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Single-cell and spatial transcriptome analysis reveals the potential therapeutic targets for testicular sex cord-stromal cell tumor

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Abstract

Testicular sex cord stromal tumor (TSCST) is a rare testicular tumor with unknown molecular mechanisms, tumor microenvironment (TME) and limited therapeutic options. To define the molecular mechanism and potential targets of TSCST, we combined single-cell RNA sequencing with single-cell nuclear RNA sequencing along with spatial transcriptomics from different TSCST tumor regions. This revealed significantly low expression of immune cell genes in tumor areas, indicating an immune-cold tumor environment and a critical epithelial-mesenchymal transition program during tumorigenesis and disease progression. Also, the study identified high expression levels of the androgen receptor (AR) and related genes in tumor cells, suggesting AR inhibitors as potential therapeutic targets. Furthermore, we identified spatial intra-tumoral heterogeneity, with high senescent characteristics. Finally, the study reports that tumor cells might interact with macrophages, promoting M2 polarization through the APP-CD74 ligand-receptor pair, which has promising therapeutic prospects for this disease. Our data provide comprehensive insights into TSCST, including its cold TME, cellular origins, spatial niches, and cell-cell interactions, highlighting that targeting AR signaling might be a potential therapeutic strategy.

Keywords Testicular sex cord stromal tumor, Androgen receptor, Epithelial-mesenchymal transition, M2 polarization, Cell-cell communication

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Introduction

Testicular sex cord-stromal tumor (TSCST) is a rare primary testicular tumor accounting for approximately 5% of all testicular tumors [1, 2]. Radical orchiectomy (RO) is the mainstream surgical treatment for TSCST. Although most patients show strong clinical prognoses, approximately 10% develop malignant behavior and recurrent metastases [3–5]. The most common site of metastasis is the retroperitoneal lymph nodes, and the removal of retroperitoneal lymph nodes may be feasible in these patients to prevent recurrence and progression [6–8]. However, patients with metastatic TSCST are likely to be resistant to radiotherapy and chemotherapy and have a worse prognosis [9, 10]. Additionally, the development of biomarkers and therapeutic targets for TSCST has impeded their relative ratio.

Molecularly, single-cell RNA sequencing (scRNA-seq) can identify novel cell types and functional features by detecting gene expression at the single-cell level [11]. Compared to traditional high-throughput sequencing, scRNA-seq can also analyze the regulatory mechanisms of cellular developmental origins, differentiation, and heterogeneity [12]. For example, our team used scRNA-seq to uncover the origin of testicular germ cell tumors (TGCT) at the single-cell level and found the involvement of the key transcription factor (TF) TFAP2C in TGCT genesis in the previous studies [13]. ScRNA-seq is widely applied in cancer research and effectively explores tumor cellular developmental origin and heterogeneity [13]. Although it's not definitive, studies have demonstrated that TSCST originates from Leydig cells, Sertoli cells, and other mesenchymal stromal cells [14].

The tumor microenvironment (TME) comprises diverse immune and stromal cells with complex interactions [13, 15]. Additionally, the positioning of these cells relative to the tumor often correlates closely with the patient's response to immunotherapy and prognosis [16]. ScRNA-Seq requires the dissociation of tissue samples into individual cells, resulting in the loss of spatial location information. However, the spatial transcriptome (ST) can map out the TME layout [17]. Regarding TME of TSCST, there have been deficits in understanding the composition, localization, and functional roles of immune and stromal cells. Also, only histopathological studies indicated that most TSCST possess anti-inhibin immunoreactivity [18]. Hence, a deeper understanding of the role of TME in TSCST is crucial for uncovering TSCST mechanisms and developing novel therapies.

In this study, we utilized single-cell nuclear RNA sequencing (snRNA-seq, a special single-cell RNA sequencing technique) and the ST to the tumor heterogeneity and tumor microenvironment of TSCST's tumor heterogeneity and tumor microenvironment. This provided us with novel perspectives on the underlying

mechanism of TSCST oncogenesis and development and serve as a theoretical basis for the precise treatment of TSCST.

Materials and methods

Sample collection

All seven fresh samples were collected from the Department of Urology at Hunan Cancer Hospital, and informed consent was obtained from all patients. All patients were pathologically diagnosed with TSCST. After the samples were collected during surgery, they were immediately washed with saline to remove blood stains, dried with absorbent paper, and placed in liquid nitrogen for rapid freezing. The remaining three paraffin samples were collected from the hospital's pathology department. This study was conducted in accordance with the Declaration of Helsinki. The Ethics Committee of the Hunan Cancer Hospital approved this study.

Extraction of single-cell nuclei

First, frozen tissues were cut into 0.5 mm³ size, placed in a 2 ml tissue homogenizer and thawed in homogenization buffer. The tissue was then crushed 20 times using a grinding rod before being separated from large pieces by passing it through a cell strainer. Next, the cells were centrifuged at 500 g for 10 min at 4 °C to precipitate the nuclei and then resuspended in 1× phosphate buffer saline (PBS) cell resuspension buffer containing 1% BSA and 0.2 U/μL RNase inhibitor. The cells were centrifuged at 500 g for 5 min, resuspended in cell resuspension buffer, and mixed 1:1 with 4',6'-diamidino-2-phenylindole (DAPI) staining solution for nuclei counting and integrity assays. Samples with a nuclear integrity above 85% are acceptable for downstream applications.

Single-cell nuclear RNA library construction and sequencing

DNBelab C4 device (MGI) was used for droplet envelopment, latex fragmentation, mRNA capture, and reverse transcription. The MGI DNBELAB C series reagent Kit (MGI, 940-000047-00) was used for cDNA amplification, library construction, and purification. A Qubit ssDNA Assay Kit (Thermo Fisher Scientific) and an Agilent Bioanalyzer 2100 were used for library quality control (QC). Finally, library sequencing was performed using the DIPSEQ T1 platform.

Align and quantification of snRNA-seq data

Filtering, comparison, and quantification of offline data were performed using DNBELAB C Series scRNA analysis software (https://github.com/MGI-tech-bioinformatics/DNBELAB_C_Series_HT_scRNA analysis software). GRCh38 was chosen as the reference genome, and the DropletUtils tool was used to remove background data.

Public scRNA-seq data download

ScRNA-seq expression matrix data of fetal testes at 6, 7, 8, 12, 15, and 16 weeks were retrieved from the Gene Expression Omnibus (GEO) dataset GSE143356 [19]. Unfractionated testicular scRNA-seq data were retrieved from the GEO dataset GSE124263, including two newborns and two adults from [20].

QC, integrated analysis and cell type annotation of scRNA-Seq and snRNA-seq data

Single-cell expression matrix data underwent quality control assessment using Seurat package (v4.2.1), and cells with $nFeature_RNA > 300$, $nFeature_RNA < 5000$, and per cent mt < 25 were retained for the following analyses, yielding a total of 28,391 cells. Neonatal testis, adult testis, and snRNA-seq data were integrated using the Harmony package (v0.1.1) in R software (v4.2.1). Cell-type annotations were performed manually based on integrated clustering and marker genes for known cell types, including macrophages (marked by MS4A6A, CSF1R, and CD68), Sertoli cells (marked by SOX9, WT1, and INHBB), endothelial cells (marked by VWF, CD34, and EPAS1), Leydig cells (marked by IGF1, IGF2, and IGFBP3), myoid cells (marked by ACTA2, MYH11, and MYL9), spermatogonia (marked by GFRA1, TCF3, and UTF1), spermatocytes (marked by SYCP1, SYCP3, and SMC1B), spermatids (marked by TNP1, PRM1, and PRM2), and tumor cells (marked by MME, CCND1, and CTNNB1) [21].

Differential expression gene analysis, cell proportion analysis and enrichment analysis of differential gene

Differential gene expression analysis, differential gene expression heatmap, dot plot, and cell type proportion analysis for each cell type were performed using the scRNAtoolVis package (v0.0.4). Differentially expressed gene calculations of macrophages were performed using the FindMarkers function of the Seurat package, and the top 100 marker genes of the two macrophage subclusters were selected for display and enrichment analysis. Differentially expressed gene enrichment analysis was performed using the Metascape online tool (<http://metascape.org/gp/index.html#/main/step1>).

Transcriptional regulon analysis

Differential transcriptional regulon analysis was performed using the R package SCENIC (v1.3.1). Based on the package's calcRSS function, the Regulon Specificity Score (RSS) was calculated for each target cell type. The plotRSS function was used to visualize the results. Cell type-specific regulons were defined using $zThreshold = 2$, and the RSS threshold was set to 0.2.

Cell trajectory analysis

Developmental trajectory and pseudotime analyses of cells were performed using Monocle2 software (v2.24.0), and Uniform Manifold Approximation and Projection (UMAP) plots were used to show the distribution of each cell subset on pseudotime trajectories. Because trajectory analysis only provides pseudotime information, we further analyzed the relationship between the degree of differentiation among cell subclusters using CytoTRACE software (v0.3.3) to explore developmental potency.

Cell communication analysis

We divided the samples into leading-edge and tumor core groups according to the source of the samples and performed intercellular communication analysis according to previously annotated cell types. Cell communication analysis was performed using Cellchat (v2.1.2) software [22].

Spatial transcriptome library and sequencing

Paraffin samples intended for spatial transcriptome sequencing were cut into 5 μ m sections on a slicer. Total RNA extraction was performed by assessing RNA concentration and integrity using an Agilent 2100 Bioanalyzer. Samples demonstrating a DV200 value $\geq 30\%$ were selected for further processing, including deparaffinization and subsequent imaging. Post-selection, tissue sections underwent de-crosslinking before hybridisation with targeted RNA probes. To facilitate spatial mapping, two standard microscope slides and a Visium capture slide containing two capture areas were placed into the CytAssist instrument, aligning tissue sections precisely with designated capture regions. Bright-field imaging, captured via the CytAssist platform, provided accurate spatial reference points necessary for downstream analysis. Finally, CytAssist transferred the probe information from the tissue sections on the slides to the Visium capture region, which captures the probe information through primers labelled with distinct locations, providing gene expression data at various spatial locations of the tissue sections.

Next, RNA was enzymatically degraded by RNase treatment, releasing the bound probes as single-stranded molecules onto the Visium capture chip. These released probes were captured via their poly(A) sequences by poly(T) oligonucleotides anchored on the Visium slide, each linked with spatial Barcode and Unique Molecular Identifier (UMI) sequences. Subsequently, spatial Barcode and UMI sequences were enzymatically extended onto the captured probes, generating probe constructs that included spatially encoded barcodes suitable for sequencing library preparation. The constructed libraries were quantified using the Qubit 4.0 fluorometer, followed by fragment size analysis employing an Agilent Fragment

Analysers system. Libraries meeting quality control standards were sequenced using the Illumina NovaSeq platform in a paired-end 150 bp sequencing configuration (PE150).

Spatial transcriptome data analysis

The raw reads were removed from adaptors and low-quality reads using FastQC, and then the 10x Genomics official analysis software, Space Ranger (v3.0), was used for data processing and quality control, probe sequence comparison, gene-spot matrix generation, and spot number evaluation and analysis. Spatial gene expression and clustering analyses of the spatial transcriptomic data were visualized using the Seurat package. Spatial gene set enrichment analysis (GSVA) was performed using the GSVA package (v1.44.5), and spatial cell communication analysis was performed using Cellchat (v2.1.2). A spatial transcriptome gradient analysis was performed using the LSGI package (v0.1.0). Except for gene expression correlation analysis and spatial clustering analysis based on spatial transcriptomic data, which were conducted at a 55 μm spot resolution, all other related analyses were performed using predefined regions (tumor core and leading edge regions).

Immunohistochemistry (IHC)

The paraffin-embedded tissues were sectioned, baked, deparaffinized, and hydrated. Sections were blocked with blocking buffer for 1 h, followed by antigen retrieval with citrate retrieval solution and endogenous peroxidase blocking with 3% H_2O_2 . Then, these tissues were incubated with primary antibody (anti-MME: #MAB-0668, MXB Biotechnologies; anti-CTNNB1: #8480, Cell Signaling Technology; anti-CD3D: #MAB-0740, anti-MXB Biotechnologies; anti-CD19: #ab134114, Abcam; anti-PDCD1: #MAB-0734, MXB Biotechnologies; anti-PBX3: #12571-1-AP, Proteintech; anti-ZEB1: #3396, Cell Signaling Technology; anti-AR: #RMA-0807, MXB Biotechnologies; anti-CDKN1A: #P23221, ProMab Biotechnologies; anti-RB1: #30592, ProMab Biotechnologies; anti-VIM: #MAB-0735, MXB Biotechnologies) overnight at 4°C and incubated with HRP-labelled secondary antibody for 1 h at room temperature, and they were stained with a Diaminobenzidine Color Development Kit (CWBIO, CW2069S). Each tissue sample was incubated with hematoxylin for several minutes, followed by washing with running water for 1–2 min. Finally, the sections were dehydrated and histochemically mounted. Images were obtained using a Zeiss microscope. Two independent pathologists, aware of the histopathological features and patient information, assessed and scored immunostaining. Scoring was determined by combining the proportion of positively stained tumor cells and the intensity of staining. The percentage of tumor cells was scored

as follows: 0 (no positive tumor cells), 1 (<10% positive tumor cells), 2 (10–35% positive tumor cells), 3 (35–75% positive tumor cells), or 4 (>75% positive tumor cells). Staining intensity was graded as follows: 1 (unstained), 2 (weakly stained = yellowish), 3 (moderately stained = tea-colored), or 4 (strongly stained = brown). The staining index was calculated as the product of the staining intensity score and percentage of positive tumor cells.

Immunofluorescence

The paraffin-embedded tissues were sectioned, baked, deparaffinized, and hydrated. Antigen retrieval was performed with citrate retrieval solution, and a circle was drawn around the tissue with a liquid blocker PAP pen and then blocked with a mounting medium for 1 h. Subsequently, a drop of primary antibody (anti-SOX9: #ab185966, Abcam; anti-MIF: #20415-1-AP, Proteintech; anti-CTNNB1: #8480, Cell Signaling Technology; anti-CD68: #66231-2-Ig, Proteintech; anti-MRC1: #18704-1-AP, Proteintech; anti-CD74: #66390-1-Ig, Proteintech) was added and incubated at 4°C overnight. Remove and rewarm, then add a drop of prepared fluorescent secondary antibody to the tissue and incubate for 1 h at room temperature. Each tissue sample was blocked with a decline of DAPI-containing mounting medium. A fluorescence microscope (Zeiss, Oberkochen, Germany) captured images.

β -Galactosidase staining

The frozen sections were rewarmed and washed twice with PBS for 5 min each. β -galactosidase fixative was added for 15 min, washed twice with PBS, and then aspirated. 1 ml of prepared staining working solution (Beyotime, C0602) was added, incubated overnight at 37°C, and blocked with a sealing film to prevent evaporation. Staining was observed under a microscope the following day.

Cut & tag-seq and data analysis

Shanghai Rainbow Genome Co. performed Cut and Tag-Seq sample processing and data analysis. Hatice et al. [23] described the method as a reference for the nucleation of frozen tissue samples in liquid nitrogen. Half a million nuclei were taken, washed with wash buffer, and incubated with ConA beads (Bangs Laboratories) for 10 min at room temperature, then the supernatant was removed. The cells were suspended with dig wash buffer while the antibody to PBX3 and IgG were added at a dilution ratio 1:50, and the cells were incubated overnight at 4°C. Following the primary antibody removal, the nuclei were incubated with a diluted secondary antibody for 1 h at room temperature. After washing, the cells were mixed with the diluted pA-Tn5 complex and incubated for another hour at room temperature. Then, the nuclei

were resuspended with tagmentation buffer and incubated at 37°C for 1 h. After digestion, the DNA was recovered using the phenol-chloroform method, and the library was constructed using the NEBNext HiFi 2× PCR Master Mix, whose size was analyzed using an Agilent 4200 TapeStation. Finally, sequencing was performed on a NovaSeq 6000 System in PE150 mode.

Quality control of the raw data was performed using Perl scripts developed by Shanghai Rainbow Genome Co. Low-quality reads were removed, and high-quality reads were retained for downstream analysis. Clean reads were compared to the reference genome using BWA software (v0.7.17), and Macs2 software (v 2.2.7.1) was used to call peaks with a threshold *q* value of <0.05. Peak annotations were performed using Homer's annotation Peaks.pl. Differential peak analysis was conducted using the DESeq2 package (v1.38.1) in R software, and $\log_2\text{FoldChange} > 1$ and adjusted *p*-value <0.01 were considered significantly different. Additionally, we restricted the analysis to peaks located only in promoter regions. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed based on the KEGG database, and pathways with *FDR* <0.05 from Fisher's exact test were considered significantly enriched [24].

Cell culture

THP-1 cells were obtained from Sun Yat-sen Memorial Hospital, Sun Yat-sen University. Mouse testicular mesenchymal stromal cell tumor cells MLTC-1 were purchased from Wuhan Pricella Biotechnology Co., Ltd. Both cell lines were cultured in the prepared medium containing RPMI-1640 basal medium plus 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and both cells were placed in a constant temperature culture at 37°C with 5% CO₂.

MLTC-1 cell culture and treatment with dihydrotestosterone (DHT), apalutamide and enzalutamide

MLTC-1 cells were cultured in RPMI 1640 complete medium containing 10% FBS (KeyGEN Biotech, KGM31800N-500, China) supplemented with 1% penicillin-streptomycin. The cells were plated in 6-well plates at a density of 6×10^5 cells/ml, which were treated with apalutamide (ARN-509) (Selleck, S2840, USA) and enzalutamide (Selleck, S1250, USA) at a concentration of 5μM, respectively, for 48 h, and then performed the following experiments.

CCK-8 assay

MLTC-1 treated with apalutamide and enzalutamide was centrifuged and resuspended, and then plated in 96-well plates at a density of 2000 cells per well. CCK-8 reagent (Life-iLab, AC11L054, China) was added at a

concentration of 1:10 per well and incubated at 37 °C for 2 h. The OD values of each concentration group were measured using a spectrophotometer for six days, and the differences over time were compared.

Clone formation

Cells were plated in 6-well plates with 10,000 cells per well and cultured for seven consecutive days to compare the difference in the number of clones formed by cells in different treatment groups.

Cell migration and invasion assay

The cells were resuspended in serum-free medium and added to the top sides of the transwell chambers at a density of 8×10^4 cells per well for the migration assay. In contrast, cells were added at a density of 16×10^4 cells per well with Matrigel matrix gel (Corning, 356234, USA) coated on the chambers for the invasion assay. Next, 10% FBS RPMI 1640 complete medium was added to the bottom of the chambers and incubated for 48 h. The cells were fixed with paraformaldehyde for 30 min, washed with water, stained with 0.1% crystal violet (Beyotime, C0121, China) at room temperature for 10 min, and photographed for observation.

RNA extraction and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

THP-1-induced macrophages treated with APP protein (MedChemExpress, Shanghai, Catalogue number: HY-P72834) were collected, and total RNA was extracted using TRIzol Reagent (Invitrogen, 15596018CN, USA). The concentration and purity of each RNA sample were determined by NanoDrop®ND-one spectrophotometer (Thermo Fisher, USA). QRT-PCR was performed using the BrightCycler Universal SYBR Green qPCR Mix with UDG (ABclonal, RK21219, USA). cDNA was synthesized from 1 μg of RNA (ABclonal, RK20433, USA), and then detected at different concentrations using a real-time fluorescent quantitative PCR system (LightCycler480, Roche, USA) to determine the relative expression of M2 macrophage marker genes in macrophages induced by THP-1 cells after treatment with different concentrations of APP, normalized by β-Actin as an internal reference gene. Threshold cycling values were analyzed using LightCycler480 software, and relative gene expression was assessed using $2^{-\Delta\Delta CT}$. The sequences of synthesized primers (Sangon Biotech, China) were as follows: β-Actin: forward: 5'-TTCCAGCCTTCCTTCCTGGG-3', reverse: 5'-TTGCGCTCAGGAGGAGCAAT-3'. MRC1: forward: 5'-GCCTCGTTGTTTTGCGTCTT-3', reverse: 5'-GAGAACAGCACCCGGAATGA-3'. CD163: forward: 5'-TCCTTGTTGGGATTGTCCTGC-3', reverse: 5'-GGTATCTTAAAGGCTCACTGGGT-3'. IL1B: forward: 5'-AACCTCTTCGAGGCACAAGG-3', reverse: 5'-AGATTC

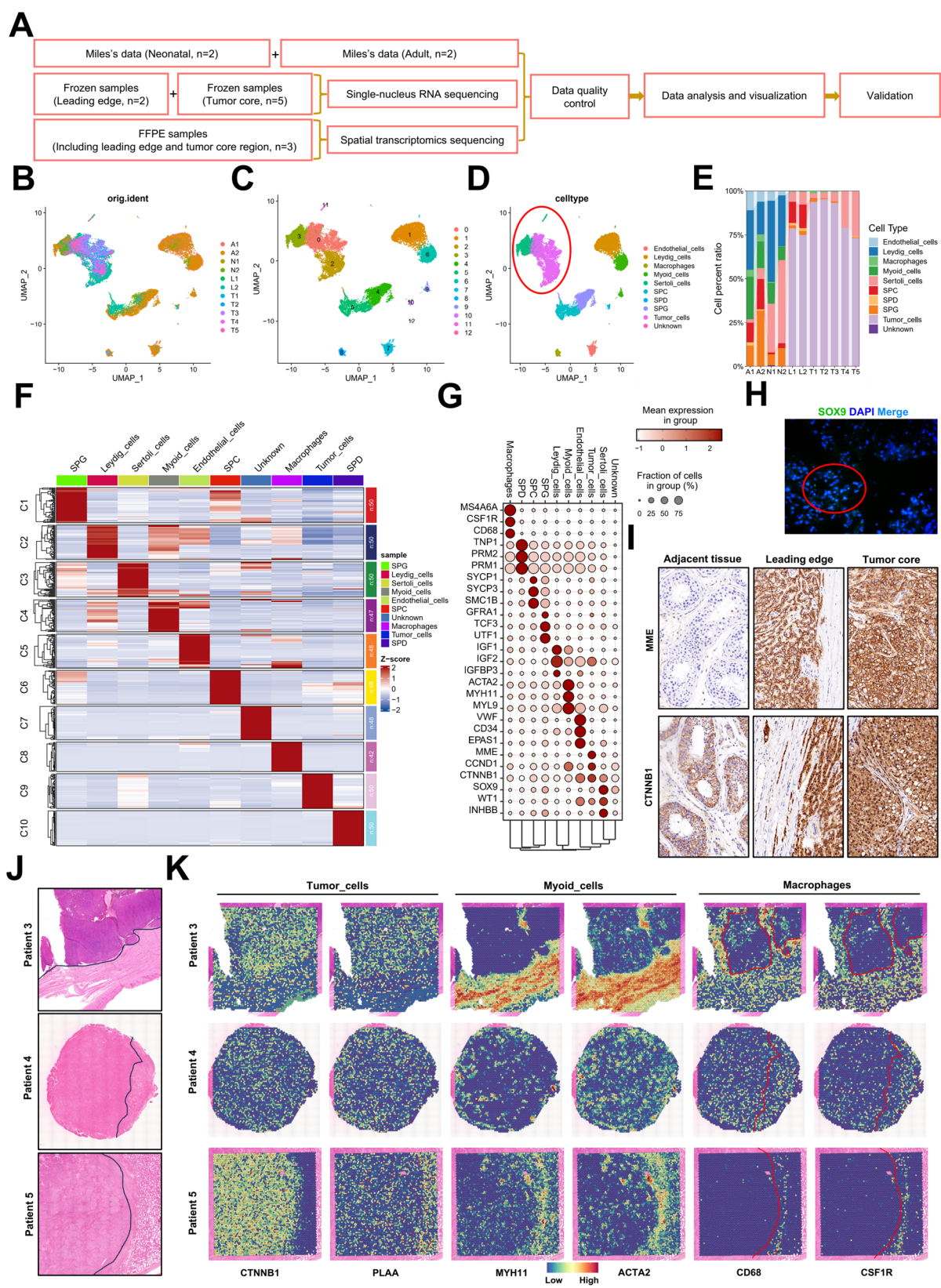


Fig. 1 (See legend on next page.)

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Fig. 1 Single-cell spatial transcriptome profile of TSCST. **(A)** Workflow of this study. **(B)** Uniform manifold approximation and projection (UMAP) of scRNA-seq and snRNA-seq cells recovered from both testicular tissues and tumor tissues labelled by patients. **(C)** UMAP of scRNA-seq and snRNA-seq cells recovered from both testicular tissues and tumor tissues labelled by Seurat clusters. **(D)** UMAP of scRNA-seq and snRNA-seq cells recovered from both testicular tissues and tumor tissues labelled by cell types. **(E)** Bar plots of the proportion of cell type by samples. Adult cases: A1 and A2; Neonatal cases: N1 and N2; Leading edge: L1 and L2; Tumor core: T1, T2, T3, T4, T5. **(F)** Heatmap showing expression levels of DEGs in each cell type. **(G)** Dot plot showing expression levels of specific markers in each cell type. SPG: Spermatogonia; SPC: spermatocyte; SPD: spermatid. **(H)** Representative immunofluorescence images showing expression of SOX9 within Sertoli cells. Nuclei were stained with DAPI ($n=4$). **(I)** IHC images of adjacent tissue, leading edge and tumor core stained for CTNNB1 and MME ($n=3$). **(J)** H & E staining of tumor tissue, and the black line indicated the interface between tumor and normal tissues. **(K)** Feature plots of select cell-type marker genes by ST

GTAGCTGGATGCCG-3'. MMP2: forward: 5'-GTCTGT GTTGTCCAGAGGCA-3', reverse: 5'-TCTGGGGCAGT CCAAAGAAC-3'. ARG1: forward: 5'-ACTTAAAGAAC AAGAGTGTGATGTG-3', reverse: 5'- GTCCACGTCTC TCAAGCCAA-3'.

Statistical analysis

Statistical analysis of data such as in vitro experiments, IHC, and immunofluorescence was performed using GraphPad Prism 5 software. Differences between two independent groups were evaluated with the independent-samples t-test; if normality was satisfied but variances were unequal, Welch's t-test was applied; when normality was violated, the Mann-Whitney test was used as a non-parametric alternative. The comparison between multiple groups was analyzed by variance analysis. Statistical analysis of single-cell sequencing data and spatial transcriptome data was performed using R software (v4.2.1). The pairwise comparison was performed using the ggpubr package (v4.2.3) based on t-test, and the differential gene analysis was performed using the Seurat package (v4.2.1) based on wilcox.test. $P < 0.05$ was considered a statistically significant difference.

Results

Single-cell spatial transcriptome profile of TSCST

In this study, we integrated testicular scRNA-seq data (GSE124263) from two neonatal (N1 and N2) and two adult (A1 and A2) cases with our snRNA-seq data from the tumor leading edge (L1 and L2) and tumor core region (T1, T2, T3, T4, and T5) of TSCST to establish a comprehensive single-cell landscape for TSCST. Additionally, we collected three formalin-fixed and paraffin-embedded samples for ST sequencing and constructed a ST profile for TSCST (Fig. 1A). All sequenced samples were subjected to hematoxylin and eosin staining, and the pathological diagnosis was reconfirmed by pathologists to verify that all samples represented tumor tissues (Fig. 1J, Supplementary Fig. 1A).

After batch-effect correction, we successfully integrated all samples (Fig. 1B). Thirteen cell clusters (0–12) were obtained after dimensionality reduction analysis (Fig. 1C). We annotated nine major cell types by leveraging known marker genes for the relevant cell types. The tumor and Sertoli cells displayed the closest positional

relationship in the UAMP plot (Fig. 1D). Furthermore, Sertoli and tumor cells were captured in the leading edge and core regions (Fig. 1E). To validate the reliability of our cell-type annotations, we also assessed the expression specificity and favourable ratio of the marker genes of these cells using a heatmap and dot plot, which showed that all these marker genes had high specificity and a high positive rate (Fig. 1F and G). To further validate the accuracy of tumor cell annotation, we performed inferCNV analysis, which revealed that tumor cells exhibited significantly higher levels of copy number variations (Supplementary Fig. 1B). Immunofluorescence staining for the Sertoli cell marker SOX9 further confirmed the presence of Sertoli cells in TSCST tissues (Fig. 1H). Additionally, according to the IHC results, tumor cells expressed the marker genes MME and CTNNB1 in both the leading edge and core regions of the results of IHC (Fig. 1I, Supplementary Fig. 1C).

To decode the spatial gene expression profile of TSCST, we conducted spatial transcriptome sequencing of paraffin-embedded tissue samples from the three patients with TSCST. Based on the pathohistological annotations, we divided the samples into tumor core and leading-edge regions (the upper-left region of Patient 3 and the left regions of Patient 4 and Patient 5 were identified as tumor core regions, Fig. 1J). Following these annotations, the tumor markers CTNNB1 and PLAA were significantly upregulated in tumor core regions, while markers MYH11 and ACTA2 associated with myoid cells, as well as CD68 and CSF1R associated with macrophages, were predominantly expressed in the leading-edge regions (Fig. 1K).

Moreover, we found that more genes were expressed in the tumor core regions than in the leading-edge areas (Supplementary Fig. 2A–C), suggesting an aberrantly activated transcriptional level in tumor cells.

TSCST exhibits an immune-cold feature, suggesting that it may have a low response to immune checkpoint blockade (ICB)

TSCST were evaluated within the context of immune-cold versus immune-hot tumor classifications, which are known to exhibit differential responses to immune checkpoint blockade (ICB). This study assessed the immune landscape of TSCST and its potential responsiveness to

ICB [24]. Here, we aimed to evaluate the tumor immune microenvironment of TSCST and predict the therapeutic benefits of ICB. We analyzed the expression of the T cell marker genes CD3D and CD3G. These genes were only weakly expressed in the non-tumor regions of the leading edge and barely represented in the tumor core (Fig. 2A-C), which has also been confirmed by IHC (Fig. 2D). These observations imply that T cells do not penetrate the tumor tissue, suggesting that TSCST can be characterized as an immune-rejecting tumor, also called an immune-cold tumor [25].

Furthermore, the B-cell marker genes CD19 and MS4A1 exhibited low expression in all samples (Fig. 2E-G). The IHC results also revealed a lack of B cells in both the tumor leading edge and core region (Fig. 2H), corroborating the results of the absence of T and B cells from our snRNA-seq. To assess the therapeutic response of TSCST to ICB, we analyzed the expression of immune checkpoint genes in different cell types using scRNA-seq. We identified their spatial expression via spatial transcriptome (ST) data (Fig. 2I-L). The results suggested that there was no or markedly low expression of ICB genes, including CD274, PDCD1, SIGLEC15, and LAG3, in tumor cells, which was consistent with the IHC result that the tumor core and leading edge were barely infiltrated by PDCD1+ cells (Fig. 2M). As one of the most promising immune therapy strategies, a PD-1/PD-L1 inhibitor is a breakthrough for the therapy of some refractory tumors; however, in all cases we tested, tumors lacked PD-1, indicating the failure of ICB treatment [26]. Taken together, TSCST is more likely an immune-cold tumor (i.e. no significant immune cell infiltration but a high cancer cell fraction) because of the minimal infiltration of T cells and B cells, and, consistently, low expression of immune checkpoints, which indicated that it might not respond to ICB therapy, but this still needs further clinical studies [27].

TSCST may originate from epithelial-mesenchymal transition (EMT)-hyperactivated fetal Sertoli cells

Previous studies proposed that TSCST originate from mesenchymal, Sertoli, and other mesenchymal stromal cells [14]. However, the origin of TSCST remains unclear. Therefore, we extracted tumor, Sertoli, macrophages, mesenchymal, endothelial, and myoid cells for the correlation analysis. Consistent with the previous UMAP plot, tumor cells exhibited the highest similarity to Sertoli cells (Supplementary Fig. 3A).

Subsequently, we extracted tumor cells and Sertoli cells for principal component analysis and found that neonatal Sertoli cells were much closer to tumor cells regarding principal components (Supplementary Fig. 3B). In addition, tumor cells hardly expressed S100A6 in mature Sertoli cells, but expressed markers associated with

immature Sertoli cells (e.g. BMPR2, TBX3, and SOX11) (Supplementary Fig. 3C and 3D). Furthermore, spatial transcriptomic data showed high expression of BMPR2 and TBX3 in the tumor region (Supplementary Fig. 3E, Supplementary Fig. 4A-B). These observations further substantiate the proximity of tumor cells to immature Sertoli cells.

Next, we analyzed the developmental relationship between tumor cells and Sertoli cells, which indicated that tumor cells were more immature than neonatal Sertoli cells (Supplementary Fig. 3F and 3G), suggesting that tumor cells may originate from more naïve cells than neonatal Sertoli cells. Therefore, we analyzed the differentially expressed genes in tumor cells and Sertoli cells and obtained 645 significantly upregulated genes in tumor cells and 1,314 upregulated genes considerably in Sertoli cells (Supplementary Fig. 3H). The significantly upregulated genes in tumor cells were mainly enriched in signaling pathways that regulate the cytoskeleton, morphology, and intercellular junctions, including cell morphogenesis, RHO GTPase cycle, regulation of GTPase activity, and cell junction organization. In contrast, the upregulated genes in Sertoli cells were mainly involved in signaling pathways such as cellular response to chemical stress, protein folding, and protein processing in the endoplasmic reticulum, which modulate the hormone stimulation and protein secretion of Sertoli cells (Supplementary Fig. 3I). These findings suggest that tumorigenesis may stem from the activation of signaling pathways associated with cellular morphology and cytoskeletal dynamics, coupled with the inadequate expression of genes essential for Sertoli cell function during differentiation from a more naïve cellular state than neonatal Sertoli cells.

To further explore the developmental origin and underlying molecular mechanisms of TSCST cells, we accessed scRNA-seq data (GSE143356) from embryonic and fetal testicular tissues at the gestational ages of 6, 7, 8, 12, 15, and 16 weeks (Supplementary Fig. 5A), followed by cell type annotation based on the marker genes expressed in each cluster (Supplementary Fig. 5B) [28]. We extracted Sertoli cells, performed an integrated analysis with our snRNA-seq data (Supplementary Fig. 5C and 5D), and investigated the pseudotime trajectory of tumor cells, Sertoli cells, and progenitor cells (Fig. 3A). The results indicated that the progenitor and Sertoli cells occupied the early stages of development (Fig. 3B). Moreover, as the pseudotime trajectory progressed, the number of progenitor and Sertoli cells gradually decreased, with tumor cells beginning to merge at the point where the progenitor and Sertoli cells exhibited the lowest levels (Fig. 3C). Evolutionary tree analysis further suggested that Sertoli cells represent a progenitor stage for TSCST tumor cells (Fig. 3D).

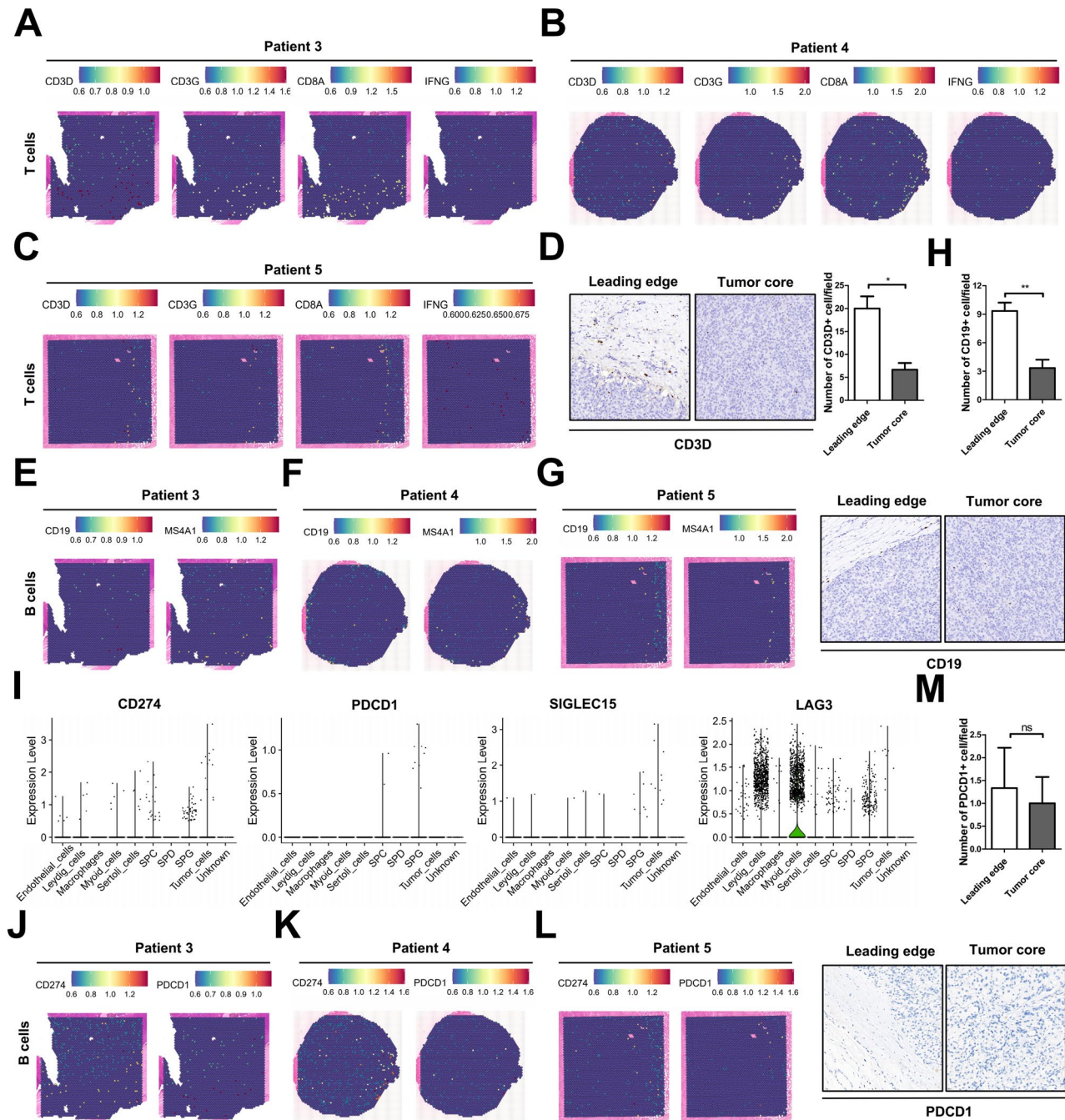


Fig. 2 TSCST exhibits an immune-cold feature and is predicted to have a low response to ICB. **(A)** Feature plots of select T cell-associated genes by ST in Patient 3. **(B)** Feature plots of select T cell-associated genes by ST in Patient 4. **(C)** Feature plots of select T cell-associated genes by ST in Patient 5. **(D)** IHC images of the leading edge and tumor core tissues stained for T-cell marker gene (n=4). **(E)** Feature plots of select B cell-associated genes by ST in Patient 3. **(F)** Feature plots of select B cell-associated genes by ST in Patient 4. **(G)** Feature plots of select B cell-associated genes by ST in Patient 5. **(H)** IHC images of the leading edge and tumor core tissues stained for B-cell marker gene (n=5). **(I)** Violin plots showing the expression level of representative immune checkpoint genes across the cell types. The y-axis shows the normalized read count. **(J)** Feature plots of select immune checkpoint genes by ST in Patient 3. **(K)** Feature plots of select immune checkpoint genes by ST in Patient 4. **(L)** Feature plots of select immune checkpoint genes by ST in Patient 5. **(M)** IHC images of the leading edge and tumor core tissues stained for PDCD1 (n=5). The P value was calculated by Mann–Whitney test. *P < 0.05, **P < 0.01, n.s: no significance

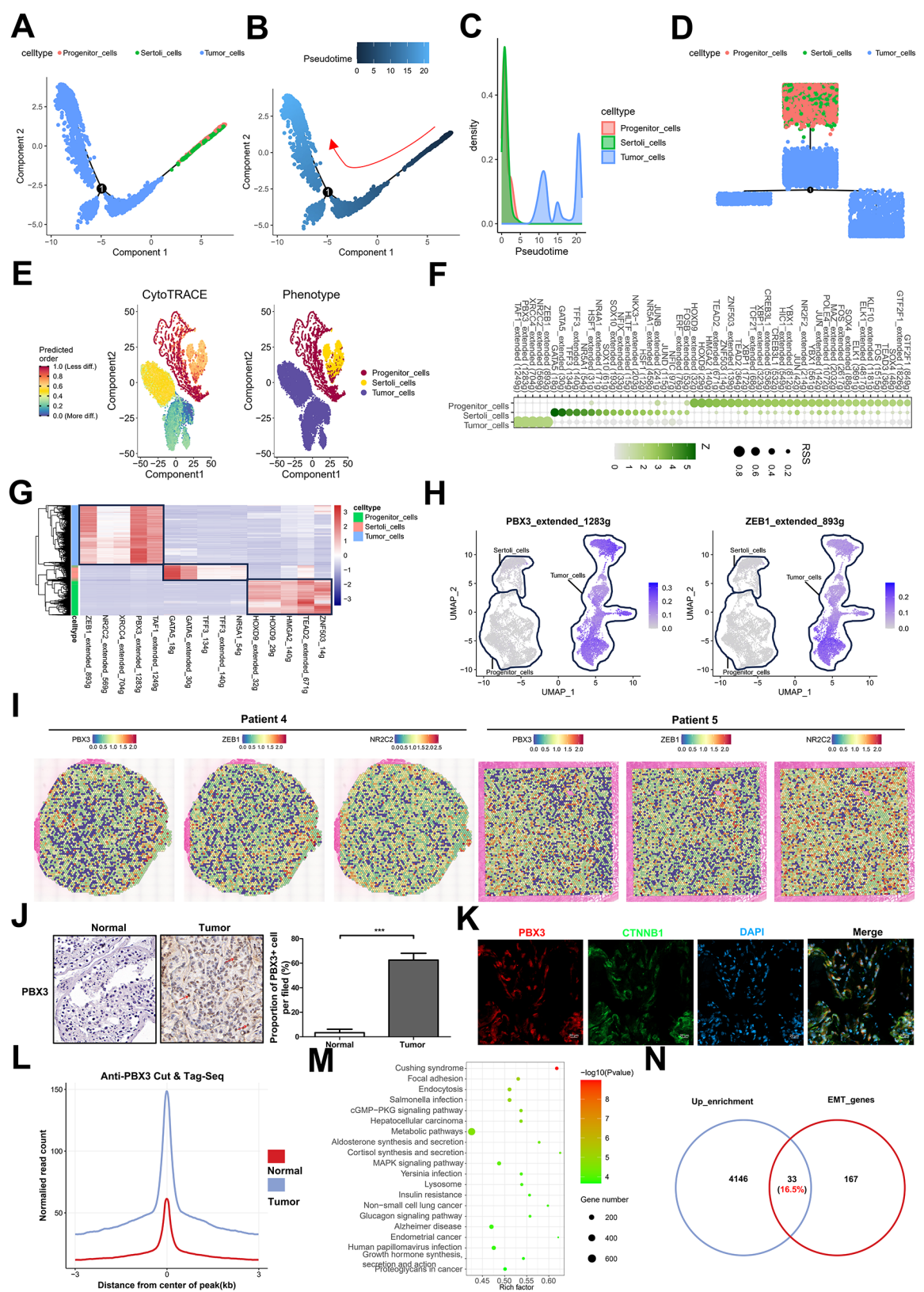


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Fig. 3 TSCST may originate from EMT-hyperactivated fetal Sertoli cells. **(A)** Pseudotime of progenitor cells, Sertoli cells, and tumor cells inferred by Monocle2. Each point corresponds to a single cell, colored by cell types. **(B)** Pseudo-time of progenitor cells, Sertoli cells, and tumor cells inferred by Monocle2. Each point corresponds to a single cell, colored by pseudotime. **(C)** Density of distinct cell types along pseudotime trajectory. **(D)** Evolution tree of progenitor cells, Sertoli cells and tumor cells. **(E)** CytoTRACE of progenitor, Sertoli, and tumor cells, colored by predicted order and cell types. **(F)** Dot plot shows regulon specificity score of TRs in progenitor, Sertoli, and tumor cells. RSS: regulon specificity score. **(G)** The top five significantly over-expressed regulon activity heatmaps in progenitor, Sertoli, and tumor cells. **(H)** AUCell-based cell-level regulon activity of PBX3_extended_1283g and ZEB1_extended_893g. **(I)** Feature plots of select tumor cells upregulated TFs by ST in Patient 4 and Patient 5. **(J)** IHC images of normal and tumor tissues stained for upregulated TF in tumor cells (PBX3, Normal: n=3, Tumor: n=3). The P value was calculated by t-test. **(K)** Immunofluorescence staining for PBX3 and the tumor cell-specific marker CTNNB1. **(L)** Distribution of PBX3 peaks in the vicinity of 3 kb upstream and downstream of the transcriptional start site. **(M)** Enrichment analysis of PBX3 targeted genes. **(N)** Venn plot showing the overlapping EMT signature and PBX3-targeted genes. The P value was calculated by t-test. *P < 0.05, ***P < 0.001, ****P < 0.001. Red arrow: Representative tumor cells with positive IHC staining

Additionally, we used CytoTRACE to assess the differentiation potential of different cell types, which revealed that progenitor cells possessed the highest probability of differentiation, followed by Sertoli cells, with tumor cells exhibiting the lowest potential (Fig. 3E). To identify the master transcription regulon (TR) responsible for the malignant transformation of tumor cells, we analyzed the differential TR across distinct cell types. Notably, the TR TAF1_extended (1249 genes), PBX3_extended (1283 genes), XRCC4_extended (704 genes), NR2C2_extended (569 genes), and ZEB1_extended (893 genes) were identified as the top five with the highest regulon specificity score in tumor cells (Fig. 3F). Moreover, both regulon activity heatmaps (Fig. 3G) and UMAP plot of AUCell-based regulon activity (Fig. 3H) showed significantly up-regulated regulon activity in tumor cells. The TFs within these TRs, including PBX3, NR2C2, and ZEB1 were expressed medially in tumor regions (Fig. 3I). Next, we performed correlation analysis of the gene expression patterns of PBX3 and ZEB1 based on the spatial transcriptomics data, which indicated a potential co-expression or co-regulatory relationship between PBX3 and ZEB1 (Supplementary Fig. 6A-C). Notably, ZEB1, recognized as a key regulator of EMT, is elevated in tumor cells, suggesting that these cells acquire an EMT signature during tumorigenesis. Furthermore, TAF1 has been identified as an oncogene in lung cancer owing to its role in promoting EMT [29]. PBX3 also serves as part of the EMT regulatory network, promoting tumor progression and poor prognosis in various cancer types [30, 31].

To validate the pivotal role of these upregulated EMT-associated TFs, we performed IHC staining of TSCST and normal testicular tissue samples. PBX3-positive cells were predominantly localized to the tumor tissues (Fig. 3J) and highly expressed in tumor cells (as shown by the red arrow in Fig. 3J). Moreover, we also confirmed the co-localization of the TF PBX3 and the tumor marker gene CTNNB1 through immunofluorescence (Fig. 3K). Given the role of PBX3 in binding to DNA regions, we conducted a CUT&Tag assay using an anti-PBX3 antibody (Fig. 3L). This analysis revealed a significant peak in the transcription start site region within tumor tissues compared to normal tissues (Supplementary Fig. 7A-C).

Enrichment analysis of PBX3-regulated genes highlighted significant pathways, including the MAPK signaling pathway and focal adhesion, which are both associated with EMT (Fig. 3M). Notably, there was 16.5% overlap between genes in the EMT signature and PBX3-upregulated genes (Fig. 3N). Previous studies have revealed that EMT signaling is highly enriched in the immune desert type and can contribute to immune escape through multiple routes, consistent with our previously described immune desert TME [32, 33]. Combined analyses of the pseudotime trajectory and TFs suggested that TSCST might originate from EMT-hyperactivated fetal Sertoli cells, in which PBX3 plays a critical role.

TSCST shows treatment potential for AR inhibitors in vitro

Testosterone is one of the most essential androgens in humans. It exerts its effects by binding to AR, thereby regulating the transcription of various genes and promoting male sexual differentiation [34], spermatogenesis [35], hair growth [36], and other physiological processes. In males, testosterone is primarily produced by Leydig cells in the testes [37]. Considering the dependence of hormone-related diseases, including cancers of the prostate, breast, ovary, and pancreas, on androgens and the importance of their target genes during spermatogenesis, we next explored the expression of androgens in the TSCST and their potential treatment options related to androgen inhibitors [38, 39]. Our results showed that tumor cells expressed higher AR, EP300, and SMAD3 levels than progenitor and Sertoli cells (Fig. 4A).

Additionally, the tumor cells expressed higher levels of AR-regulated genes, including ACPP, USP9Y, and DDX3Y (Fig. 4B). Consistent with the transcriptomic results, ST showed significantly distinct expression of these genes in the tumor regions (Fig. 4C-D). Moreover, we analyzed the correlation between AR and EP300 with ACPP and DDX3Y, which suggested a positive correlation between AR and EP300 and their target genes (Fig. 4E). Although AR was expressed in almost all patients with TSCST, the expression levels of AR showed high inter-patient heterogeneity (Fig. 4F). Given the high expression of AR and its target genes, patients with TSCST may exhibit growth

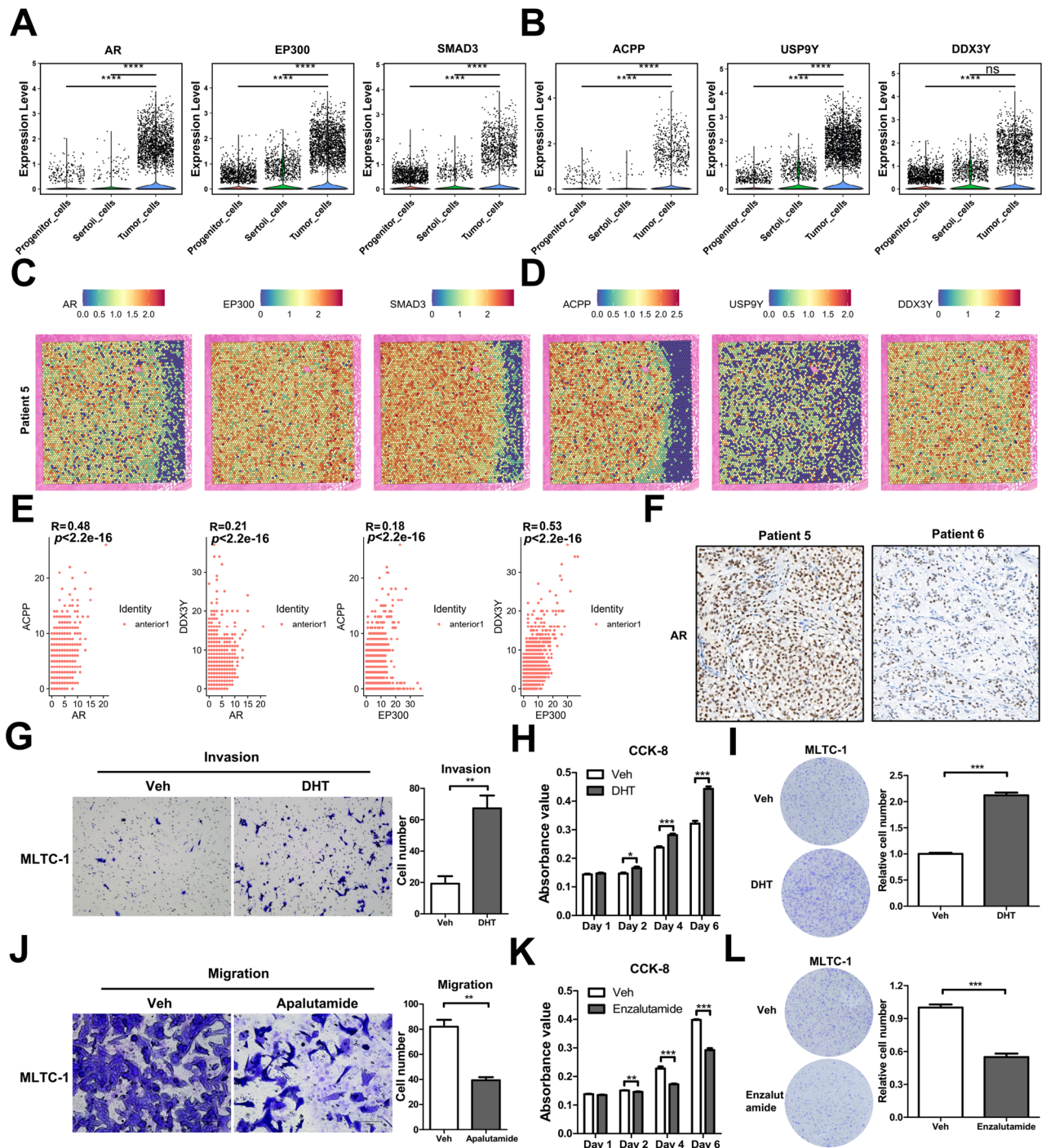


Fig. 4 TSCST shows treatment potential for AR inhibitors. **(A)** Violin plots showing the expression level of AR and representative AR-related genes (EP300, SMAD3) across the cell types. The y-axis shows the normalized read count. **(B)** Violin plots showing the expression level of AR targeted genes (ACPP, USP9Y, DDX3Y) across the cell types. The y-axis shows the normalized read count. **(C)** Feature plots of AR and representative AR-related genes (EP300, SMAD3) by ST. **(D)** Feature plots of AR targeted genes (ACPP, USP9Y, DDX3Y) by ST. **(E)** Correlation analysis of AR-related TFs and targeted genes. **(F)** IHC staining (n=3) for AR in Patient 5 and Patient 6. **(G)** Representative results of the trans-well invasion assays show DHT's effects on the invasion abilities of MLTC-1 cells (n=3). **(H)** Bar plot showing the effects of DHT on the proliferation of MLTC-1 cells (n=4). **(I)** Representative result showing the effects of DHT on the colony formation abilities of MLTC-1 cells (n=3). **(J)** Representative results of the trans-well migration assays showing the effects of Enzalutamide on the migration abilities of MLTC-1 cells (n=3). **(K)** Bar plot showing the effects of DHT on the proliferation of MLTC-1 cells (n=4). **(L)** Representative result showing the effects of Enzalutamide on the colony formation abilities of MLTC-1 cells (n=3). The P value was calculated by t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

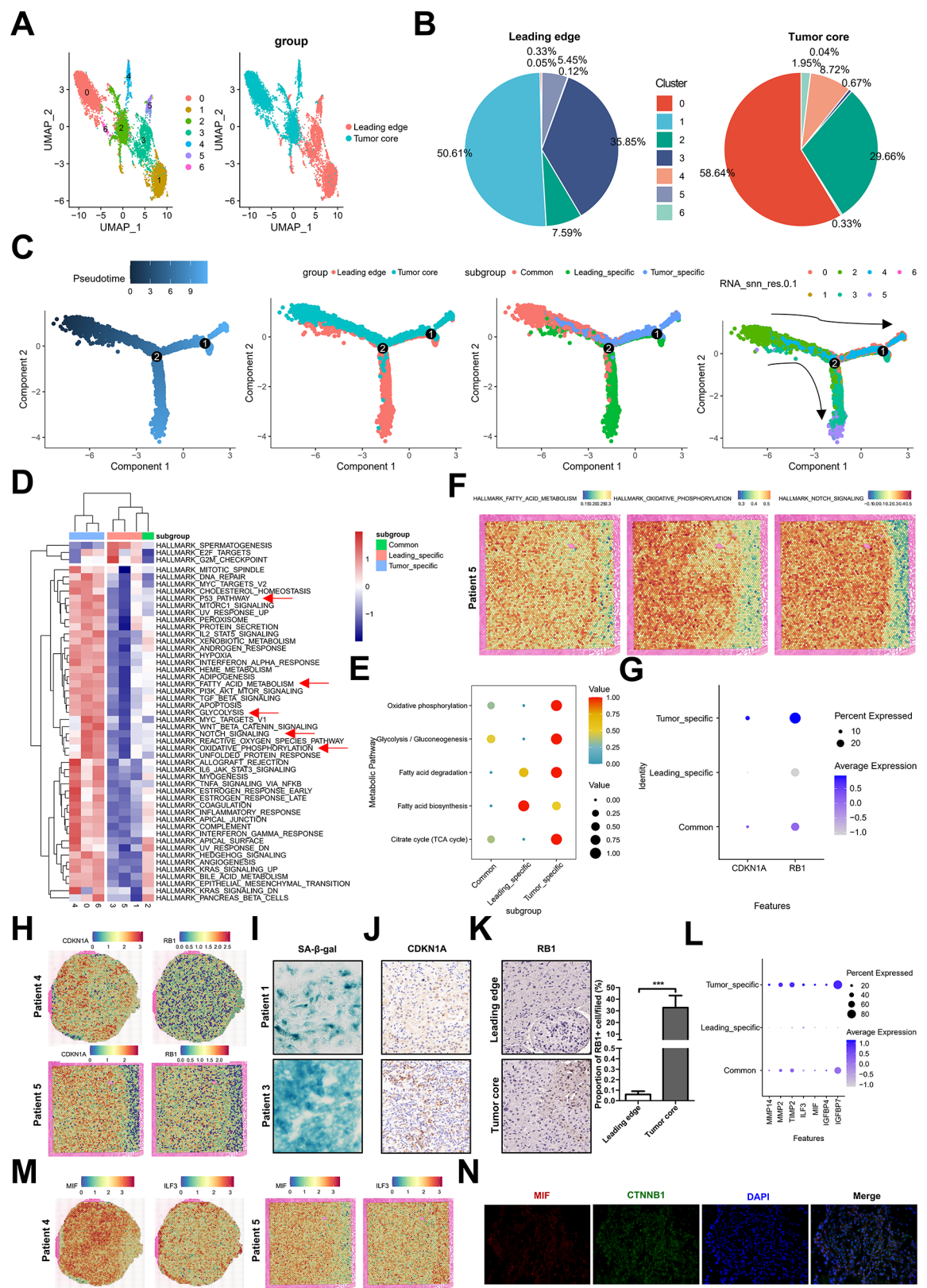


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Fig. 5 Heterogeneity analysis of tumor cells in TSCST. **(A)** UMAP of all tumor cells labelled by Seurat clusters and tissue locations. **(B)** Pie chart showing the proportion of distinct clusters in the leading edge and the tumor core, respectively. **(C)** Pseudo-time of leading edge and tumor core cells inferred by Monocle2. Each point corresponds to a single cell, colored pseudotime, tissue locations, subgroups and Seurat clusters. **(D)** The heat map shows the Hallmark terms enriched in distinct subgroups, and the red arrow indicates metabolism-related pathways. **(E)** Dot plot showing the metabolic pathways grouped by subgroups. **(F)** Feature plots of select metabolic hallmark signatures by ST in Patient 5. **(G)** Dot plot showing the normalized expression of CDKN1A and RB1 grouped by subgroups. **(H)** Feature plots of CDKN1A and RB1 by ST in Patient 4 and Patient 5. **(I)** β -galactosidase staining in Patient 1 and Patient 3 ($n = 2$). **(J)** IHC images of tumor tissues from Patient 1 and Patient 3 stained for CDKN1A ($n = 3$). **(K)** IHC images of tumor tissues of the leading edge and the tumor core stained for RB1 ($n = 3$). **(L)** Dot plot showing the normalized expression of senescence-associated secreted genes grouped by subgroups. **(M)** Feature plots of senescence-associated secreted genes by ST in Patient 4 and Patient 5. **(N)** Representative immunofluorescence images showing expression of MIF and CTNNB1 within tumor cells. Nuclei were stained with DAPI. T-test, *** $P < 0.001$

dependence on androgens and show therapeutic potential for AR inhibitors.

To further verify the biological roles of androgen in TSCST cells, we used DHT, which is the most potent hormone among the androgens and is considered a pure androgen, as it cannot be converted into estrogen, to treat mouse Leydig tumor cells (MLTC-1) in vitro [40]. We found that cells treated with DHT showed a higher invasion ability of MLTC-1 cells (Fig. 4G). Moreover, DHT significantly promoted the proliferation and colony formation abilities of MLTC-1 cells (Fig. 4H–I). The AR inhibitor, enzalutamide, consistently showed an opposite biological function to DHT in MLTC-1 cells (Fig. 4J–L) [41]. These results suggest that TSCST cells show higher expression levels of the AR gene than normal cells, indicating the treatment potential of AR inhibitors in vitro.

Heterogeneity analysis of tumor cells in TSCST

Tumor heterogeneity is frequently associated with tumor metastasis and drug resistance [42]. We thoroughly extracted the tumor cells for re-clustering to investigate the tumor cells' heterogeneity in TSCST and obtained seven clusters ranging from cluster 0 to 6 (Fig. 5A). Cluster 2 (common), shared by the two groups of samples, was identified by analyzing the percentage of each cluster in the leading edge and tumor core regions (Fig. 5B). Clusters 1, 3, and 5 were unique to the leading edge group and are named "Leading_specific". Clusters 0, 4 and 6 were unique to the tumor core group and are named "Tumor_specific".

Furthermore, we analyzed the developmental trajectories of these cells. We observed that either the cluster specific to the tumor leading edge or that particular to the tumor core region was generated from cluster 2 (Fig. 5C). GSVA enrichment analysis of the tumor-specific cluster, leading-specific cluster, and standard cluster in the former two areas showed that the tumor-specific cluster was significantly enriched in the P53 signaling pathway, fatty acid metabolism, glycolysis, the NOTCH signaling pathway, and oxidative phosphorylation (Fig. 5D). This indicated that these cells may have metabolic disorders. Furthermore, we analyzed the enrichment of metabolic pathways in these clusters using the scMetabolism package. We found that metabolic pathways such as oxidative

phosphorylation, glycolysis, fatty acid degradation, and the tricarboxylic acid cycle were all significantly enriched in the tumor-specific cluster (Fig. 5E).

Furthermore, spatial transcriptome data showed enrichment of fatty acid metabolism, oxidative phosphorylation, and NOTCH signaling pathways (Fig. 5F, Supplementary Fig. 8A). Meanwhile, the P53 and NOTCH signaling pathways have been proven to be classical signaling pathways that regulate cellular senescence [43, 44]. Therefore, we hypothesized that these cells may be heading toward senescence. The senescence-associated marker genes CDKN1A and RB1 were also prominently overexpressed in tumor-specific clusters (Fig. 5G), and the same trend was observed in the spatial transcriptomic data (Fig. 5H, Supplementary Fig. 8B). Subsequent β -galactosidase staining also confirmed that tumor cells in the core region exhibited a senescent phenotype (Fig. 5I). IHC also revealed a high expression of CDKN1A and RB1 in the tumor core region (Fig. 5J and K). We also found that the senescence-associated secretory factors MIF, ILF3, MMP2, and IGFBP7 were elevated in the tumor core region (Fig. 5L and M, Supplementary Fig. 8C). Finally, we verified the expression of MIF in the tumor region using immunofluorescence (Fig. 5N). These data indicated the transition of tumor cells in the tumor core region of TSCST to a senescence-like state.

Tumor cells in the invasive zone exhibit a more malignant phenotype

Cells located at the leading edge of the tumor often exhibit distinct characteristics compared with cells at other sites [45]. We first analyzed the cell cycle characteristics of all tumor cells. We found that, compared to the leading-specific and common clusters, a greater proportion of tumor-specific cells entered the G2/M phase (Fig. 6A), and these cells highly expressed the G2/M phase-related genes TOP2A, SMC4, CTCE, CBX5, and CKAP5 (Fig. 6B). Subsequently, we found that three genes, TOP2A, SMC4, and CKAP5, were expressed in the tumor core region and leading edge by analyzing the spatial transcriptome data (Fig. 6C). After clustering the spatial spots (Fig. 6D), it was demonstrated that tumor cluster 0, which was closer to the leading edge, expressed higher levels of TOP2A, SMC4, and CKAP5 than tumor

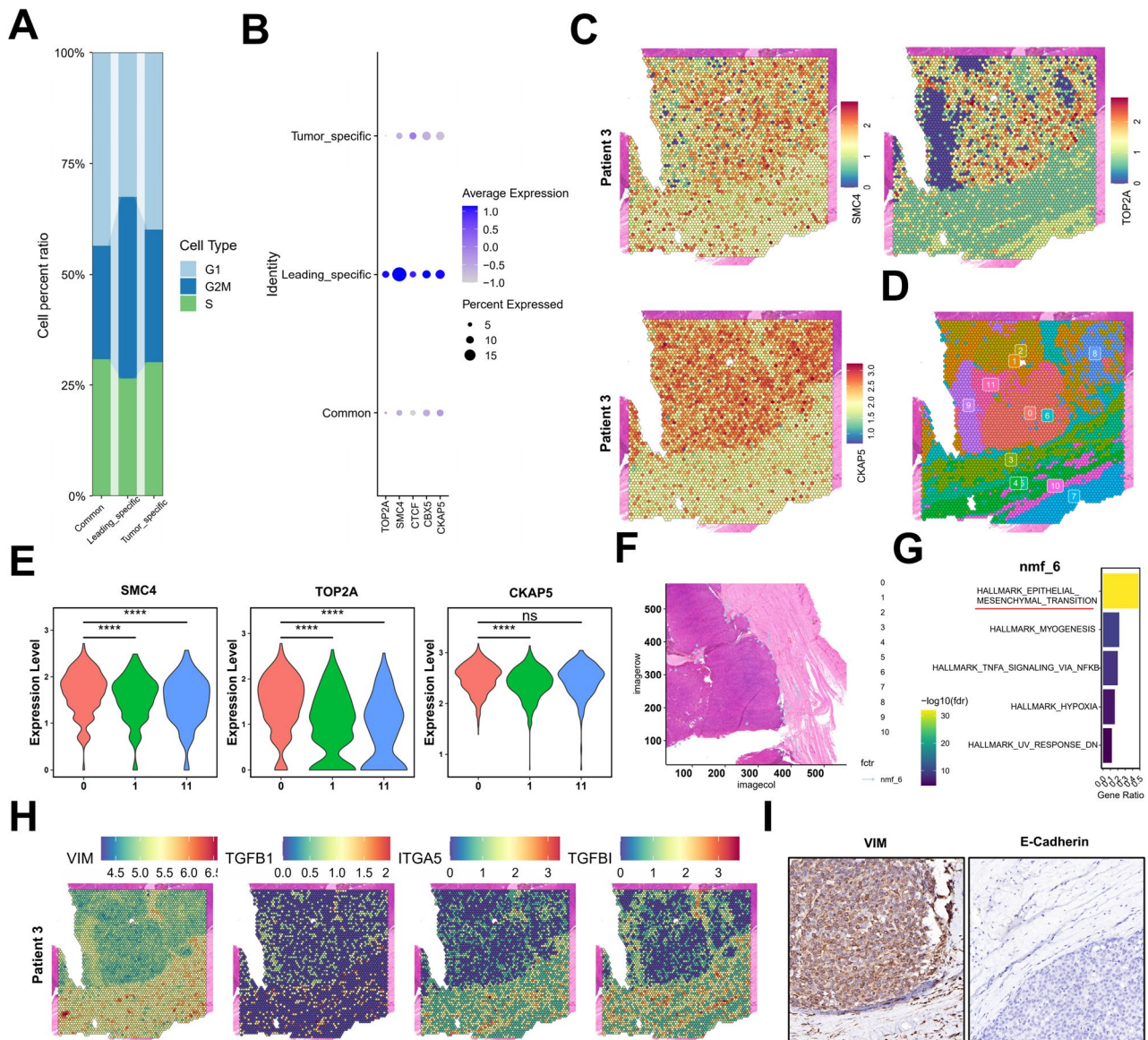


Fig. 6 Tumor cells in the leading edge exhibit a more malignant phenotype. **(A)** Bar plots the proportion of different cell cycle cells across all subgroups. **(B)** Dot plot showing the G2M phase-related genes TOP2A, SMC4, CTCF, CBX5 and CKAP5 in distinct tumor cell subgroups. **(C)** Feature plots of G2M phase-related genes by ST in Patient 3. **(D)** Unbiased clustering of ST spots. **(E)** Violin plots showing the expression level of G2M phase-related genes across the Seurat clusters. The y-axis shows the normalized read count. T-test, **** $P < 0.0001$, ns: no significance. **(F)** Spatial ecological niche of the TSCST tissue samples employing NMF. **(G)** Bar plot showing the top 5 enriched hallmark terms upregulated in nmf_6. **(H)** Feature plots of EMT-related genes by ST in Patient 3. **(I)** IHC images of tumor tissues (n=4) from the leading edge stained for CDH1 and VIM

clusters 1 and 11, which were further away from the leading edge (Fig. 6E).

We also analyzed the spatial ecological niche of TSCST tissue samples using non-negative matrix factorization (NMF). We obtained 11 containing 0–10 nmfs, one of which, nmf_6, were notably abundant at the tumor-normal interface (Fig. 6F). Enrichment analysis revealed a remarkable enrichment of EMT signals (Fig. 6G). In the spatial transcriptomics data, the EMT-related genes VIM, TGFB1, ITGA5, and TGFBI were highly expressed at the leading edge (Fig. 6H, Supplementary Fig. 9A and

9B). Finally, IHC confirmed that VIM, which is related to the mesenchymal cell-like phenotype, was present at the leading edge. In contrast, E-cadherin, which is associated with the epithelial cell-like phenotype, was not detected in this area (Fig. 6I). These data suggest that tumor cells at the leading edge exhibit more robust proliferation and EMT phenotypes.

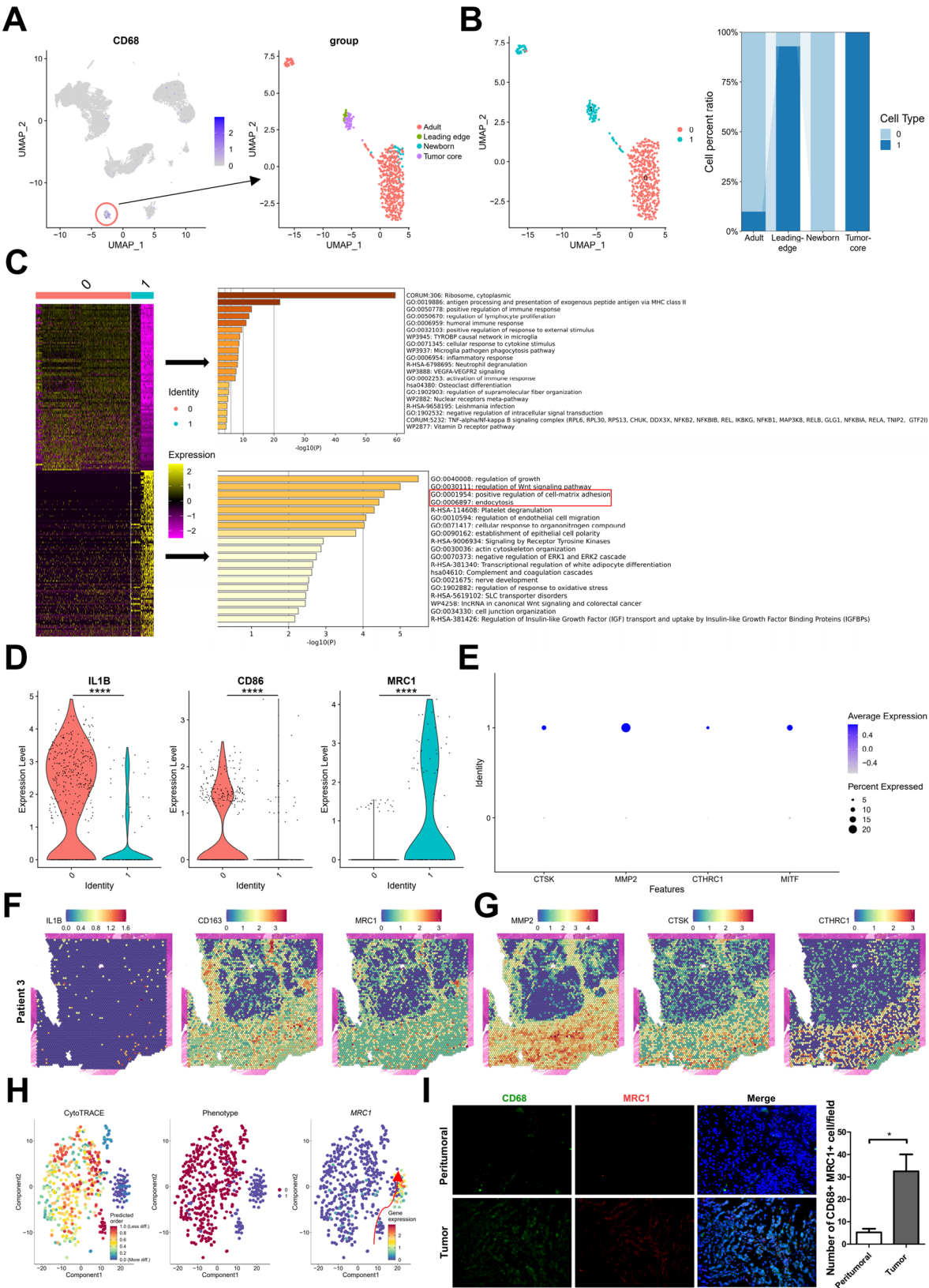


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Fig. 7 Macrophages in TSCST tend to polarize toward the M2 phenotype. **(A)** UMAP of all cells and re-clustering of CD68+ macrophages colored by samples. **(B)** Left, UMAP of all macrophages colored by Seurat clusters. Right, Bar plots the proportion of different cell cycle cells across all samples. **(C)** The heatmap shows the DEGs in distinct clusters and the GO and KEGG enrichment analysis, respectively. **(D)** Violin plots showing the expression level of M1 and M2-associated genes across the Seurat clusters. The y-axis shows the normalized read count. Wilcoxon-test, **** $P < 0.0001$. **(E)** Dot plot showing the normalized expression of phagocytosis-related genes CTSK, MMP2, CTHRC1 and MITF across different Seurat clusters. **(F)** Feature plots of M1 and M2-associated genes by ST in Patient 3. **(G)** Feature plots of phagocytosis-related genes by ST in Patient 3. **(H)** CytoTRACE of all macrophages colored by predicted order, Seurat clusters, and expression of MRC1. **(I)** Representative immunofluorescence images showing expression of CD68 and MRC1 within macrophages. Nuclei were stained with DAPI. ($n = 4$), The P value was calculated by t-test, * $P < 0.05$

Macrophages in TSCST might tend to polarize toward the M2 phenotype

Macrophages play an essential role in regulating the tumor microenvironment. Our previous studies found that macrophages were enriched in the TSCST in the invasive zone, suggesting they may have a necessary function in tumor progression. We separately extracted CD68-positive macrophages for re-clustering (Fig. 7A). We obtained two clusters, clusters 0 and 1. Macrophages in the core and invasive zones were predominant in cluster 1 (Fig. 7B). Enrichment analysis of the marker genes of Cluster 1 revealed that the functions of this cluster were mainly enriched in positively regulated signaling pathways, such as extracellular matrix adhesion and endocytosis (Fig. 7C). Classical M1 macrophage markers IL1B and CD86 were barely expressed in cluster 1, whereas M2 macrophage marker MRC1 was significantly overexpressed in cluster 1 (Fig. 7D). These data suggest that cluster 0 might be an M1 macrophage, and cluster 1 might be an M2 macrophage.

Additionally, the macrophage phagocytosis-related genes CTSK, MMP2, CTHRC1, and MITF were overexpressed in cluster 1 (Fig. 7E). Based on the spatial transcriptomic data, we also found that the M1 macrophage (IL1B) was rarely expressed in the macrophage region. In contrast, the marker genes of M2 macrophages (CD163 and MRC1) were upregulated in the macrophage region (Fig. 7F, Supplementary Fig. 10A). Consistent with the scRNA-seq data, three macrophage phagocytosis-associated genes, MMP2, CTHRC1, and MITF, exhibited comparable enrichment levels within the macrophage region (Fig. 7G, Supplementary Fig. 10B). Moreover, the marker gene CD68 of macrophages in the spatial transcriptome data significantly correlated with the markers of M2 macrophages and macrophage phagocytosis-related genes (Supplementary Fig. 10C and 10D). These data suggest that macrophages in tumor tissues and their surrounding tissues may be polarized toward the M2 phenotype and exhibit enhanced phagocytosis. Furthermore, macrophages differentiated from cluster 0 to cluster 1 according to developmental potential analysis, and MRC1 expression was also elevated along the developmental trajectory (Fig. 7H). Finally, immunofluorescence confirmed the co-localization of CD68 and the M2 macrophage marker MRC1 (Fig. 7I).

Disordered cellular communication in TSCST

Abnormalities in cellular communication are involved in developing a wide range of tumors [13, 46, 47]. We first analyzed the differences in the number and intensity of cellular communications between the leading-edge and tumor core groups using CellChat software. We found that the number and intensity of cellular communication were significantly higher in samples from the tumor core group (Fig. 8A and B). There was stronger communication between endothelial cells and tumor cells, as well as between Sertoli cells and tumor cells, in the samples of the tumor core group (Fig. 8C). Furthermore, we found that APP-CD74 signaling received by macrophages was stronger in both the leading-edge and tumor core groups (Fig. 8D and E). In the tumor core region samples, macrophages sent stronger MDK-NCL signals to the tumor cells (Fig. 8F). For spatial transcriptome data, we found that APP and NCL were relatively highly expressed in the tumor region, while CD74 and MDK were relatively highly expressed in the macrophage-enriched region (Fig. 8G and H). Moreover, the APP and MK signaling pathways have complex communications between cell clusters in different spatial locations (Fig. 8I). In both the tumor core and leading-edge groups, APP was mainly expressed in tumor cells, and CD74 was mainly expressed in macrophages (Fig. 8J). This suggests that APP-CD74 communication between tumor cells and macrophages is essential in tumor development.

Subsequently, immunofluorescence staining confirmed that tumor cells (CTNNB1) and macrophages (CD74) were adjacent to each other (Fig. 8K). Based on these data and our previous analysis showing that macrophages might tend to polarize to the M2 phenotype, we speculated that APP-CD74 signaling may be involved in this process. To verify our hypothesis, we treated THP-1-induced macrophages with APP and detected markers of M1 and M2 macrophages using qRT-PCR. We found that APP promoted the expression of M2 macrophage markers MRC1 and ARG1 and inhibited the expression of M1 macrophage marker IL1B (Fig. 8L). Additionally, APP promoted the expression of the macrophage phagocytosis-associated gene MMP2 (Fig. 8M).

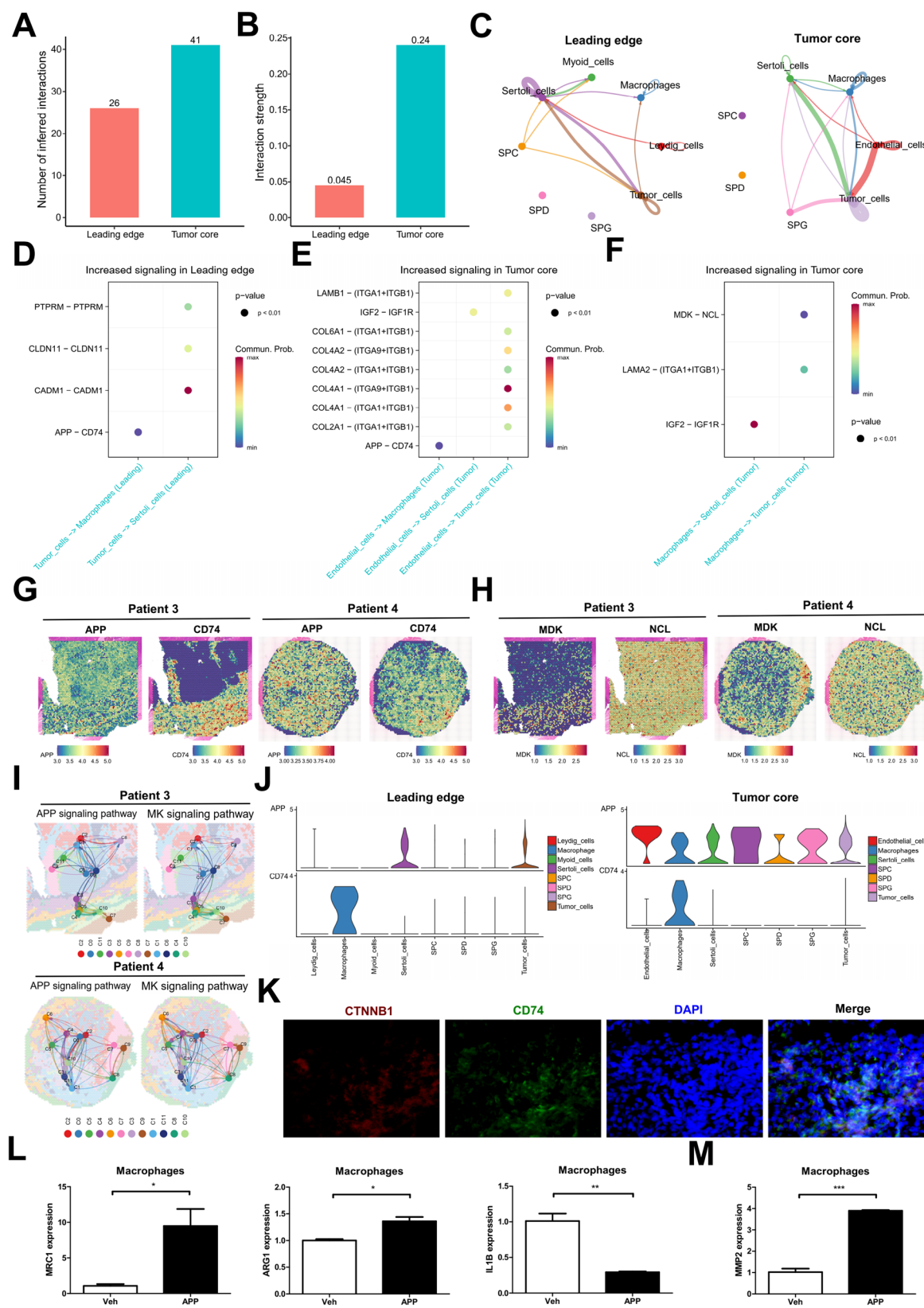


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Fig. 8 Disordered cellular communication in TSCST. **(A)** Bar plot showing the number of inferred interactions in the leading edge and the tumor core. **(B)** Bar plot showing the interaction strength in the leading edge and the tumor core. **(C)** Circle plots display ligand-receptor interaction weights between distinct cellular components in the leading edge and the tumor core. Colors distinguish cell types. The circle size is proportional to the number of cells in each cluster, and the edge width represents communication probability. **(D)** Dot plots show the increased ligand-receptor interactions on the leading edge. **(E)** Dot plots showing the increased ligand-receptor interactions in the tumor core. **(F)** Dot plots show increased signaling in macrophages to tumor and Sertoli cells. **(G)** Feature plots of APP-CD74 by ST in Patient 3 and Patient 4. **(H)** Feature plots of MDK-NCL by ST in Patient 3 and Patient 4. **(I)** Mapped SPP and MK signaling pathways in ST, colored by Seurat clusters. **(J)** Violin plots showing the expression level of APP-CD74 genes across the cell types in the leading edge and the tumor core, respectively. The y-axis shows the normalized read count. **(K)** Representative immunofluorescence images showing expression of CTNNB1 and CD74 ($n=4$). Nuclei were stained with DAPI. **(L)** Relative mRNA expression of MRC1 and ARG1 (M2-type macrophage markers) and IL1B (M1-type macrophage marker) in macrophages treated with APP. **(M)** Relative mRNA expression of MMP2 (a marker associated with phagocytic function in macrophages) in macrophages treated with APP. The P value was calculated by Welch's t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Discussion

TSCST is a rare testicular tumor whose morphological, molecular, and cellular characteristics remain unclear and require further study [48]. In this study, we developed a comprehensive single-cell and spatial transcriptomic atlas of TSCST, revealing significant insights into these tumors' cellular and molecular landscapes. Our findings indicate that TSCST exhibits a striking similarity between tumor and immature Sertoli cells. Our pseudo-time trajectory analysis further elucidated the developmental relationship between tumor and Sertoli cells, suggesting that tumor cells may adopt an EMT signature during malignant transformation. This raises intriguing questions regarding the role of EMT in TSCST progression and the potential for targeting EMT-related pathways as a therapeutic strategy. Interestingly, we observed that CTNNB1 was localized in the nuclei of tumor cells, whereas in non-tumor cells it was predominantly cytoplasmic. Recent studies have shown that SOX9 can interact with the transcriptional coactivator YAP to promote its nuclear translocation [49]. Moreover, SOX9 has been reported to regulate the CTNNB1 signaling in tumors [50]. Therefore, we speculate that SOX9 may also contribute to the nuclear translocation of CTNNB1 in our study samples.

Few studies have focused on the immune microenvironment of TSCST. Spatial transcriptomic analysis revealed a pronounced immune cold in TSCST, marked by the absence of T and B cell infiltration. This suggests that TSCST may employ immune evasion mechanisms, which could hinder the effectiveness of immunotherapy. Previous studies have shown that ICB effectively treats metastatic tumors associated with TSCST. These findings suggest that patients with and without metastatic lesions should be treated differently [51]. Additionally, the lack of immune cells in the tumor microenvironment highlights an area for further exploration of how these tumors evade immune surveillance and what this means for treatment resistance.

Understanding intratumor heterogeneity (ITH) in human tumor is crucial for understanding tumor progression, treatment failure, and drug resistance [52]. Notably, TSCST exhibited a high degree of spatial ITH.

The tumor cores displayed distinct metabolic rewiring compared to the edge regions, which facilitated the re-stratification of the tumor microenvironment. Furthermore, the leading zone of the tumor exhibits more malignant and invasive characteristics. A highly heterogeneous TME allows selective survival under targeted treatment [53]. AR expression has been observed in multiple cancers, including prostate cancer [54] and breast cancer [55] and is associated with the clinical subtypes of affected patients. Our findings indicate that tumor cells in the TSCST express higher and heterogeneous levels of AR and associated target genes, suggesting a potential reliance on androgens for tumor growth.

AR signaling plays a critical role in the initiation and progression of prostate cancer. AR signaling activation promotes tumor cell proliferation, differentiation, and metastasis. As a result, targeting the AR signaling pathway has long been considered an important therapeutic strategy for prostate and breast cancers [56, 57]. Enzalutamide and other AR signaling inhibitors have been widely used in the treatment of prostate and breast cancers [58] and can partially inhibit disease progression. Our study found that testosterone may promote the proliferation, invasion, and colony formation of the mouse Leydig tumor cell line MLTC-1. In contrast, inhibition of AR signaling by enzalutamide significantly suppressed these malignant phenotypes. These findings strongly suggest that AR signaling plays a key role in maintaining the malignant phenotype of TSCST cells and opens new avenues for therapeutic interventions using AR inhibitors.

Except for the significant upregulation of EMT features during TSCST tumorigenesis, tumor cells exhibited high enrichment of NOTCH-induced senescence, a cellular response to various stress signals. This response is characterized by a stable withdrawal from the cell cycle and significant changes in cell morphology and physiology [59]. The overexpression or activation of cell cycle inhibitors, such as p21 (encoded by *CDKN1A*) and p53 (encoded by *TP53*), which are key markers of senescence, is notably increased in the core regions of the tumor compared to the leading-edge areas. Senescence provides tumor cells with a strong antitumor barrier. The secretory phenotype of tumor cells creates an inflammatory

and catabolic microenvironment that promotes tumor growth and metastasis. Furthermore, the substances secreted by these cells contribute to a more immunosuppressive microenvironment, consistent with the low immune cell infiltration observed in TSCST [60, 61]. This evidence suggests that targeting senescent tumor cells may be a promising therapeutic strategy for TSCST.

Additionally, we found that APP is highly expressed in tumor cells, whereas its receptor is mainly expressed in macrophages. This suggests that tumor frequently interact with immune cells in the TME. Moreover, we previously discovered that the MIF-CD74 axis promotes testicular tumor progression [13]. Notably, we identified for the first time that APP facilitates the polarization of macrophages from IL1B+ M1 to MRC1+ M2. Reprogrammed M2 macrophages promote tumor progression and shape the TME.

Similar to our findings, Chen et al. identified, through single-cell RNA sequencing analysis, that the APP-CD74 axis may play an immunosuppressive and tumor-promoting role in testicular tumors (mainly Leydig cell tumors) [62]. The APP-CD74 axis is one of the most prominent cell-cell communication pathways between tumor cells and tumor-associated macrophages (TAMs), and the associated transcriptional changes are significantly correlated with patient prognosis. APP-CD74 signaling has been shown to modulate the immunosuppressive function of macrophages in the TME and their tumor-promoting capacity [63]. CD74-related signaling pathways are also enriched in the macrophages of ovarian cancer patients [64], and blocking CD74 prevents inflammatory macrophage differentiation [65]. These findings support the idea that the APP-CD74 axis may serve as an essential immunomodulatory mechanism for tumor cells to remodel macrophages within the TME, providing new insights for developing immunotherapeutic strategies targeting solid tumors.

However, this study has some limitations. First, because TSCST is a rare testicular tumor, we were unable to collect a sufficient number of samples. Therefore, our conclusions need to be validated in larger patient cohorts. Second, the inhibitory effect of the identified AR inhibitor on TSCST, as well as the role of APP in promoting macrophage polarization are based on cell line models, requires further validation in *in vivo* animal models. Moreover, these above conclusions are based primarily on the analysis of sequencing data; they remain at a very preliminary stage and have yet to be validated experimentally on multiple levels.

In conclusion, we present the first single-cell and spatial transcriptome atlas of TSCST, offering a comprehensive understanding of its cellular composition and TME. Additionally, we emphasize that AR and APP-CD74

signaling might be potential targets for the treatment of TSCST.

Abbreviations

AR	Androgen receptor
DAPI	4',6-diamidino-2-phenylindole
DHT	Dihydrotestosterone
EMT	Epithelial-mesenchymal transition
FBS	Fetal bovine serum
GEO	Gene Expression Omnibus
GSVA	Gene set enrichment analysis
ICB	Immune checkpoint blockade
IHC	Immunohistochemistry
ITH	Intratumor heterogeneity
KEGG	Kyoto Encyclopedia of Genes and Genomes
NMF	Non-negative matrix factorization
PBS	Phosphate buffer saline
QC	Quality control
QRT-PCR	Quantitative real-time reverse transcription polymerase chain reaction
RSS	Regulon Specificity Score
ScRNA-seq	Single-cell RNA sequencing
SnRNA-seq	Single-cell nuclear RNA sequencing
ST	Spatial transcriptome
TF	Transcription factor
TGCT	Testicular germ cell tumors
TME	Tumor microenvironment
TR	Transcription regulon
TSCST	Testicular sex cord stromal tumor
UMAP	Uniform Manifold Approximation and Projection
UMI	Unique Molecular Identifier

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40364-026-00924-0>.

Supplementary Material 1

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Author contributions

Lei Chen, Yu Xie, Liqing Fan, Jian Cao and Hao Bo designed the experiments; Chunlin Ou, Jian Cao, Zhizhong Liu and Hao Bo collected the samples; Lin Du, Jian Cao and Lei Chen performed the experiments; Lei Chen, Jingyu Fan, Jian Cao and Hao Bo analyzed the data; Lei Chen, Jingyu Fan, Lin Du, Zailong Qin and Hao Bo wrote the paper; Lei Chen, Jingyu Fan, Zailong Qin, Liqing Fan and Hao Bo revised the manuscript. All the authors have read and approved the final version of the manuscript.

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Data availability

Data are available from the authors upon reasonable request.

Declarations

Ethics approval and consent to participate

This study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Hunan Cancer Hospital (ID: 2021KYKS-46). All patients provided written informed consent to participate.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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