



# Single-cell transcriptome profiling reveals the heterogeneity of ossification of the posterior longitudinal ligament (OPLL) and its immune microenvironment

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## Abstract

**Background** Ossification of the posterior longitudinal ligament (OPLL) is a heterotopic ossification process of the posterior longitudinal ligament (PLL) that can compress the spinal cord and nerve roots, yet its cellular heterogeneity and pathogenesis remain unclear.

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**Methods** We performed single-cell RNA sequencing (scRNA-seq) with integrative computational analyses and histological validation on 4,683 cells from surgically resected cervical OPLL lesions obtained from human patients.

**Results** We identified 15 major cell subsets, including chondrocyte-lineage populations-progenitor/proliferative fibrocartilage chondrocytes (ProFCs), pre-hypertrophic chondrocytes (preHTCs), and hypertrophic chondrocytes (HTCs)-as well as osteoblasts, endothelial cells, and diverse immune cells. Pseudotime analysis suggested an inferred trajectory from cartilage progenitor-like cells through inflammatory/reactive and hypertrophic states toward

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osteogenic programs, with progressive activation of ossification, extracellular-matrix, and inflammation-related pathways. Cell–cell communication analyses showed coordinated immune–stromal crosstalk, with FGF signaling enriched in earlier pseudotime states and SPP1-CD44 signaling enriched in later states. SPP1 signals originated predominantly from HTC, whereas macrophages were prominently involved in the broader inflammatory niche and immune-stromal communication within OPLL lesions. Consistently, SPP1/CD44-positive cells were enriched in OPLL tissues and further increased by IL-1 $\beta$  stimulation in vitro and in an Enpp1-driven OPLL mouse model.

**Conclusions** This exploratory single-cell atlas suggests that an inflammatory niche may couple chondrocyte hypertrophy with osteogenic activation and highlights candidate immune-stromal pathways for future biomarker and therapeutic exploration in OPLL.

## Highlights

- Single-cell profiling resolved stromal and immune heterogeneity in human OPLL.
- Chondrocyte-lineage cells showed staged progression toward hypertrophic and osteogenic states.
- Immune-stromal communication featured early FGF and late SPP1-CD44 signaling modules.
- SPP1/CD44 activation was supported by tissue, in vitro, and mouse-model validation.

**Keywords** OPLL · Single-cell RNA sequencing · Immune microenvironment · Osteogenic differentiation · SPP1-CD44 axis

## Introduction

Ossification of the posterior longitudinal ligament (OPLL) is a common chronic condition that most frequently affects the cervical spine, particularly the mid-cervical levels (C4–C6). Epidemiological studies report a prevalence of approximately 0.4%–9% in East Asian populations, with a male predominance (about 2–3:1) (Ito et al. 2024; Moghaddamjou and Fehlings 2019; Alaa et al. 2025). The heterotopic ossification (HO) proceeds slowly, and the incidence increases with age. Most patients develop myelopathy

when the spinal cord is severely compressed (He et al. 2023a). Despite the increasing number of reports on OPLL, effective measures to alter early diagnosis and outcome are still lacking, conservative treatment of patients often has unsatisfactory efficacy, and surgical treatment is generally required in the late stage. However, the operation of OPLL is complex and extremely high risk, mainly due to its unique anatomical and pathophysiological factors (Park et al. 2020; Liu et al. 2024a). It is therefore crucial to better understand its pathophysiology and the underlying mechanisms of action of various cell subsets in the processes leading to OPLL.

Early clinical and epidemiological studies have shown that the underlying causes of OPLL disease are multifactorial; its pathogenesis involves both familial inheritance (genetic factors) and non-genetic factors. Genetic factors include familial inheritance and specific susceptibility genes, such as those encoding RUNX2, BMP2, COL6A1, ENPP1, and IFN, etc. (Yan et al. 2013; Wang et al. 2020; Kato et al. 2023; Liu et al. 2024b). Non-genetic factors include age, BMI, hyperlipidemia, obesity, diabetes, diet, and mechanical strain on the posterior longitudinal ligament (PLL) (Fujita et al. 2023; Oshima et al. 2020; Koike et al. 2023; Takahata et al. 2025). Over the past decades, various genetic investigations, including pedigree studies, twin studies, and detailed molecular analysis, have documented many genes or loci related to the molecular and genetic pathobiology regulating OPLL. GWAS studies have also identified CCDC91 and RSPO2 as susceptibility genes (Nakajima et al. 2016, 2023). In addition to genetic factors, systemic hormones and growth factors, such as IL-1 $\beta$ , IL-6, LepR, and TGF- $\beta$ , are believed to play roles in OPLL development (Saito et al. 2023; Chen et al. 2018; Fay et al. 2023). The pathological mechanism of OPLL involves endochondral ossification, followed by vascular invasion, which promotes osteogenesis. Connective tissue ossification also occurs, characterized by fibroblast proliferation and vascular invasion throughout the degenerated ligament, ultimately leading to ectopic ossification (Sakai et al. 2022). Despite these findings, the composition of cell types in the PLL, the biochemical markers that can effectively predict OPLL, and the cellular heterogeneity that leads to the progression of OPLL remain unclear.

Emerging evidence suggests that cellular heterogeneity within the PLL microenvironment plays a

critical role in OPLL pathogenesis, yet the composition of these cell types, their transcriptional states, and their contributions to disease progression remain poorly characterized (Tian et al. 2019; Huang et al. 2025). Here, we applied single-cell RNA sequencing (scRNA-seq) to surgically resected OPLL lesions to (i) define stromal and immune cell landscapes, (ii) infer chondro-osteogenic progression states and regulators, and (iii) map immune–stromal communication circuits that may drive ectopic ossification.

## Results

### scRNA-seq reveals the cellular landscape of human OPLL lesions

A total of 4,683 high-quality cells from two human OPLL samples were included in the final single-cell dataset. Clustering analysis of the integrated dataset at a resolution of 0.6 identified 18 transcriptionally distinct clusters (Fig. 1a–b; sFig. 1c). After integration, major clusters were observed in both samples without overt sample-restricted major populations (Fig. 1c). Based on canonical marker genes and reference atlases, transcriptionally related clusters with shared cell identities were consolidated into 15 annotated cell populations (Fig. 1d, e). These populations included progenitor/proliferative fibrocartilage chondrocytes (ProFCs), pre-hypertrophic chondrocytes (preHTCs), hypertrophic chondrocytes (HTCs), osteoblasts, endothelial cells, multiple immune subsets (T cells, B cells, macrophages, mast cells, neutrophils, myelocytes, and promyelocytes), stem/stromal cells, and a small “others” group. Representative marker expression patterns supporting cell-type annotation are shown in Fig. 1e, h, and Supplementary Table S3.

We next examined the distribution of each cell population across samples. Overall, OPLL1 and OPLL2 exhibited broadly similar cellular landscapes, with preHTCs representing the largest population in both samples (Fig. 1f, g). The unequal cell numbers between OPLL1 and OPLL2 likely reflect differences in tissue quality, dissociation efficiency, and cell survival during sample preparation. Despite this imbalance, the major cell populations were detected in both samples after integration. Beyond the dominant preHTC compartment, several stromal and immune populations showed modest proportional

differences between OPLL1 and OPLL2, indicating limited inter-sample heterogeneity. To corroborate the single-cell findings at the tissue level, we performed histological and immunofluorescence analyses on PLL sections. Hematoxylin and eosin (H&E) staining revealed marked structural differences between OPLL lesions and non-OPLL controls from cervical spondylotic myelopathy (CSM) patients. Compared with the uniformly aligned fibrous architecture in CSM specimens, OPLL tissues showed disrupted ligament organization, fibrocartilage-like changes, and ectopic ossified regions (Fig. 1i). Consistently, immunofluorescence staining demonstrated increased CD31 and RUNX2 signals in OPLL tissues compared with CSM controls (Fig. 1j, k).

### Chondrocyte heterogeneity and chondro-osteogenic transition in OPLL

Chondrocyte-lineage cells and extracellular matrix (ECM) remodeling are important features of OPLL progression. To delineate chondrocyte heterogeneity, we reanalyzed the chondrocyte-lineage populations identified in Fig. 1, including ProFCs, preHTCs, and HTCs. Unsupervised subclustering identified seven transcriptionally distinct chondrocyte subpopulations (Fig. 2a; Supplementary Table S3), including MSMP<sup>+</sup>CHI3L1<sup>+</sup> chondrocytes, HTCs, homeostatic chondrocytes (HomCs), cartilage progenitor-like cells (CPCs), undecided chondrocytes (uChond), reactive chondrocytes A (rChond A), and reactive chondrocytes B (rChond B). MSMP<sup>+</sup>CHI3L1<sup>+</sup> chondrocytes were characterized by high MSMP and CHI3L1 expression, consistent with an inflammation-associated and matrix-remodeling state. HTCs exhibited a hypertrophic transcriptional program, characterized by elevated expression of COL10A1, IBSP, and SPP1. HomCs expressed FOS, JUN, and SNHG12 and exhibited a relatively balanced, non-hypertrophic transcriptional profile. CPCs were enriched for ASPN and PRG4, consistent with a cartilage progenitor-like phenotype. uChond lacked strong enrichment of the principal marker genes highlighted in Fig. 2b–e and did not display a clear subtype-defining transcriptional signature, suggesting an intermediate or transitional state. Two reactive chondrocyte subsets were also identified: rChond A, marked by S100A8/S100A9, and rChond B, enriched for immune-associated genes including PTPRC, IL7R, and TNFAIP3





**Fig. 1** scRNA-seq delineates the heterogeneity of cell subpopulations in OPLL. **(a)** Workflow of the present study, including single-cell dissociation, library construction, RNA sequencing, and downstream bioinformatics analysis. **(b)** Clustering tree showing the complete scRNA-seq map at various resolutions, with an arrow indicating the selected resolution. **(c)** t-SNE plot of cells derived from OPLL tissues. **(d)** t-SNE plot showing the major annotated clusters at a resolution of 0.6. **(e)** Dot plot showing the top 3 marker genes for the annotated cell types. **(f)** The proportions of each cluster across the samples are depicted. **(g)** Balloon plot with corresponding cell counts showing the distribution of each annotated cluster across the two OPLL samples. **(h)** Violin plots showing the expression of representative marker genes across the annotated cell clusters. **(i)** Representative H&E staining images of surgically removed PLL tissues from patients with CSM and OPLL. The osteogenic region in the OPLL tissue is indicated by dashed lines. Scale bar, 100  $\mu$ m. **(j)** Representative immunofluorescence staining of RUNX2 (red), CD31 (green), and DAPI (blue) in CSM and OPLL tissues. Scale bars, 100  $\mu$ m in the overview images and 50  $\mu$ m in the enlarged images on the right. **(k)** Quantitative analysis of the relative fluorescence intensities of CD31 and RUNX2 in CSM and OPLL tissues. OB indicates ossified bone. Data are presented as mean  $\pm$  SD. \*\* $p < 0.01$ , \*\*\* $p < 0.001$

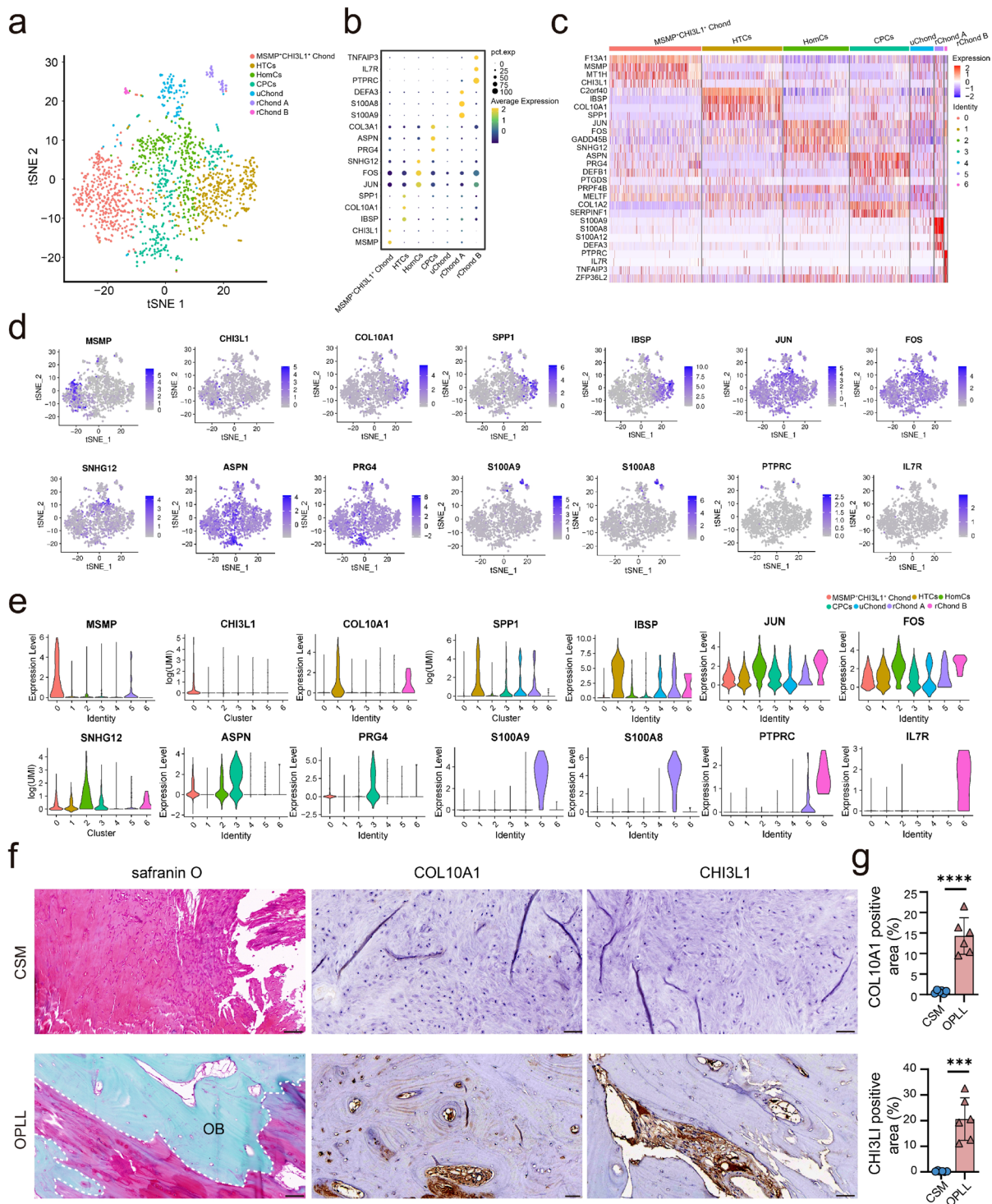
(Fig. 2b–e). Notably, rChond B retained chondrocyte-associated features while expressing immune-response genes, consistent with an inflammation-activated reactive state rather than a canonical immune lineage.

Histological validation further supported these findings. Safranin O/Fast Green staining showed increased Safranin O-positive cartilage-like matrix deposition and expanded fibrocartilage-like regions in OPLL tissues compared with CSM controls. In parallel, immunohistochemistry and quantitative analysis demonstrated increased COL10A1 and CHI3L1 positivity in cartilage and ossification regions of OPLL specimens (Fig. 2f, g). Collectively, these data reveal pronounced chondrocyte heterogeneity in OPLL, encompassing progenitor-like, homeostatic, hypertrophic, undecided, and reactive/inflammation-associated states during ectopic ossification.

In OPLL lesions, stromal subpopulations exhibited distinct functional enrichment patterns. GO enrichment analysis showed that osteoblasts were enriched in ossification- and osteoblast differentiation-related terms. In contrast, ProFCs, preHTCs, and HTCs were predominantly associated with extracellular matrix (ECM) organization,

extracellular structure organization, and related matrix-remodeling processes (Fig. 3a). Notably, compared with ProFCs and preHTCs, HTCs showed enrichment for bone mineralization- and ossification-related terms, consistent with a more advanced chondro-osteogenic transitional state. KEGG analysis further showed that the chondrocyte-lineage subsets (ProFCs, preHTCs, and HTCs) were commonly enriched for PI3K-Akt signaling, focal adhesion, ECM-receptor interaction, and proteoglycans in cancer (Fig. 3b–d). In contrast, osteoblasts exhibited broader pathway enrichment, including PI3K-Akt, MAPK, TNF, IL-17, TGF- $\beta$ , and NF- $\kappa$ B signaling pathways (Fig. 3e). Consistent with these enrichment patterns, protein–protein interaction (PPI) analysis identified an SPP1-centered interaction module associated with ECM remodeling, cartilage maturation, and endochondral ossification-related processes (Fig. 3f). In this network, each node represents a protein, and the colored sectors within nodes indicate the enriched biological processes or pathways annotated for that protein. Edges represent predicted protein–protein associations derived from the STRING database, and edge colors denote different evidence types supporting these interactions. Collectively, these results delineate stage-specific ECM-remodeling, inflammatory, and osteogenic programs across OPLL stromal subpopulations and position HTCs at the chondro-osteogenic interface.

HTCs and osteoblasts formed two clearly separated clusters in the tSNE projection, indicating distinct transcriptional states between these two cell populations (Fig. 4a). SCENIC analysis further revealed divergent transcriptional regulatory programs between HTCs and osteoblasts in OPLL lesions. In HTCs, regulons centered on CEBPG, FOXA2, TFF3, KDM4B, and CREB3 were preferentially activated, indicating an HTC-specific transcriptional regulatory program (Fig. 4b, d). In contrast, osteoblasts displayed preferential activation of osteogenesis-associated regulons, including DLX5, DLX6, PRRX1, STAT2, and EBF1 (Fig. 4c, d). DLX5 and DLX6 are established regulators of endochondral bone formation and osteogenic differentiation, consistent with their enrichment in osteoblast-lineage cells in OPLL. PRRX1, a marker associated with osteoprogenitor populations, was also activated at the regulon level,



**Fig. 2** Characterization of chondrocyte subclusters in human OPLL. **(a)** t-SNE plot at a resolution of 0.6 showing 7 distinct chondrocyte subclusters: MSMP<sup>+</sup>CHI3L1<sup>+</sup> chondrocytes, HTCs, HomCs, CPCs, uChond, rChond A, and rChond B. **(b)** Dot plot showing representative differentially expressed genes in each chondrocyte subcluster. **(c)** Heatmap showing the top four differentially expressed genes in each chondrocyte subcluster. **(d)** t-SNE plots showing the expression of representative marker genes in chondrocytes. **(e)** Violin plots showing the expression levels of representative marker genes across chondrocyte subclusters. **(f)** Representative SOFG staining of CSM and OPLL tissues and IHC staining of COL10A1 and CHI3L1 in CSM and OPLL tissues. The ossified region in the OPLL tissue is labeled as OB. Scale bar, 50  $\mu$ m. **(g)** Quantitative analysis of the COL10A1-positive area and CHI3L1-positive area in CSM and OPLL tissues. Data are presented as means  $\pm$  SD. \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$

suggesting enhancement of a PRRX1<sup>+</sup> osteogenic program during ectopic ossification. Immunofluorescence staining and quantitative analysis further validated these findings, showing significantly increased PRRX1 and RUNX2 signals in OPLL lesions compared with CSM controls (Fig. 4e, f). Quantification confirmed that the relative fluorescence intensities of both PRRX1 and RUNX2 were significantly elevated in OPLL tissues, supporting enhanced osteoprogenitor activation and osteogenic differentiation during ectopic ossification.

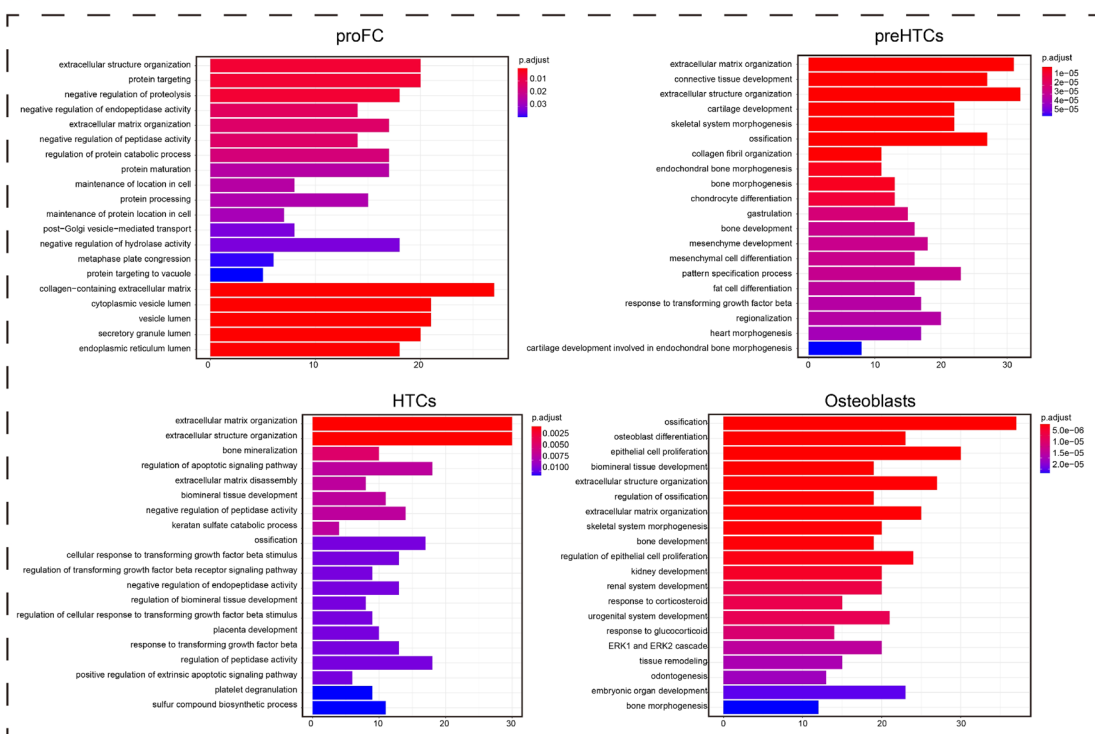
#### Pseudotime analysis suggests a staged chondro-osteogenic trajectory in OPLL

To infer a putative chondro-osteogenic progression in OPLL, we performed pseudotime trajectory analysis using Monocle2 (Fig. 5a). Trajectory ordering was computationally inferred and does not constitute direct lineage tracing. The reconstructed trajectory contained four branch points and diverged into two principal terminal paths (Fig. 5a, b). CPCs, HomCs, HTCs, and MSMP<sup>+</sup>CHI3L1<sup>+</sup> chondrocytes were broadly distributed along the shared trajectory backbone, whereas osteoblasts became progressively enriched along one terminal path, particularly beyond the late branch point (Branch 4) (Fig. 5c). Notably, HomCs and a subset of CPCs were present along both principal paths, indicating that these intermediate chondrocyte states were not restricted to a single

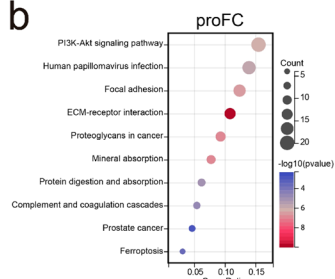
trajectory branch. MSMP<sup>+</sup>CHI3L1<sup>+</sup> chondrocytes were mainly localized to the intermediate pseudotime region bridging CPC-enriched and preHTC-enriched states and retained partial CPC-associated features (e.g., PRG4 and ASPN), suggesting an inflammation-activated transitional state rather than an independent lineage.

Branch-state mapping and branch-dependent heatmaps further supported a staged chondrocyte-to-osteoblast transition (Fig. 5d, f). Additional branch-state maps and branch-specific heatmaps are provided in sFig. 1d-f and sFig. 2a. The earlier branch points (Branches 1 and 2) likely reflected intrachondrocytic diversification events before terminal lineage commitment, as supported by the Branch1/2 heatmaps showing divergence among matrix-, inflammatory-, and cartilage-associated transcriptional modules (sFig. 1e, f). In contrast, the later branch points, especially Branch 4, were more closely associated with the bifurcation toward cartilage-maintaining versus osteogenic differentiation states. Among all branch points, Branch 4 appeared to be the most prominent late fate-decision node, because it showed the clearest transcriptional segregation between the two terminal fates (Fig. 5f). At the terminal end of the osteoblast-directed path, osteogenesis- and matrix-remodeling-associated genes, including SPP1, IBSP, PDGFRA, PDGFRB, and PRRX1, were preferentially represented, whereas the HTC-directed path retained cartilage-related programs marked by genes such as PRG4, ENPP1, COL2A1, SOX9, and SCX (Fig. 5f; sFig. 2a, b). Consistently, pseudotime trends showed a progressive decline in chondrogenic/ligament-associated genes, including COL2A1, SOX9, ENPP1, and SCX, accompanied by late upregulation of PDGFRA, PDGFRB, and PRRX1 along the osteogenic path (Figs. 5e; sFig. 2b). KEGG enrichment of the Branch 4 transcriptional program highlighted coordinated activation of ECM-receptor interaction, focal adhesion, PI3K-Akt signaling, and inflammation-related pathways, including TNF and IL-17 signaling, consistent with this node representing a key late-stage bifurcation toward osteogenic maturation (Fig. 5g). Additional KEGG analyses for earlier branch-associated modules are shown in sFig. 2c.

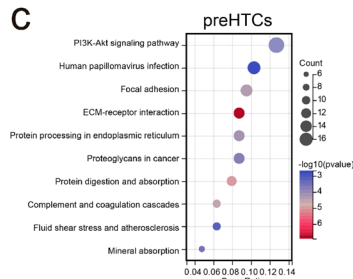
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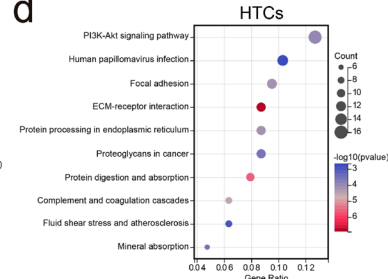
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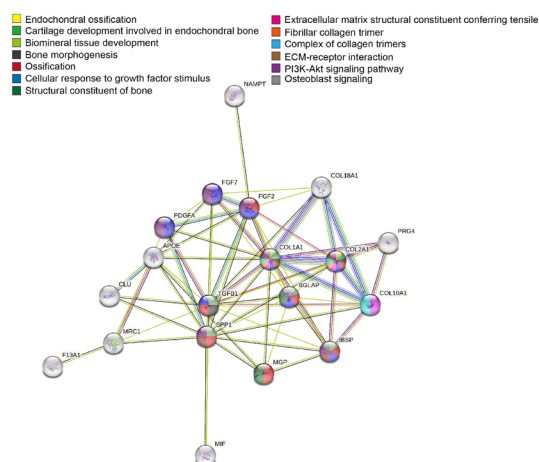
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**Fig. 3** Functional enrichment and interaction analysis of chondrocytes and osteoblasts. **(a)** GO enrichment analysis showing the enriched biological process (BP) terms in proFC, preHTCs, HTCs, and osteoblasts. **(b–e)** KEGG pathway enrichment analysis of differentially expressed genes in proFC **(b)**, preHTCs **(c)**, HTCs **(d)**, and osteoblasts **(e)**. **(f)** PPI network analysis of representative regulated genes in chondrocytes and osteoblasts, edge colors correspond to different sources of interaction evidence in the STRING database

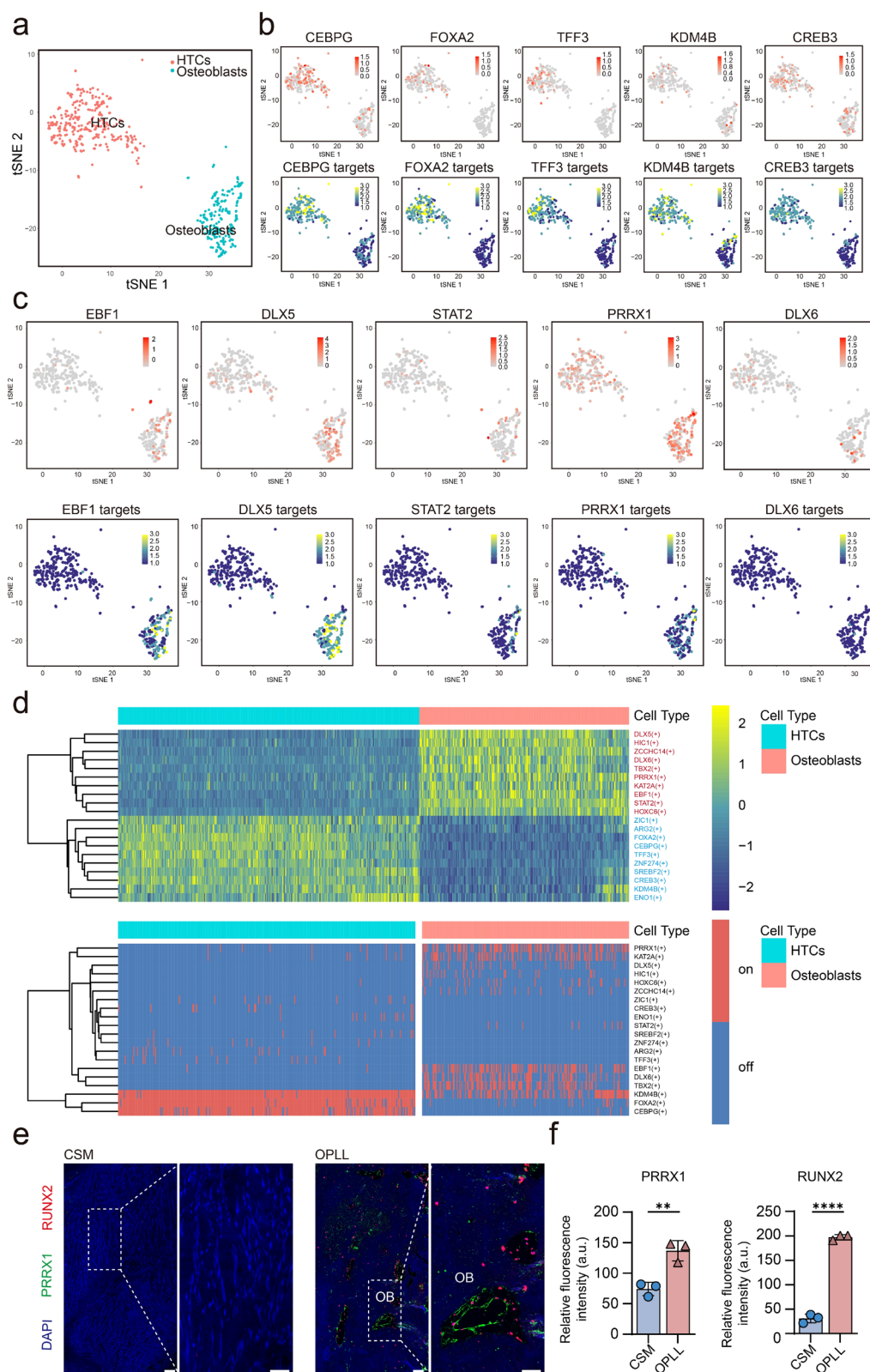
### Macrophage-stromal signaling coordinates chondrocyte hypertrophy and osteogenic activation in OPLL

Cell–cell communication is thought to contribute to lineage specification during ectopic ossification, and inflammatory signaling has been implicated in OPLL progression. To further explore intercellular signaling within OPLL lesions, we applied CellChat to the major stromal subsets together with macrophages (ProFCs, preHTCs, HTCs, osteoblasts, and macrophages), because previous studies of heterotopic ossification have identified macrophages as important inflammatory regulators of chondrogenic and osteogenic differentiation, and related studies of spinal ligament ossification have further suggested that macrophage-associated inflammatory signals can promote osteogenic activation in ligament-derived cells. In addition, inflammatory studies in OPLL have reported increased monocyte/pro-inflammatory cytokine signatures, supporting the biological relevance of macrophage-associated signaling in this disease context (Huang et al. 2021; Sun et al. 2025a, b). The inferred network showed extensive interactions among macrophages and stromal/chondrocyte-lineage subsets, with particularly prominent communication involving macrophages, HTCs, preHTCs, and osteoblasts (Fig. 6a, b). Ligand–receptor analysis highlighted several prominent signaling axes (Fig. 6c, d), including FGF2-FGFR1 and FGF7-FGFR1, which were enriched in stromal/chondrocyte-lineage communication, and SPP1-CD44, which was preferentially represented in HTC-associated signaling. Macrophage-associated ligand–receptor pairs, including TGFB1-(TGFB1+TGFB2) and NAMPT-(ITGA5+ITGB1), represented additional signaling routes linking the inflammatory niche to stromal

cell states. Pattern analysis further organized these pathways into coordinated communication modules, including “incoming” programs that reflect how target cells receive and integrate signals and “outgoing” programs that reflect how sender populations contribute to niche-wide signaling (Fig. 6e, f). Together, these data suggest that macrophages participate prominently in immune-stromal communication with chondrocyte-lineage cells in OPLL. The inferred network further supports a model in which FGF-related signaling is associated with stromal/chondrocyte-lineage communication, whereas SPP1-CD44 signaling is preferentially represented in later HTC-associated communication, potentially contributing to hypertrophy-associated ossification.

To further define functional signaling within the OPLL niche, we examined pathway-specific CellChat networks centered on SPP1, FGF, ANGPTL, and RESISTIN, which emerged as representative signaling programs in the inferred communication network and were supported by specific ligand–receptor pairs shown in Fig. 7a–f. In the SPP1 signaling network, HTCs were the major signal-sending population and participated in both autocrine and paracrine communication. This pathway involved SPP1 together with its cognate receptors/integrin complexes, including CD44, ITGA5-ITGB1, ITGAV-ITGB1, and ITGAV-ITGB5, with SPP1-CD44 representing the top-contributing ligand–receptor pair (Fig. 7a). ProFCs and preHTCs were among the principal receiver populations in this network. Immunofluorescence staining further showed increased SPP1 (OPN) and CD44 signals in OPLL lesions compared with CSM controls, and quantitative analysis confirmed that the relative fluorescence intensities of both markers were significantly elevated in OPLL tissues (Fig. 7b, c). In the FGF signaling network, ProFCs, preHTCs, and HTCs constituted the principal ligand-producing populations. The major ligands included FGF1, FGF2, and FGF7, whereas the corresponding receptors included FGFR1 and FGFR3, with FGF2-FGFR1 representing the strongest ligand–receptor pair and osteoblasts serving as prominent receiver cells (Fig. 7d). The ANGPTL signaling network suggested a complementary stromal communication program in which HTCs and preHTCs served as major source populations,





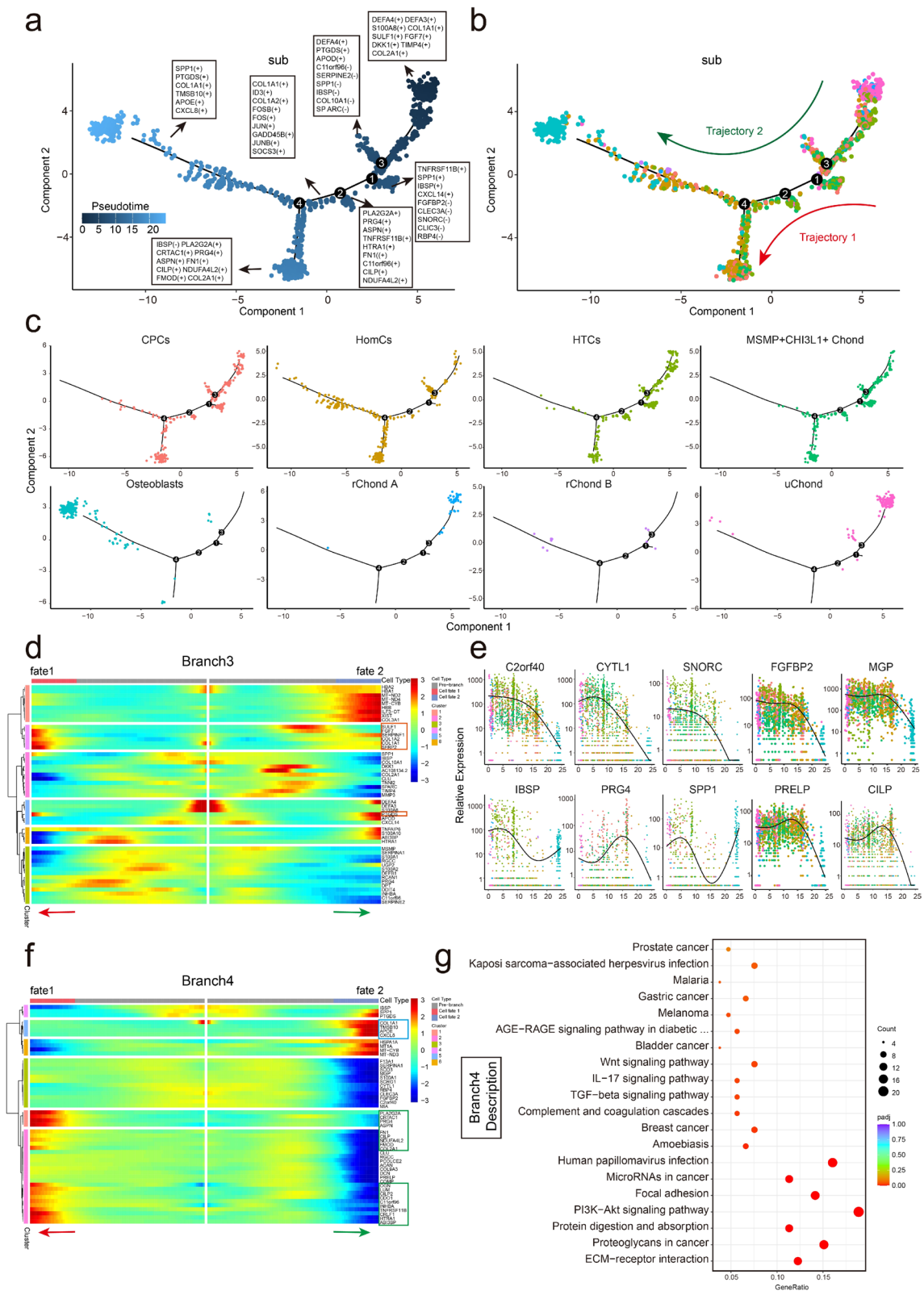
**Fig. 4** Transcription factor regulatory network analysis in HTC and osteoblasts. **(a)** t-SNE plot showing the distribution of HTCs and osteoblasts. **(b)** t-SNE plots showing the expression of representative transcription factors (upper) and the activity of representative regulons (lower) in HTCs. **(c)** t-SNE plots showing the expression of representative transcription factors (upper) and the activity of representative regulons (lower) in osteoblasts. **(d)** Heatmaps showing SCENIC-derived regulon activity in HTCs and osteoblasts, including continuous AUC scores (upper) and binarized regulon activity states (lower), where “on” indicates an active regulon and “off” indicates an inactive regulon. **(e)** Representative IF staining of RUNX2 (red), PRRX1 (green), and DAPI (blue) in CSM and OPLL tissues. The ossified region in the OPLL tissue is indicated as OB. Scale bars, 100  $\mu$ m in the overview images and 50  $\mu$ m in the enlarged images. **(f)** Quantitative analysis of the relative fluorescence intensities of PRRX1 and RUNX2 in CSM and OPLL tissues. Data are presented as mean  $\pm$  SD.  $**p < 0.01$ ,  $****p < 0.0001$

whereas ProFCs were among the principal targets (Fig. 7e). This pathway included ANGPTL2 and ANGPTL4 together with receptors or receptor-associated complexes such as ITGA5-ITGB1, SDC2, SDC4, CDH11, and TLR4, indicating that ANGPTL signaling may contribute to matrix remodeling and microenvironmental activation during early ossification. Finally, the RESISTIN signaling network was largely macrophage-associated, with preHTCs representing the principal receiver population (Fig. 7f). This pathway was defined mainly by RETN and its receptor pairs RETN-CAP1 and RETN-TLR4, suggesting an additional inflammatory communication axis linking macrophage-associated signaling to stromal/chondrocyte-lineage cells. Integrating pseudotime with ligand-receptor localization, we observed a staged communication pattern: FGF2-FGFR1 signaling was enriched in early ProFC/preHTC states, consistent with osteogenic priming, whereas SPPI-CD44 interactions were concentrated between HTCs and mature osteoblasts, suggesting a downstream reinforcement of hypertrophy-associated ossification. Together, these pathway-level analyses highlight coordinated inflammatory, osteogenic, and angiogenic signaling modules that couple macrophage activation to chondrocyte hypertrophy and osteoblast differentiation in OPLL.

## Bulk transcriptome analysis reveals immune-related genes and inflammatory pathways in OPLL

To externally validate the scRNA-seq findings and extend the analysis beyond the limited single-cell sample size, we analyzed two public bulk transcriptome datasets of OPLL and non-OPLL posterior longitudinal ligament tissues. This integrative approach enabled us to assess immune-associated transcriptional changes at the cohort level and compare them with the single-cell findings. After batch correction and integration, the combined bulk transcriptome dataset comprised 5 OPLL samples and 5 non-OPLL controls. Differential expression analysis identified 60 up-regulated and 401 down-regulated genes, and the top differentially expressed genes are shown in the heatmap (sFig. 3a, b). Protein interaction network analysis indicated that MAPK8, SRC, EGFR, and NF- $\kappa$ B-associated nodes occupied central positions within the DEG network, while CD44 was connected to a smaller module including COL4A1, COL4A2, COL6A1, COL6A2, LAMA3, LAMC3, and VAV2 (sFig. 3c).

To define immune-associated transcriptional changes, we intersected the bulk DEGs with immune-related gene sets and identified 34 immune-related DEGs (IMM-DEGs) (sFig. 3d), including both up-regulated and down-regulated genes (sFig. 3e). GO and KEGG enrichment analyses of the IMM-DEGs highlighted inflammation-related biological processes and pathways, including MAPK, RAS, NF- $\kappa$ B, IL-17, and T-cell receptor signaling (sFig. 3f, g). Notably, several immune-associated genes identified in the bulk analysis, including TNFAIP3, HCK, and LCN1, were also elevated in OPLL samples at the cohort level (sFig. 3h) and showed cell-type-specific expression in the scRNA-seq dataset (sFig. 3i). TNFAIP3 and HCK were mainly enriched in preHTCs/ProFC-related populations, whereas CD44 showed broader expression across stromal subsets, including preHTCs, ProFCs, HTCs, and osteoblasts (sFig. 3i). These findings support a link between immune-associated transcriptional activation and stromal cell-state heterogeneity in OPLL.



**Fig. 5** Trajectory analysis reveals distinct differentiation states of chondrocytes and osteoblasts in OPLL. **(a)** Pseudotime trajectory of chondrocytes and osteoblasts reconstructed by Monocle analysis. **(b)** Pseudotime trajectory overlaid with cluster identities and lineage branches. **(c)** Distribution of each cell subtype along the pseudotime trajectory. **(d)** Heatmap of branch-dependent genes at Branch 3 identified by Monocle branch expression analysis. **(e)** Expression dynamics of representative genes along pseudotime. **(f)** Heatmap of branch-dependent genes at Branch 4 identified by Monocle branch expression analysis. **(g)** KEGG enrichment analysis of genes associated with Branch 4

### Validation of inflammation-associated SPP1-CD44 signaling in OPLL

Because our computational analyses implicated the SPP1-CD44 axis in OPLL-associated ossification, we next sought experimental validation *in vivo* and *in vitro*. We first examined an early-stage *Enpp1<sup>fllox/fllox</sup>/EIIa-Cre* mouse model of spinal ligament ossification, which was generated based on the same locus implicated in the classical ttw model (sFig. 4). Micro-CT demonstrated prominent ectopic ossification along the posterior longitudinal ligament region in *Enpp1<sup>fllox/fllox</sup>/EIIa-Cre* mice, confirming the presence of ligament-associated ossification lesions (Fig. 8a). Histological analysis further demonstrated ectopic ossified lesions in *Enpp1<sup>fllox/fllox</sup>/EIIa-Cre*. H&E staining showed abnormal ossified tissue formation adjacent to the posterior longitudinal ligament, whereas Safranin O/Fast Green staining revealed cartilage-like matrix accumulation within the ossification area (Fig. 8b). Immunofluorescence staining showed markedly increased SPP1 (OPN) and CD44 signals in ossification areas of *Enpp1<sup>fllox/fllox</sup>/EIIa-Cre* mice compared with WT controls (Fig. 8e, f). In parallel, CD31 staining was increased in the ossification layer of *Enpp1<sup>fllox/fllox</sup>/EIIa-Cre* mice, consistent with increased vascularization within developing lesions (Fig. 8c, d).

To assess inflammatory activation of this axis *in vitro*, primary human OPLL-derived ligament cells were stimulated with IL-1 $\beta$ . After 24 h of IL-1 $\beta$  stimulation, immunofluorescence staining showed significantly increased RUNX2, SPP1, CD44, and COL10A1 signals in primary human OPLL-derived ligament cells (Fig. 8g, i). Western blot analysis

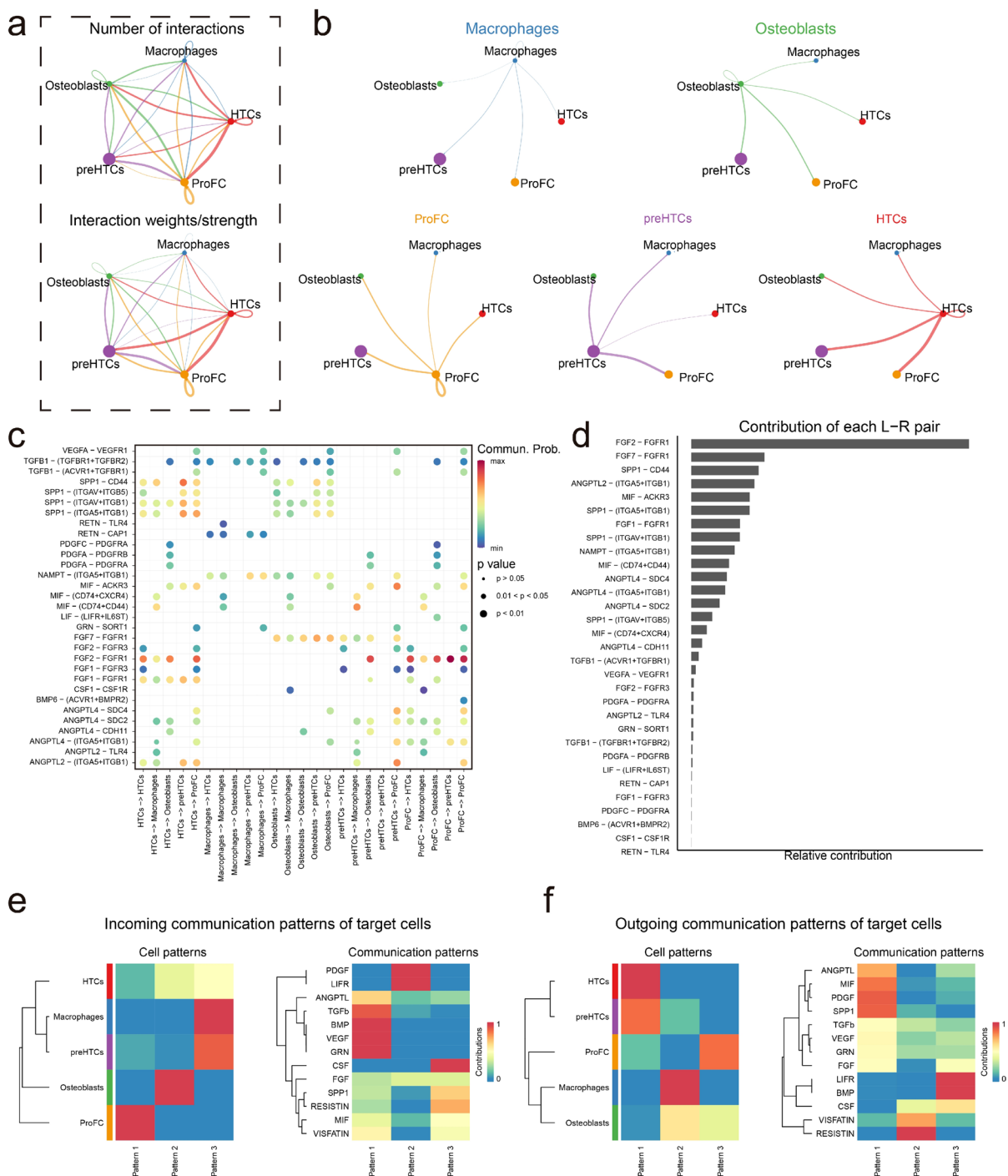
further confirmed increased protein expression of CD44, SPP1, and COL10A1 (Fig. 8h, j). Together, these *in vivo* and *in vitro* data support that SPP1 and CD44 are elevated in ossification lesions and can be further enhanced by inflammatory stimulation in primary human OPLL-derived ligament cells. These findings are consistent with a model in which inflammation promotes hypertrophic and osteogenic activation in association with the SPP1-CD44 signaling axis.

### Discussion

OPLL remains difficult to treat, in part because the stromal heterogeneity and inflammatory niche within the posterior longitudinal ligament are still poorly understood. Although access to truly normal PLL tissue is limited, we used clinically available non-OPLL PLL specimens from CSM patients as controls and processed them using matched sampling and histological workflows. By integrating an exploratory scRNA-seq atlas from two OPLL donors with external bulk transcriptomics, tissue staining, *in vitro* assays, and the mouse model, we identified consistent stromal and immune cell states and propose a staged model of lesion progression. Specifically, trajectory and regulon analyses support a CPC-to-hypertrophic continuum that progressively acquires osteogenic programs, whereas CellChat analysis suggests an inflammatory niche in which early FGF-related signaling and downstream SPP1-CD44 signaling couple immune activation to chondrocyte hypertrophy and osteogenic activation.

Distinct from previous OPLL studies that focused mainly on osteogenic cells, our single-cell data indicate that chondrocyte-lineage heterogeneity is a central feature of lesion progression. We resolved CPCs, homeostatic and hypertrophic chondrocytes, reactive subsets, and an MSMP<sup>+</sup>CHI3L1<sup>+</sup> population with inflammatory features. In pseudotime analysis, the MSMP<sup>+</sup>CHI3L1<sup>+</sup> subset was mainly localized to an intermediate region between CPC-enriched and pre-HTC-enriched states and retained partial CPC markers, supporting an inflammation-activated transitional





**Fig. 6** Overview of the crosstalk networks among cell populations in OPLL. **(a)** Overall number of interactions and interaction strength among macrophages, chondrocytes, and osteoblasts in OPLL. **(b)** Cell-specific communication networks for macrophages, osteoblasts, proFC, preHTCs, and HTCs. **(c)** Dot plot showing the communication probability and significance of ligand–receptor pairs among the indicated cell populations.

**(d)** Relative contribution of individual ligand–receptor pairs to the overall intercellular communication network across the five included cell populations (macrophages, osteoblasts, preHTCs, HTCs, and ProFC) in OPLL. **(e–f)** Heatmaps showing the incoming **(e)** and outgoing **(f)** communication patterns of the indicated cell populations in OPLL.

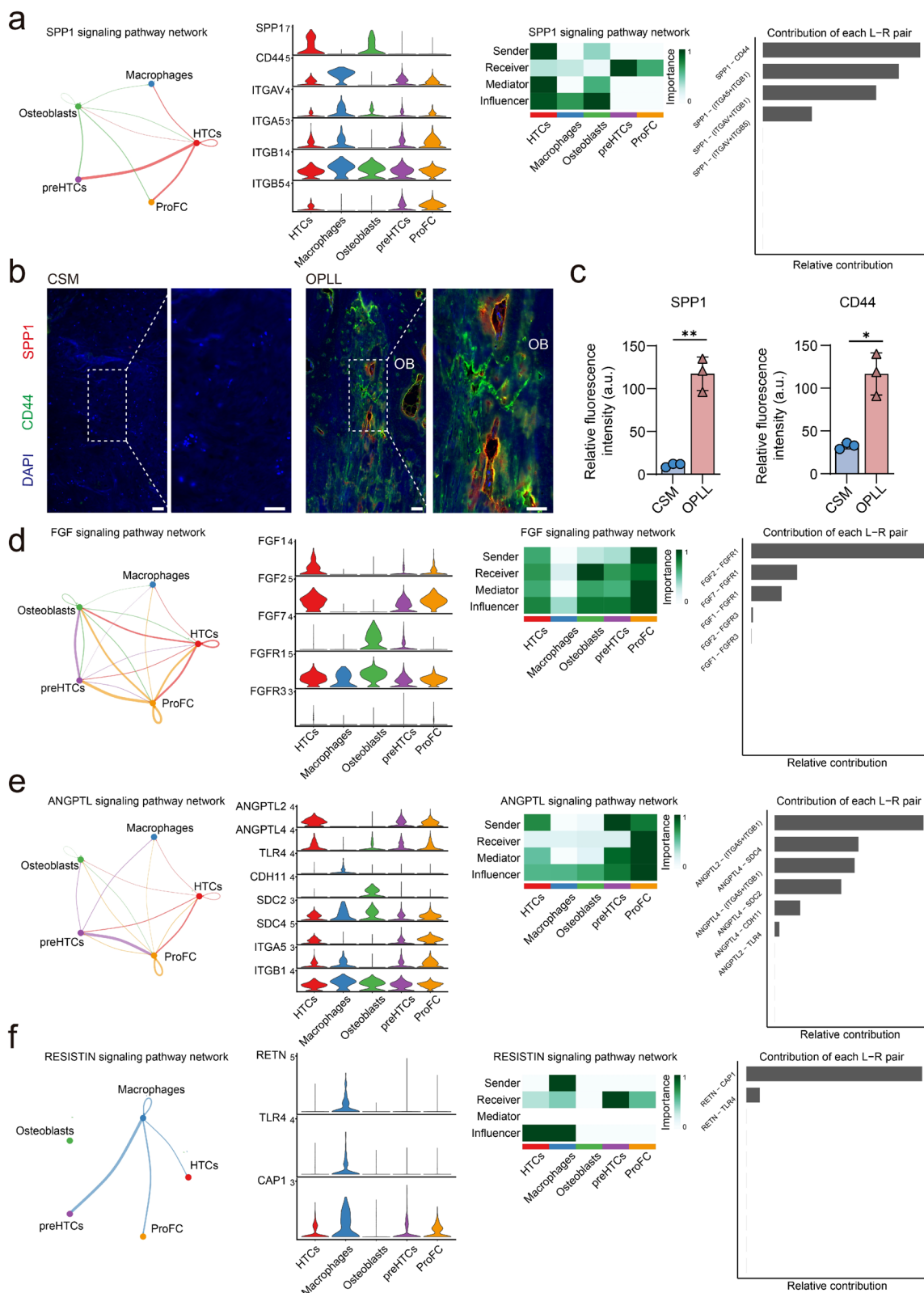
state rather than an independent lineage (Cherif et al. 2022; Väänänen et al. 2014; Li et al. 2023). Together with the strong mineralization/ossification programs in HTC, these results suggest a staged process in which inflammatory activation promotes chondrocyte hypertrophy and calcification, thereby predisposing to osteogenic conversion during OPLL (Gan et al. 2021; Sun et al. 2020). Our subclustering analysis further suggested that inflammation-associated chondrocytes in OPLL are not a uniform population but instead comprise multiple transcriptionally distinct reactive states, including a matrix-remodeling MSMP<sup>+</sup>CHI3L1<sup>+</sup> population, a stress-reactive population characterized by high S100A8/S100A9 expression, and an immune-associated reactive population enriched for PTPRC, IL7R, and TNFAIP3. This finding implies that inflammatory activation of chondrocytes during OPLL may occur in different functional states or stages.

Consistent with an endochondral-like mechanism, we observed vascular ingrowth at ossification fronts and increased CD31/PECAM1-positive endothelial signals, indicating that angiogenic remodeling accompanies and likely facilitates ossification under inflammatory conditions (Lin et al. 2023; Ling et al. 2021). In parallel, we identified an osteoblast subset enriched for osteogenesis-related genes (e.g., COL1A1, BGLAP, SPP1, TGFB1), and pathway analyses across chondrocyte- and osteoblast-lineage cells converged on PI3K-Akt, TGF- $\beta$ , MAPK, IL-17, and TNF signaling, linking ECM remodeling, inflammation, and osteogenic differentiation. Collectively, these data refine the prevailing model by positioning inflammation-driven chondrocyte hypertrophy, together with vascular and immune cues, as an essential upstream event that licenses osteoblast activation and ectopic bone formation in OPLL (Liu et al. 2017; Tang et al. 2022; Cai et al. 2020).

We next sought to define key transcriptional regulators of the chondrocyte-osteoblast transition in OPLL using scRNA-seq-based regulon analysis. Compared with HTCs, osteoblast-lineage cells showed higher activity of osteogenic transcription factors, including DLX5, DLX6, PRRX1, STAT2,

and EBF1, whereas HTCs preferentially enriched FOXA2, CEBPG, CREB3, and TFF3. DLX5/6 are well-established drivers of osteoblast differentiation and endochondral ossification, and their upregulation in OPLL osteoblasts is consistent with prior reports linking DLX5 to stretch-responsive osteogenesis in ligament-derived cells (Tang et al. 2023). PRRX1, a mesodermal lineage factor associated with osteoprogenitor programs and heterotopic ossification, was likewise activated in our osteoblast subset. These TF signatures, together with our tissue validation, indicate that OPLL lesions engage a coordinated osteogenic regulatory network downstream of chondrocyte hypertrophy and inflammation (Lu et al. 2011; Hu et al. 2024).

Ectopic ossification is commonly attributed to aberrant activation of progenitor-lineage cells. Recent single-cell studies in tendon and traumatic HO have identified progenitor subsets marked by RSPO2, TPPP3, PDGFRA, and SCX family programs. In our dataset, RSPO2 expression was negligible, whereas TPPP3, PDGFRA, and PDGFRB were prominently expressed in chondrocyte and osteoblast lineages, and SCX declined toward late pseudotime. Consistently, trajectory analysis showed that an osteoblast-fated branch was characterized by induction of SPP1, PTGDS, COL1A1, TMSB10, APOE, and CXCL8, implicating inflammatory cues and altered lipid/ECM programs in osteogenic commitment. In contrast, cells remaining in the chondrocyte trajectory retained high COL2A1, SOX9, PRG4, ASPN, and FMOD, reflecting maintenance of cartilage identity. Notably, SPP1-high chondrocytes have been reported as transitional states in osteoarthritis, paralleling the hypertrophic stage we observe in OPLL. Together, these findings support an inferred model in which CPC-derived chondrocytes undergo inflammation-linked hypertrophy and progressively acquire osteogenic programs, potentially culminating in osteoblast-like states during ectopic ossification. (Tachibana et al. 2022; Feng et al. 2020; Lin et al. 2022; Yea et al. 2023; Harvey et al. 2019; Qu et al. 2023).



**Fig. 7** Key signaling pathways mediating the interactions among macrophages, chondrocytes, and osteoblasts. **(a)** Cell–cell communication analysis of the SPP1 signaling pathway. **(b)** Representative immunofluorescence staining of SPP1 (red), CD44 (green), and DAPI (blue) in CSM and OPLL tissues. Scale bars, 100  $\mu$ m in the overview images and 50  $\mu$ m in the enlarged images. **(c)** Quantitative analysis of the relative fluorescence intensities of SPP1 and CD44 in CSM and OPLL tissues. Data are presented as mean  $\pm$  SD. \* $p$  < 0.05, \*\* $p$  < 0.01. **(d–f)** Cell–cell communication analysis of the FGF **(d)**, ANGPTL **(e)**, and RESISTIN **(f)** signaling pathways

Inflammatory activity is increasingly recognized as a key contributor to OPLL. Clinically, higher systemic inflammation correlates with more extensive ossification patterns, as evidenced by associations between hs-CRP levels and vertebral morphology in OPLL, and by links between paravertebral ossification burden and serum IL-17 in our previous work (Kawaguchi et al. 2017; He et al. 2023b). However, how specific macrophage programs translate inflammatory cues into aberrant stromal differentiation has remained unclear. By integrating CellChat with pseudotime, our study identifies a prominent immune–stromal signaling niche in which SPP1–CD44 signaling, largely associated with HTC and macrophages, and FGF2/FGF7–FGFR1 signaling represent major communication axes linking inflammatory activity to chondrocyte hypertrophy and osteogenic commitment. SPP1, a macrophage-derived matrix-interacting cytokine involved in fracture repair and macrophage polarization, is well positioned to link inflammation with angiogenesis and endochondral-like ossification. Its receptor CD44, a broadly expressed adhesion and signaling molecule in bone and vascular biology, provides a plausible conduit through which macrophage–stromal crosstalk reinforces pathological mineralization (Wang et al. 2024; Weng et al. 2022).

Our data support this model at multiple levels. SPP1 was among the most highly expressed macrophage-associated ligands in OPLL and formed a central interaction module with CD44 in HTC and osteoblasts, while pathway analyses placed this axis downstream of inflammatory activation. In vivo,

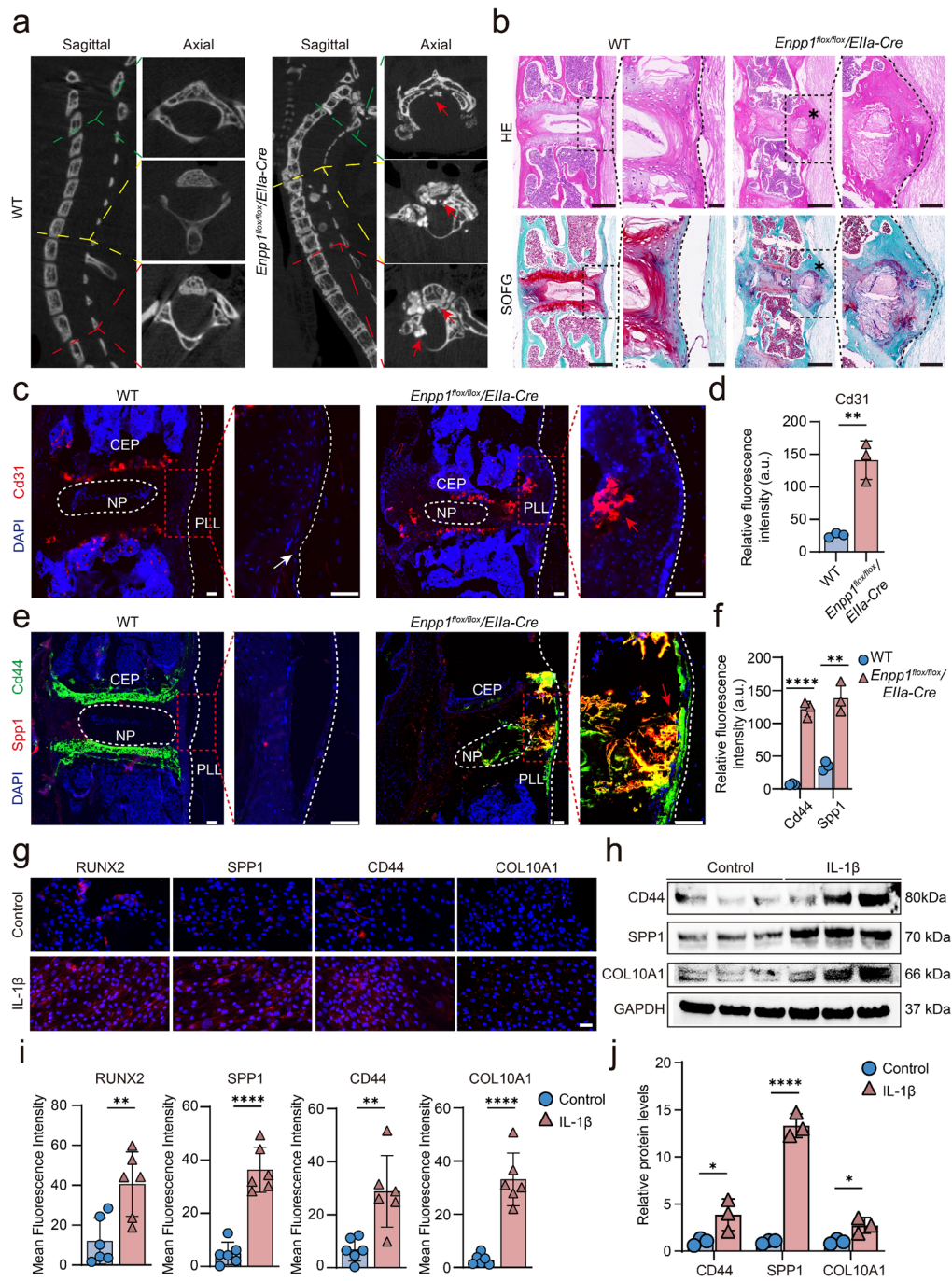
OPLL lesions showed expansion of OPN<sup>+</sup> and CD44<sup>+</sup> cells within ossification fronts, and in vitro IL-1 $\beta$  stimulation further increased SPP1/CD44 together with hypertrophic and osteogenic markers, indicating that inflammatory cues amplify this signaling loop. These findings align with broader evidence that SPP1–CD44 governs immune–stromal communication and matrix remodeling across diverse contexts, and here we extend that concept to OPLL as a mechanistic bridge between macrophage activation, cartilage hypertrophy, and ectopic bone formation.

These findings suggest that SPP1–CD44 signaling likely contributes to osteogenic activation under inflammatory stimulation; however, functional validation is warranted to confirm its direct role in OPLL pathogenesis. Despite the limited number of patient samples, which partly reflects the technical difficulty of dissociating ossified OPLL tissues into high-quality single-cell suspensions (sFig. 5), the integration of external transcriptomic data, trajectory analysis, and experimental validation consistently supports a model in which inflammation-induced activation of CPC-derived chondrocytes promotes endochondral ossification in OPLL. The MSMP<sup>+</sup>CHI3L1<sup>+</sup> subset likely represents an inflammatory transitional phenotype within this process. Moreover, we propose that FGF2–FGFR1 may act as an upstream inducer of osteogenesis, whereas SPP1–CD44 represents a downstream feedback signal that reinforces ossification and macrophage stromal communication. These refinements strengthen the mechanistic interpretation and provide a framework for targeted intervention in OPLL.

## Conclusion

Overall, this exploratory single-cell and multi-modal analysis provides insights into dynamic stromal heterogeneity and immune–stromal coupling in OPLL. Our data support an inferred model in which inflammation-activated CPC-derived chondrocytes undergo hypertrophy and acquire osteogenic





**Fig. 8** Role of the SPP1-CD44 axis and inflammatory factors in OPLL. **(a)** Representative sagittal and axial Micro-CT images of the spine in WT and *Enpp1<sup>fllox/fllox</sup>/Ella-Cre* mice. **(b)** Representative H&E and SOFG staining images in WT and *Enpp1<sup>fllox/fllox</sup>/Ella-Cre* mice. Scale bars, 500  $\mu$ m in the overview images and 200  $\mu$ m in the enlarged images. **(c)** Representative IF staining of Cd31 (red) and DAPI (blue) in WT and *Enpp1<sup>fllox/fllox</sup>/Ella-Cre* mice. Scale bar, 100  $\mu$ m. **(d)** Quantification of Cd31 fluorescence intensity. **(e)** Representative immunofluorescence staining of Spp1 and Cd44 in WT and *Enpp1<sup>fllox/fllox</sup>/Ella-Cre* mice. Scale bar, 100  $\mu$ m. **(f)** Quantification of Spp1 and Cd44 fluorescence intensities. **(g)** Representative immunofluorescence staining of RUNX2, SPP1, CD44, and COL10A1 in OPLL cells after 24 h IL-1 $\beta$  treatment. Scale bar, 50  $\mu$ m. **(h)** Representative western blot analysis of CD44, SPP1, and COL10A1 in OPLL cells after 24 h IL-1 $\beta$  treatment. **(i)** Quantification of fluorescence intensities in **(g)**. **(j)** Quantification of relative protein levels in **(h)**. Data are presented as mean  $\pm$  SD. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.0001

programs, reinforced by a late SPP1-CD44 signaling module. These findings highlight immune-stromal interactions as potential targets for earlier diagnosis and intervention in OPLL.

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**Author contribution** He ZY, Zhou JX, and Wang FA: conceptualization, methodology, investigation, writing original draft. Guo P, Li JF, Tang T, Zhu ZY, Tung NTC, Kawaguchi Y: methodology, data analysis, and figures preparation. Liu SY: conceptualization, resources, supervision, project administration, and funding acquisition. Liu XZ, Gao MM: conceptualization, supervision, writing, editing, and provided funding support. Zhou ZY: conceptualization, supervision, project administration, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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**Data availability** All data generated or analyzed during this study are available from the corresponding author on reasonable request.

## Declarations

**Ethics approval and consent to participate** Ethical approval for the collection and use of human tissue specimens (No. 2020SYSUSH-055) was granted by the Ethics Committee of the Seventh Affiliated Hospital, Sun Yat-sen University. All animal procedures complied with the International Guide for the Care and Use of Laboratory Animals and were authorized by the Research Ethics Committee of the Sun Yat-Sen University (No. 2020000147).

**Patient consent for publication** Written informed consent was obtained from the patient.

**Clinical trial number** Not applicable.

**Competing interests** The authors declare no competing interests.

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