proportion of SPP1+cells in RegC, ProC, and preHTC was the highest (0.78, 0.44, and 0.43) in the OA group (Fig. 5B). By analyzing the DEGs between SPP1- and SPP+cells in OA group, we observed that the hemoglobin-related genes, including HBB, HBA1, and HBA2, were the genes with the most pronounced differences (Fig. 5C-D), and we also observed that the SPP1+cells were mainly expressed in cells that highly expressed with HBB (Fig. 5E). Also, we observed that mTOR-related "mTOR signaling pathway" and "insulin signaling pathway" were enriched in SPP1 high-expressing cells (Fig. 5F), suggesting that SPP1 might exert its influence on HBB+cells by mediating mTOR-related signaling to modify the energy and oxygen redistribution. In addition, we observed that the expression level of mTOR pathway-associated molecules such as MTOR, IGFBP1 and EIF4EBP2 were significantly higher in the SPP1 high-expressing cell population (Fig. 5G). Finally, we observed the distribution of hemoglobin and SPP1 mice of sham group and OA group. Safranin O staining was used to assess the extent of cartilage damage and to validate the successful establishment of the OA model (Supplementary material 1, Figure S1). It is observed that the amount of hemoglobin distributed in articular cartilage in OA group is significantly higher than that in sham group (Fig. 5H, J). SPP1 is mainly distributed in the deep layer of articular cartilage, immunofluorescence staining illustrated that the amount of SPP+cells is significant higher in OA tissue (Fig. 5I, K).

Discussion

With the aging of society, increased economic burden, and growing population of obese people, there is an urgent need for more new strategies for the early diagnosis and treatment of OA in order to reduce the number of joint replacement surgeries [48–50]. Recently hemoglobin was found in articular cartilage, and was believed to be related with hypoxic adaptation of chondrocytes [20]. However, a detailed understanding of the expression of hemoglobin in OA arthritis is urgently needed. Here, we investigated hemoglobin in chondrocytes in normal people and patients with OA at single-cell resolution using comprehensive gene expression profiling. We identified the expression difference of hemoglobin in normal and OA articular tissue. We also verified hemoglobin expression level in different chondrocyte subpopulations, and compared the DEGs and cell-cell interactions between HBB high-expression chondrocytes and HBB low-expression chondrocytes. Notably, we identified ProC, FC and PreHTC as the chondrocytes subpopulations that express the most hemoglobin in OA based on scRNA-seq analysis. We found oxidative phosphorylation pathway was the most prominent expression pathway in HBB-low expression cell population, and HIF-1α pathway and mTOR pathway were the top-ranking expression signaling pathways in HBB-high expression cell population. Importantly, we identified SPP1 as a potential gene that is specifically expressed in HBB high-expressed cells and intimately involved in the cell-cell interaction in HBB high-expressing population. In this study, we tried to explore the expression characteristics of hemoglobin in chondrocytes and provide new insights for the diagnosis and therapeutic treatment of human OA.

As a central pivot of cellular metabolism, the oxidative phosphorylation is source of ATP production, and could generate intermediate metabolites and precursor structures for biosynthetic pathways [51]. The pathological process of OA is associated with altered metabolic pathways. glycolysis, cholesterol metabolism, and various other metabolic pathways have altered expression of regulatory enzymes and generate interaction with each other [52]. Under pro-inflammatory conditions, cells generally switch between oxidative phosphorylation and glycolysis in order to meet energy requirement [53, 54], and chondrocytes in the pro-inflammatory microenvironment are enhanced for anaerobic glycolytic processes [55]. In this study, the result of KEGG showed the pathway of oxidative phosphorylation was prominently expressed in the HBB low-expressive cell population, for chondrocytes with high HBB expressing, the HIF-1α pathway which

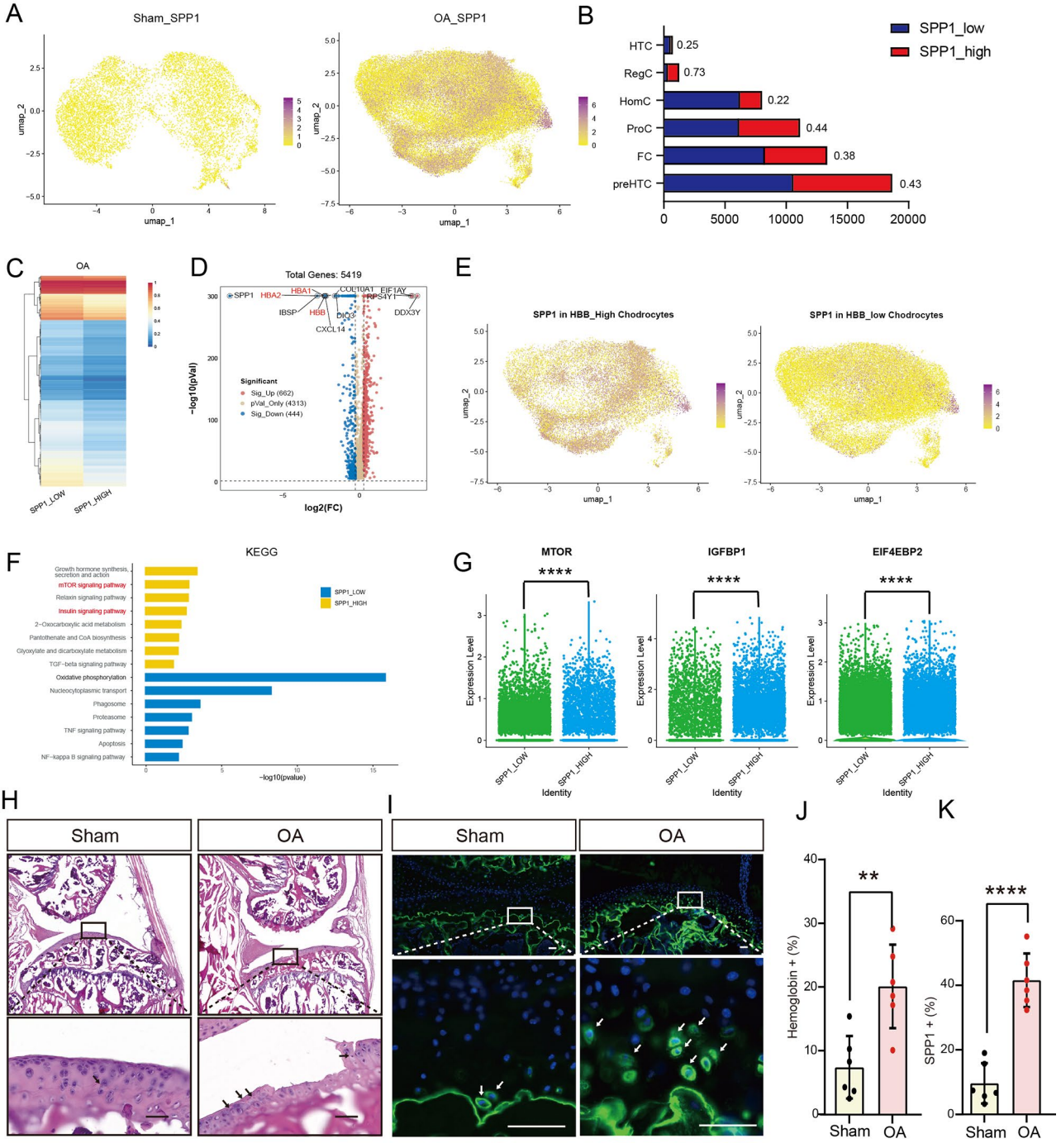

Fig. 5 Characteristics of SPP1 high-expressing chondrocytes in control and osteoarthritis (OA) tissue. **(A)** The expressing pattern of SPP1 in chondrocytes in control and OA tissue. Dot plots showing the expression of SPP1 in OA chondrocytes is higher than that in control chondrocytes on the tsne map. **(B)** The proportions of SPP1 high-expressing cells in 7 subpopulations in OA tissue, and HBB is highest expressed in ProC, FC and preHTC chondrocyte subgroups. **C-D.** Heatmap and volcano plot of differentially expressed genes between SPP1 low-expressing and SPP1 high-expressing chondrocytes. **E.** Dot plots showing the expression of SPP1 in HBB high-expression chondrocytes is higher than that in HBB low-expressing chondrocytes on the tsne map. **F.** KEGG biological process enrichment analysis between SPP1 low-expressing and SPP1 high-expressing chondrocytes, and mTOR signaling pathway and insulin signaling pathway are enriched in SPP1 high-expressing chondrocytes. **G.** The expression level of representative genes including MTOR, IGFBP1 and EIF4EBP2 of mTOR pathway is significantly higher in SPP1 high-expressing chondrocytes compared with SPP1 low-expressing chondrocytes. **H**, **J.** The percent of hemoglobin positive chondrocytes is higher in OA chondrocytes compared with control chondrocytes. **I**, **K.** The percent of SPP1 positive chondrocytes is higher in OA chondrocytes compared with control chondrocytes. Data is expressed as mean ± SD, $^{**}p < 0.01$, $^{****}p < 0.0001$. Scale bar: 50 μm

associated with glycolytic process is the most remarkable pathway. Results of our study and previous studies indicate that the HBB high-expressing cells in OA might have a low oxygen consume themselves by enhancing their anaerobic respiration level. Reversely, HBB low-expressing cells could get enough O_2 and have a normal oxygen support in this way.

Rapamycin (TOR) is a critical pivot that targets the signaling cascade pathway responsible for regulating cell growth and metabolic nutrient sensing in cells [56]. As the target of TOR effect, mTOR consists of two complexes, mTORC1 and mTORC2, which had different biological functions. mTORC1 inhibits autophagy, and it is a downstream target of the PI3K/Akt pathway [57]. Previous study showed that IL-1β inhibited the cell cycle and proliferation rate of rat chondrocytes and reduced autophagy by intervening PI3K/Akt/mTOR pathway [58]. Inhibition of the PI3K/AKT/mTOR signaling pathway could promote chondrocyte autophagy and attenuates the inflammatory response in rats with OA [58]. Upregulation of mTOR in OA cartilage was accompanied by an increase in chondrocyte apoptosis and a decrease in the expression of key autophagy genes during OA [59]. In addition, it is also reported that the modulation of the ULK1/AMPK pathway by mTOR may be partially responsible for the regulation of autophagy signaling and the balance between catabolic and anabolic factors in articular cartilage [59]. Epigenetic regulation targeting the mTOR signaling pathway has been shown to regulate chondrocyte viability, autophagy, and apoptosis [60]. In this study, using KEGG analysis, we observed that the mTOR pathway is specifically high expressed in the HBB high-expressing chondrocytes and SPP1 high-expressing chondrocytes. In SPP high-expressing chondrocytes, the expression of MTOR and IGFBP1, which are mTOR pathway related genes, are significantly high expressed. Above results indicate that the modification of mTOR pathway in HBB high-expressing chondrocytes might be associated with the balance of cell proliferation and autophagy in OA, which could potentially regulate the oxidative and energy metabolic processes in chondrocytes.

SPP1 codes for the expression of the protein OPN (osteopontin), which is widely distributed in a variety of tissues, and is associated with multiple biological functions including migration, proliferation, and inflammation [30]. OPN has been previously reported to be associated with osteogenic and chondrogenic processes, and could play an important role in the development of OA [61, 62]. OPN was previously found to be highly expressed in OA cartilage, but its function and action mode in the OA process are still unclear [32, 33]. Studies have shown that SPP1 is highly expressed in the subchondral bone and synovium of people with OA [63]. Injection of OPN could alleviate the progression of OA in mice with DMM model through PI3K signaling pathway [64]. Recent studies have shown that OPN inhibits autophagy in OA through CD44/MAPK pathway [65]. In addition, a recent study conducted by Qu et al. [34] demonstrated that the number of SPP1+chondrocytes increased with the degree of OA, and that chondrocytes with high expression of SPP1 had greater angiogenic capacity and senescence characteristics, and were involved in the transition between chondrocyte subpopulations. Besides, previous studies have initially explored the relationship between SPP1 and hemoglobin. Study by Kang et al. found that in erythrocytes SPP1 helps to increase erythrocyte pressure volume and hemoglobin levels, and that OPN mediates the phosphorylation and activation of a number of proteins in erythrocytes [66]. Aprile et al. also found that SPP1 may regulate haematopoietic stem cell function through interactions with parathyroid hormone, and that supplementation of SPP1 levels would produce activation of PTH signalling with rescue of haematopoietic stem cell function [67]. However, we are afraid that we have found few studies that made a further in-depth exploration of the relationship between the two molecules in OA. Considering that both of them play an important role in OA, we speculate that there may be some correlation between the roles played by the two, which might need more investigation in the future study. Our study found that expression of SPP1 gene was different between different cell populations after OA, and was consistent with the cell profile expression of hemoglobin. KEGG analysis suggests that SPP1 high-expressing cells may be involved in energy and anabolic-catabolic metabolism after OA. Previous studies and our results suggest that SPP1 high-expressing chondrocytes may play an important role in the development of OA, and further exploration of this type of cells could be a target to reveal new mechanisms in OA development.

The result in this study showed that the cell populations of ProC, preHTC and FC significantly expressed more hemoglobin than other populations. It has been previously reported that ProC is mostly distributed in the proliferative region of cartilage, which is abundant and near the start of trajectory [21]. The large number of cells with high expression of haemoglobin contained in ProC may provide the needed oxygen to other dividing cells in time during the proliferation process to ensure that normal proliferation proceeds in a hypoxic state. Both preHTC and FC are cell populations expressed in the late OA stage, and pairwise correlation analyse indicate a strong link between the two cell populations [21]. PreHTC uniquely expresses a gene signature that related with bioconjugation and multicellular bioprocesses [21], and FC is a population of cells in cartilage that thought to contribute to the pathophysiology of OA through cellular molecular processes that disturb the integrity of

extracellular matrix and enhance cartilaginous fibrosis [27, 28]. The higher proportion of hemoglobin-overexpressing cells contained in preHTC and FC might provide oxygen for the massive synthesis of matrix in late OA, safeguarding the energetic vital activity of other matrix protein-synthesizing chondrocytes. In summary, through the phenomenon of high hemoglobin expression in ProC, preHTC and FC cells, we speculate that hemoglobin-overexpressing cells in OA may be mainly involved in the process of cell proliferation and matrix protein synthesis, which guarantees the normal conduct of life activities by lowering the level of its own oxidative phosphorylation and providing oxygen to the cells that have an exuberant proliferative or synthetic process.

Conclusion

In conclusion, our single-cell analysis of normal and OA cartilage tissues allowed the characterization of hemoglobin distribution in OA chondrocyte populations at both single-cell resolution and transcriptome-wide scales. Our analysis identified the expression characteristics, intercellular interactions, highly enriched pathways and potential key markers of hemoglobin-overexpressing chondrocytes, and preliminarily speculated on the potential correlation between high-expressing hemoglobin and cellular energy metabolism. The present study opens up new ideas and possibilities for diagnostic and therapeutic strategies in OA.

Supplementary Information

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Supplementary Material 1

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Not applicable.

Author contributions
Z.Z., H.W., and H.Y. conceived the idea of manuscript, Z.Z. and T.H. designed the study. H.G., Y.Z., and Y.H. performed the in vitro experiments. T.H., S.T., K.H., and Z.Z. performed the in vivo experiment. Z.Z., H.G. and T.H. wrote the manuscript. H.W., and H.Y. review the manuscript and provided guidance for the project.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Animal procedures in this study were approved by the Northern Theater General Hospital Animal Medical Research Ethics Committee, and conducted according to the fundamental principles of the Basel Declaration and the ethical guidelines of the International Council for Laboratory Animal Science (ICLAS).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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