

**Multi-omics analysis of dynamic cellular and transcriptional characteristics in pig liver
across physiological and developmental states**

Yu-Jing Li¹, Jia-Hao Tang¹, Fu-Jie Li¹, Yi-Fei Wang¹, Ting Zheng¹, Xing-Long Xu¹,
Meng-Jiao Bai¹, Zheng-Yin Gong¹, Xing-Fa Han¹, Rong Yuan², Yan-Zhi Jiang^{1,3,*}

¹*Department of Zoology, College of Life Science, Sichuan Agricultural University, Ya'an,
Sichuan 625014, China*

²*Chengdu Livestock and Poultry Genetic Resources Protection Center, Chengdu, Sichuan
610081, China*

³*State Key Laboratory of Swine and Poultry Breeding Industry, College of Animal Science and
Technology, Sichuan Agricultural University, Chengdu, Sichuan 611130, China*

*Corresponding author, E-mail: jiangyz04@163.com



ABSTRACT

The liver undergoes profound metabolic adaptations during development, pregnancy, and castration, yet the underlying cellular and transcriptional dynamics remain poorly understood. Here, we performed single-nucleus RNA-seq on livers of Chenghua sows across developmental (youth, D30; adulthood, Y1) and physiological (late-pregnancy at Y1, PY1; neonatal castration, CY1) states, integrated with spatial transcriptomics and targeted metabolomics for validation. From youth to adulthood, hepatocytes shifted from proliferation to enhanced fatty acid metabolism, evidenced by *FASN*⁺/*LIPG*⁺ subtype (HepIII) and elevated levels of these metabolites NADP⁺, NADPH, and NAD⁺ in Y1. Pseudo-time analysis revealed a functional progression from cell cycle regulation to catabolic metabolism and immune regulation. Pregnancy and castration induced distinct hepatocyte remodeling. PY1 hepatocytes were enriched in lipid catabolism-associated subtypes (Hep1, *ABHD5*⁺; Hep3, *AIG1*⁺, *AR*⁺) with ATF6/TCF7L2 activation promoting fatty acid oxidation, while CY1 hepatocytes were characterized by ribosomal protein-enriched subtypes (Hep5, *RPSA*⁺, *RPL10*⁺) and BACH1 with enhanced glycolysis and elevated NADP⁺, suggesting heightened fatty acid metabolism. Beyond hepatocyte-intrinsic changes, pregnancy triggered a marked increase in cell-cell communication, with PY1-specific ligand-receptor pairs (e.g., ANGPTL family, WNT signaling) connecting hepatocytes to non-parenchymal cells, while immune cell subsets (T and NK cells) exhibited state-dependent compositions. Spatial transcriptomics proved hepatocyte-NK cell co-localization in specific niches. Cross-species analysis revealed both conserved liver cell types and species-specific metabolic gene expression profiles across adult pigs, humans, and mice. This work delineates the cellular and transcriptional dynamics of liver homeostasis, supports the pig model for human hepatic research, and provides a valuable multi-omics resource.

Keywords: Pig; Liver; SnRNA-seq; Pregnancy; Castration

INTRODUCTION

The liver plays a vital role in systemic metabolism and energy homeostasis, regulating glycolysis, fatty acid levels, and glucose balance in the body (Andrews et al., 2022; Hou et al., 2021; Liang et al., 2022; Yang et al., 2025). It is also essential for protein synthesis, immune responses, and detoxification (Andrews et al., 2022; Hou et al., 2021). Hepatocytes, as the main parenchymal cells, are the primary executors of these diverse hepatic functions, serving as the central hub of liver physiology.

The pig is an ideal model for studying liver biology due to its anatomical and metabolic similarities to humans (Rao et al., 2023). Unlike rodents, the pig liver consists of six lobes, including the right lateral, right medial, left lateral, left medial, quadrate, and caudate lobes, with lobular organization similar to that of the human liver (Ekataksin & Wake, 1991). These lobes are formed by repetitive units termed liver lobules, and during hepatocytes maturation, lobules organize into zonation structures that perform distinct functions along the lobular axis (Hildebrandt et al., 2021; Liang et al., 2022). Beyond its value as a biomedical model, the pig is a major livestock species, and understanding its liver metabolism has direct implications for animal production and health.

A critical period of liver functional maturation is postnatal development. In mice, hepatocytes proliferate rapidly after birth and revert to a quiescent state during maturation (Yang et al., 2017). The liver exhibits different transcriptional and metabolic profiles during the transition from birth to adulthood (Li et al., 2009; Nakagaki et al., 2018). In pigs, a study has revealed some specific transcription factors (TFs) involved in hepatocyte maturation and circadian rhythm regulation (Rao et al., 2023). However, the specific transcriptional and metabolic changes that occur during postnatal maturation in mammals remain poorly characterized at single-cell resolution.

Beyond normal development, the liver also undergoes remarkable adaptations in response

to physiological challenges. Pregnancy represents a state of heightened anabolic demand, with increased liver volume and hepatocyte proliferation, followed by involution after weaning (Barllett et al., 2021; Yang et al., 2025). Single-cell transcriptomic analysis in mice has revealed that the liver undergoes metabolic adaptations during pregnancy, with a cyclical hepatocyte trajectory governing these changes and reverting to the pre-pregnancy state after weaning (Yang et al., 2025). In contrast, castration eliminates sex hormone signaling. A study in rats has shown that neonatal castration feminizes sex-differentiated liver metabolism, particularly cytochrome P450-mediated pathways (Blanck et al., 1986). While in mice, castration alters hepatic fatty acid composition and abundance and increases the protein level of isocitrate dehydrogenase 2, a rate-limiting enzyme in the tricarboxylic acid cycle (Yao et al., 2024). These two perturbations, pregnancy (anabolic challenge) and castration (hormonal deprivation), provide complementary models to investigate liver metabolic plasticity across species. However, the metabolic adaptation characteristics of the liver during pregnancy and castration remain poorly characterized at the single-cell level in pigs, a species with greater anatomical and metabolic similarity to humans.

The liver undergoes profound phenotypic and functional changes across different developmental and physiological states, requiring intricate cellular crosstalk between hepatocytes and non-parenchymal cells (NPCs). To investigate these processes at cellular resolution, single-nucleus RNA sequencing (snRNA-seq) technology has the advantage of capturing hepatocytes and has been successfully applied to construct liver development maps and study hepatic cell heterogeneity (Andrews et al., 2022; Rao et al., 2023). It has also been used to characterize mouse hepatocytes with different ploidy levels (Richter et al., 2021), revealing functional crosstalk between liver zonation and ploidy, and to characterize the hepatocyte adaptations during mouse pregnancy and postpartum (Yang et al., 2025).

Hepatocytes express different receptors, translocators, and enzymes along the lobular axis,

and their spatial organization is critical for liver function (Liang et al., 2022). Spatial transcriptome sequencing (stRNA-seq) provides an ideal platform to map gene expression in its native tissue context. Previous studies have utilized this technology to map extensive spatial gene expression patterns and delineate lobular zonation in mouse liver (Hildebrandt et al., 2021), to systematically identify nine cell types and define their developmental pathways in the human fetal liver (Hou et al., 2021), and to reveal distinct immune cell and fibroblast profiles between primary and liver metastatic colorectal cancer (Wang et al., 2023). However, spatial transcriptomic characterization of the pig liver under different physiological states, particularly how hepatic cell types and their interactions are spatially organized, remains lacking.

In this study, to investigate how liver cells dynamically remodel their transcriptional, metabolic, and cellular interaction landscapes to adapt to maturation, pregnancy, and castration, we performed snRNA-seq on livers of Chenghua sows, a fat-type Chinese native pig breed, across developmental (youth, 30 days old, D30; adulthood, 1 year old, Y1) and physiological (late-pregnancy at Y1, PY1; neonatal castration sampled at Y1, CY1) states, complemented by stRNA-seq and targeted energy metabolomics. Using this multi-omics approach, we characterized the transcriptional and metabolic changes across different developmental and physiological states at cellular and molecular levels, profiled the dynamics of hepatic cell populations (primarily hepatocytes and immune cells) and their cell-cell communication networks, proved the spatial organization of key hepatic cell types and interactions, and uncovered the conservation of liver cellular behavior and species-specific metabolic gene expression profiles across pig, human, and mice. This work provides a valuable resource for our understanding of hepatic cell heterogeneity and offers insights into the dynamic balance of liver energy metabolism and spatial organization.

MATERIALS AND METHODS

Ethics approval and consent to participate

All experimental procedures were approved by the Animal Care and Ethics Committee of Sichuan Agricultural University, China (permit No. 20240692). The study was conducted in accordance with national guidelines and institutional policies for the care and the use of experimental animals. Animal welfare standards were strictly maintained throughout all tissue sampling procedures.

Animal sample collection and pretreatment

All pigs used in this study were obtained from the Chengdu Livestock and Poultry Genetic Resources Protection Center (Chengdu, China). A total of 12 Chenghua sows were selected from four groups, including youth (30 days old, D30), adulthood (1 year old, Y1), late-pregnancy (1 year old, PY1), and castration (1 year old, CY1; castrated at 7 days of age), with three biological replicates per group.

Blood was collected from the precaval vein before slaughter, and serum was separated by centrifugation and stored at -80°C for ELISA. After humane slaughter, the right lateral lobes of the liver were quickly collected and processed for different downstream applications (Supplementary Figure S1). Tissue blocks (5 mm long $\times$ 5 mm wide $\times$ 5 mm high) were excised and fixed in 4% paraformaldehyde for H&E and Oil Red O staining ($n=3$ per group). Approximately 100 mg of liver tissue was snap-frozen for ELISA ($n=3$ per group). For snRNA-seq, tissue pieces (2 mm long $\times$ 2 mm wide $\times$ 2 mm high) were washed with cold Dulbecco's phosphate-buffered saline (DPBS) (Solarbio, China) and snap-frozen in liquid nitrogen ($n=3$ per group). For targeted energy metabolomics, approximately 50 mg of tissue was snap-frozen; six replicates per group were analyzed, three from individual pigs (same as snRNA-seq) and three from pooling equal amounts of tissue from two animals within the same group. For

stRNA-seq, tissue blocks (10 mm long×10 mm wide×5 mm high) were frozen in optimal cutting temperature (OCT) compound (Sakura, USA) with one replicate per group for Y1, PY1, and CY1. All samples were stored at –80°C.

Single nucleus isolation and snRNA-seq

Nuclei were isolated from liquid nitrogen frozen liver tissue. The minced tissue was transferred to a pre-cooled homogenizer containing homogenization buffer. The resulting mixture was filtered through 40 µm and 30 µm strainers into a 15 mL centrifuge tube and centrifuged at 500 ×g for 5 min at 4°C. The supernatant was discarded, and the pellet was resuspended in blocking buffer and centrifuged again. The supernatant was discarded, and the precipitated nuclei were resuspended in cell resuspension buffer. Nucleus activity and concentration were determined using DAPI staining and a hemocytometer, respectively.

The Chromium Single Cell 3'Reagent Kit v3 (10× Genomics, USA) was used to construct the snRNA-seq library according to the manufacturer's instructions. Raw sequencing reads were filtered and demultiplexed using PISA (v1.1.0), and the reads were aligned to the *Sus scrofa* reference genome (Sscrofa11.1) using STAR (v2.7.4a). Finally, PISA was used to obtain the cell-gene UMI count matrix of each library.

SnRNA-seq data processing

Raw snRNA-seq data were processed using the R package Seurat (v5.1.0) (Hao et al., 2024). Low-quality cells and genes were filtered out for downstream analysis. In brief, genes expressed in fewer than three cells, cells with fewer than 200 or more than 6 000 genes, and cells with more than 5% mitochondrial reads (percent.mt) were removed from the gene expression matrix. Doublet cells were identified and removed using the R package DoubletFinder (v2.0.4) with default parameters (Mcginnis et al., 2019). Cells were classified

as 'Singlet', 'Doublet-Low Confidence', or 'Doublet-High Confidence' based on the optimized pK parameter and pANN (proportion of artificial nearest neighbors) thresholds. To balance the doublet removal sensitivity against the risk of discarding authentic cells, we retained singlets and low-confidence doublets, while excluding only high-confidence doublets from subsequent analyses. Multi-sample snRNA-seq datasets were initially merged using the merge function, and batch effects were corrected using Harmony (v1.2.1) (Korsunsky et al., 2019). All sequencing data were scaled and dimensionally reduced using the NorMalizeData (scale.factor=10 000), FindVariableFeature (nfeatures=2 000), ScaleData and RunPCA functions in Seurat. Cell clustering was performed using the FindClusters function, and Uniform Manifold Approximation and Projection (UMAP) visualization was performed using the RunUMAP function. The FindAllMarkers function was used to detect the marker genes for each cluster, and the cell types were manually annotated based on the established marker genes from previous publications (Aizarani et al., 2019; MacParland et al., 2018; Popescu et al., 2019; Rao et al., 2023; Watson et al., 2025; Wesley et al., 2022) (Supplementary Table S1).

Identification of differentially expressed genes and enrichment analysis

After cell type annotation, differentially expressed genes (DEGs) were identified using the FindAllMarkers and FindMarkers functions in Seurat (logfc.threshold>0.25, min.pct>0.1, adjusted $P<0.05$). Pseudobulk data were also analyzed using DESeq2 for DEG identification across conditions. Enrichment analysis of DEGs was performed using the enrichGO function and enrichKEGG functions in the R package clusterProfiler (v4.10.1).

Hepatocyte development trajectory analysis

To construct the hepatocyte developmental trajectory, we applied the R package Monocle2 (v2.30.1) to hepatocytes from D30 and Y1. Using the DDRTree method, highly variable genes

were identified with the FindVariableFeature function, and cells were clustered and ordered using the reduceDimension function. The orderCells function was then used to infer the pseudo-temporal transition process. The earliest state was manually specified as the starting point through the root_state parameter based on the identification results of hepatocyte subtypes. DEGs along pseudo-time were identified using the DifferentialGeneTest function and significantly changed genes were clustered into modules, followed by heatmap visualization and functional enrichment analysis.

Transcription factor-target gene network analysis

Transcription factor (TF) regulation was analyzed using the Python package pySCENIC (v0.12.1). Based on the snRNA-seq expression matrix and a TF factor annotation list, the GRNBoost2 algorithm was used to infer co-expression modules, CisTarget was used to identify direct target genes and enriched motifs, and AUCell was used to quantify regulator activity per cell. The resulting TF regulatory activity matrix was imported into the Seurat object, and hepatocyte subtype-specific regulons were identified based on regulon specificity scores.

Cell-cell communication analysis

Cell-cell communication networks were analyzed using CellChat (v1.6.1) with default parameters for each snRNA-seq sample. The human ligand-receptor database was used, conserved homologous genes between pigs and humans were selected. Significant ligand-receptor interactions (P -value<0.05) between hepatocytes and NPCs were identified based on co-expression patterns of cell-type-specific genes.

Single-cell Disease Relevance Score (scDRS) analysis

We performed scDRS analysis to evaluate associations between metabolic traits and liver cell

types. Six metabolic traits were obtained from the IEU OpenGWAS database, including cholesterol (ukb-d-30690_raw), high-density lipoprotein cholesterol (HDL; ukb-d-30760_raw), glycated hemoglobin (HbA1c; ieu-b-103), homeostatic model assessment of insulin resistance (HOMA-IR; ieu-b-118), triglyceride (ukb-d-30870_raw), and fasting glucose (ebi-a-GCST90002232). SNPs were mapped to ± 100 kb of transcription start sites (hg19), and conserved homologous genes between pigs and humans were selected. scDRS (v1.0.2) was used to calculate correlation scores (1 000 gene sets), and average standardized scores were calculated per cell type per sample. *P* values were corrected using the Benjamini-Hochberg false discovery rate (FDR) method, with $FDR < 0.05$ considered statistically significant. Results were visualized as heatmaps showing average standardized scores for each cell type-trait pair.

10× Visium spatial transcriptomics

Spatial transcriptome sequencing was performed on Y1, PY1, and CY1 liver samples that had been embedded in isopentane and stored at -80°C . Frozen sections (10 μm thick) were prepared, followed by tissue staining and imaging. Next, tissue permeabilization and cDNA synthesis were carried out, followed by Visium spatial library preparation and sequencing. cDNA libraries were constructed using the Visium Spatial Library Construction Kit (10× Genomics, USA) according to the manufacturer's instructions, and library concentrations were measured using an Agilent 2100 (Agilent, USA).

Integrated analysis of snRNA-seq and stRNA-seq data

Cell2location is a Bayesian-based algorithm designed to resolve fine-scale cell type distributions in spatial transcriptome data. Here, we generated high-precision cell abundance estimation maps by integrating snRNA-seq and stRNA-seq data from Y1, PY1, and CY1. Using the cell types identified from snRNA-seq data as a reference, we ran cell2location (v0.1.3)

in the Python environment (v3.9.19) to determine the cellular composition of each spot in the stRNA-seq data. Model training was set to “max_epochs=30 000”. Additionally, using the cell2location deconvolution outputs, the abundance of hepatocyte subtypes and the expression of marker genes were visualized on H&E images using the sc.pl.spatial function in Scanpy for spatial localization.

Spatial niche analysis

Spatial niches were identified based on the cell type proportion matrix derived from cell2location deconvolution. Following the method of Kuppe et al. (2022), we converted the estimated cell type proportions for each spatial transcriptomics spot and slice into isometric log-ratios (ILRs) and clustered the spots into groups. Shared nearest neighbor graphs were constructed using the BuildSNNGraph function from the R package scan (v1.30.2) with varying neighbor counts, followed by Louvain clustering. Cellular abundance within each niche was quantified as the median of proportion of each cell type across spots within the niche.

Constructing spatial map of cell dependencies

The R package mistyR (v1.10.0) was used to evaluate the importance of each major cell type's abundance in explaining the abundance of other major cell types. Spatial cell type abundances estimated by cell2location deconvolution for all slices were modeled in a multiview framework that included three views: (1) the intrinsic view, measuring relationships between deconvolution estimates within a single spot; (2) the juxta view, summing deconvolution estimation of adjacent cells with a maximum distance of 5 spots; and (3) the para view, applying weighted deconvolution estimation to farther neighbors with an effective radius of 15 spots.

Spatial cell-cell interaction analysis

COMMOT (COMMunication analysis by Optimal Transport) is a method for inferring cell-cell communication in spatial transcriptomics that accounts for competition between different ligand-receptor pairs and spatial distance between cells (Cang et al., 2023). Here, we used COMMOT (v0.0.3) in the Python environment (v3.8.15) to explore the abundance of specific ligand-receptor interactions between hepatic cell types in space.

Cross-species comparison of pig, human, and mouse hepatocyte atlases

Published snRNA-seq data from healthy human and mouse livers were downloaded, including adult human liver samples (GSM5616007) and adult mouse liver samples (GSM5764242, GSM5764243, GSM5764244) (Andrews et al., 2024; Guilliams et al., 2022). Pig liver samples from the Y1 stage were selected for cross-species analysis. Homologous genes across species were converted, and data processing was performed using Seurat (v5.1.0). Cells expressing more than 200 genes were retained, and batch effects between species were corrected using Harmony (v1.2.1; group.by.var="species") (Korsunsky et al., 2019). Cluster-specific genes and hepatocyte-specific DEGs among the three species were identified using FindAllMarkers (logfc.threshold=0.25, min.pct=0.25), and unique DEGs were visualized using Venn diagrams. Gene Ontology (GO) enrichment analysis was performed on hepatocyte cluster-specific genes, and selected terms were visualized using heatmaps and gene set scoring (AddModuleScore) violin plots.

Targeted energy metabolomics by LC-MS

Targeted energy metabolomics was performed on frozen liver tissue, with six biological replicates per group. Metabolites were separated using an Agilent 1290 Infinity LC ultra-high performance liquid chromatography system coupled with an LC-MS detection method.

Chromatographic separation was performed on an ACQUITY UPLC HSS T3 column (2.1×100 mm, 1.7 μm, Waters, USA). Metabolites were kept in an autosampler at 4°C, and the column temperature was maintained at 35°C. Mobile phase A consisted of 50 mM ammonium acetate aqueous solution with 1.2% ammonium hydroxide, and mobile phase B consisted of 1% acetylacetone in acetonitrile. The flow rate was 300 μL/min, and the injection volume was 2 μL. The mobile phase gradient was as follows: 0–1 min, 70% B; 1–10 min, 70% to 60% B; 10–12 min, 60% to 30% B; 12.1–15 min, 30% B; 15–15.5 min, 30% to 70% B; 15.1–22 min, 70% B.

Mass spectrometry analysis was performed using a 5500 QTRAP mass spectrometer (SCIEX, USA) in negative ion mode. The conditions were set as follows: source temperature 450°C, Ion Source Gas1 (Gas1): 45, Ion Source Gas2 (Gas2): 45, Curtain Gas (CUR): 30, IonSpray Voltage Floating (ISVF): –4 500 V. Multiple reaction monitoring (MRM) was used to detect the target ion pairs. The MRM peak areas and retention times were extracted using Multiquant 3.0.2 software.

H&E staining

Liver tissues were fixed in 4% paraformaldehyde (Servicebio, China) for 24 h, dehydrated through an ethanol series, and embedded in paraffin. Paraffin sections were cut, and stained with hematoxylin solution for 3–5 min, dehydrated with ethanol, and then stained with eosin solution for 5 min. After clearing and sealing, the sections were observed under a microscope.

Oil Red O staining

Frozen liver sections were removed from the –20°C refrigerator, brought to room temperature, fixed with tissue fixing solution (Servicebio, China) for 15 min, washed with tap water, and air-dried. The slices were then immersed in Oil Red O solution (Servicebio, China) for 8–10

min protected from light. The sections were removed, held for 3 s, and then immersed in two changes of 60% isopropyl alcohol for differentiation (3 s and 5 s, respectively). The slides were then washed in two changes of distilled water for 10 s each. Hematoxylin counterstaining was performed, and slides were sealed with glycerin gelatin. Images were captured under a microscope.

Enzyme-linked immunosorbent assay (ELISA)

ELISA kits (RuiXin Biotech, China) were used for determination. Serum concentrations of progesterone (PROG), glucocorticoid receptors α (GR- α), and glucagon (GC) were quantified according to the manufacturer's instructions. PROG was detected by competitive ELISA, whereas GR- α and GC were detected by sandwich ELISA. Concentrations of free fatty acids (FFAs) and triglycerides (TGs) in frozen pig liver tissues were also determined using a microplate method. A four-parameter logistic (4-PL) curve was used for calibration. A standard equation was generated based on the concentrations of standard samples and their corresponding optical density (OD) values, and sample concentrations were then calculated accordingly.

Statistical analysis

Statistical analysis was performed using R, Python, and GraphPad Prism 8.0. Specific details are provided in the figure legends.

RESULTS

Establishing the liver cell atlas from youth to adulthood

To establish the baseline cellular landscape of liver development, we performed snRNA-seq on the Chenghua sow livers at 30 days (D30, youth) and 1 year after birth (Y1, adulthood)

(Figure 1A). H&E staining showed that the Y1 liver lobular structure was more complete and clearer than at that D30 (Supplementary Figure S2). After quality control, normalization, and batch effect correction, we retained 87 145 high-quality nuclei for downstream analysis (Figure 1B; Supplementary Figure S3A; Supplementary Table S2). Based on manually selected canonical marker genes, we identified nine major cell types, including hepatocyte (Hep), endothelial cell (EC), Kupffer cell (KC), T cell (TC), NK cell (NK), B cell (BC), dendritic cell (DC), hepatic stellate cell (HSC), and cholangiocyte (Chol) (Supplementary Figure S3B; Supplementary Table S3). The proportion of each cell type varied markedly between the two time points (Figure 1C). Compared with D30, the proportion of Heps in Y1 showed a significant decrease (Figure 1C), and the liver organ index of Y1 was also significantly lower than that of D30 (Supplementary Figure S3C).

We then re-clustered 11 500 Heps and identified nine Hep subtypes (HepI–HepIX) with distinct functional marker genes (Figure 1D, E). Notably, the HepIII subtype was mainly derived from Y1, whereas three other Hep subtypes (HepI, HepVI, and HepVIII) were mainly derived from D30 (Figure 1E; Supplementary Figure S3D). Remarkably, HepVI exhibited proliferative capacity, as evidenced by high expression of the proliferation marker genes *TOP2A* and *TPX2* (Figure 1E). HepVIII specifically expressed *DDIT3* (DNA Damage Inducible Transcript 3) (Figure 1E), a key regulator of cellular stress response (Osman et al., 2023). HepI specifically expressed *ASB4* (ankyrin repeat and SOCS box containing 4) and *LIPC* (lipase C, hepatic type) (Figure 1E); *LIPC* is an enzyme involved in triglyceride and phospholipid hydrolysis (Rufibach et al., 2006). Additionally, HepII was characterized by high expression of marker genes associated with protein synthesis activity, including *RPL36*, *RPS20* and *RPLP1* (Figure 1E).

To explore the transcriptomic dynamics of Hep maturation, we performed pseudo-time analysis using Monocle2 (Qiu et al., 2017) on Heps from D30 and Y1. The inferred trajectory

consisted of three branch points and divided all Heps into seven states (Figure 1F; Supplementary Figure S4A, B), with pseudo-time ordering largely consistent with chronological age (Figure 1G; Supplementary Figure S4C–E). Along this trajectory, we identified five gene expression modules that revealed a progressive functional shift (Figure 1H; Supplementary Table S4). Module 3 was associated with cell cycle regulation (e.g., *CCAR2*, *CCNBI*, *CCNF*) and peptide/amide metabolic processes. Module 2 was enriched in catabolic pathways, including the carboxylic acid catabolic process (e.g., *ABAT*, *ACAT1*, *ARG1*) and fatty acid degradation. Modules 4 and 5 transitioned into antigen processing and presentation (e.g., *SLA-1*, *SLA-2*, *SLA-3*) and immune system regulation (e.g., *CCL5*, *IRF1*, *KLRK1*), respectively. Module 1, while not significantly enriched in GO terms, was associated with insulin, glucagon, and FoxO signaling pathways.

Next, GO analysis of differentially expressed genes (DEGs) between Y1 and D30 revealed that genes up-regulated in Y1 were enriched in pathways such as organic acid metabolic process, oxoacid metabolic process, and carboxylic acid metabolic process, as well as in fatty acid metabolism (Supplementary Figure S4F; Supplementary Table S5). Similar results were obtained by pseudobulk analysis (Supplementary Figure S4G; Supplementary Table S6). Interestingly, targeted energy metabolomics showed that among the significant differential metabolites (DMs) between the two groups, NADP⁺ (nicotinamide adenine dinucleotide phosphate), NADPH (reduced nicotinamide adenine dinucleotide phosphate), and NAD⁺ (nicotinamide adenine dinucleotide), which are related to fatty acid metabolism (Chen et al., 2024), were highly expressed in Y1 (Figure 1I; Supplementary Table S7). Moreover, ELISA results showed that free fatty acids (FFA) levels in the liver were much higher in Y1 than in D30, while triglyceride levels were similar between the two groups (Supplementary Figure S4H). These results indicate significant differences in fatty acid metabolism between the livers of young and adult pigs, with adulthood characterized by enhanced fatty acid metabolic

capacity.

Pregnancy and castration remodel the adult liver cell landscape

To dissect the cellular transcriptome profiles of the pig liver during pregnancy and after castration, we isolated liver cell types from Chenghua sow livers at three adult stages, including normal adulthood (Y1, 1 year after birth; serving as the control from the developmental study), late pregnancy (PY1, 1 year after birth), and castrated adulthood (CY1, 1 year after birth, castrated at 7 days of age). All isolated single nuclei were used for snRNA-seq on a 10× Genomics platform (Figure 1A; Supplementary Table S2). H&E staining of liver tissue is shown in Supplementary Figure S2. After quality control, normalization, clustering, and dimension reduction, we retained a total of 125 120 high-quality single nuclei for Uniform Manifold Approximation and Projection (UMAP) analysis after multi-sample integration (Figure 2A; Supplementary Figure S5A). We identified ten major cell types based on manually selected well-known markers, including Hep, EC, KC, TC, NK, BC, DC, HSC, Chol, and proliferating cell (PC), which were not detected in the developing liver (Figure 2B; Supplementary Table S3). The proportions of these cell types showed distinct variations across different physiological stages (Figure 2C). Notably, CY1 contained almost no PCs, whereas the proportion of Heps increased in both PY1 and CY1 compared to Y1.

Comparison between PY1 and Y1 revealed that, in Heps, genes up-regulated in PY1 were significantly enriched in oxoacid, carboxylic acid, and organic acid metabolism processes. Conversely, genes down-regulated in PY1 Heps were primarily associated with translation, peptide metabolic process, and peptide biosynthetic process, which was highly consistent with the up-regulated genes observed in other cell types from PY1 (Figure 2D; Supplementary Table S5). In CY1 compared to Y1, up-regulated genes in TC and NK cells were associated with negative regulation of coagulation and hemostasis, wound healing, complement activation,

and lipid transport capabilities, whereas down-regulated genes were involved in catabolic activity of proteins and macromolecules (Supplementary Table S5). Interestingly, similar to the up-regulated genes in PY1 Heps (PY1 vs. Y1), the down-regulated genes in CY1 Heps (CY1 vs. Y1) were significantly enriched in acid metabolism processes and fatty acid catabolic pathways (Figure 2E; Supplementary Table S5). Beyond differential gene expression, we further performed association analysis between metabolic traits and hepatic cell types using scDRS (Supplementary Figure S5B). Fasting glucose was enriched in Heps, with enhanced expression during both PY1 and CY1. HbA1c was significantly enriched in HSCs. Compared to Y1, HOMA-IR and HDL traits enriched in PCs were significantly enhanced during PY1, whereas triglyceride enrichment was significantly decreased. Furthermore, cholesterol enrichment in NK cells was significantly reduced in CY1 compared to Y1.

Given that Heps are the major functional cell type (Yang et al., 2023), we extracted 16 973 Heps from the snRNA-seq data of Y1, PY1, and CY1 and re-clustered them into ten Hep subtypes (Hep1–Hep10) with distinct functional marker genes (Figure 3A, B). Interestingly, we found that Hep1 and Hep3 primarily originated from PY1 (Figure 3B; Supplementary Figure S5C). Hep1 specifically expressed *ABHD5* (abhydrolase domain-containing protein 5) (Figure 3B), which is involved in the positive regulation of lipid catabolism and triglyceride catabolism, serving as a key regulator of lipid and energy homeostasis (Badin et al., 2012; Lass et al., 2006; Schratter et al., 2022). Hep3 highly expressed *AIG1* (Androgen Induced 1) (Figure 3B), which is involved in the long-chain fatty acid catabolic process, and also highly expressed androgen receptor (*AR*) (Figure 3B). ELISA results confirmed that progesterone (PROG) and glucocorticoid receptor α (GR- α) were significantly expressed in PY1 (Supplementary Figure S5D). Another Hep subtype, Hep7 (corresponding to Hep III identified in the developing liver), was primarily derived from Y1 and absent in CY1, and showed high expression levels of *FASN* (fatty acid synthase) and *LIPG* (endothelial lipase) (Figure 3B; Supplementary Figure S5C).

Moreover, Hep5, primarily derived from CY1, specifically expressed ribosomal proteins such as ribosomal protein SA (*RPSA*) and ribosomal protein L10 (*RPL10*) (Figure 3B; Supplementary Figure S5C). GO enrichment analysis indicated that DEGs in Hep5 were predominantly enriched in translation, peptide metabolic process, and amide metabolic process (Supplementary Figure S5E). These GO terms were similarly enriched in the DEGs of Hep1 and Hep3 (Supplementary Figure S5F, G). These results reveal the functional heterogeneity of Heps under different physiological conditions.

We identified two immune-like Hep subtypes, Hep6 (corresponding to HepVII from the developing liver) and Hep8 (corresponding to HepV from the developing liver), both of which co-expressed immune cell marker genes. Notably, Hep6 specifically expressed *CD244* (Figure 3B), a cell surface protein typically expressed by NK cells and T cells (Sun et al., 2021). Additionally, we identified a growth-type Hep subtype, Hep10 (corresponding to HepIX from the developing liver), which showed high expression of hepatocyte growth factor (*HGF*) and specific expression of pleiotrophin (*PTN*) (Figure 3B). *PTN* belongs to the heparin-binding growth factor subfamily (Xu et al., 2014). Furthermore, we found that Hep2 specifically expressed the glucagon receptor (*GCGR*) (Figure 3B), and ELISA results showed that glucagon (GC) expression was significantly increased in PY1 (Figure 3C). Hep4 specifically expressed estrogen sulfotransferase (*SULT1E1*) (Figure 3B), which regulates estrogen homeostasis inactivation (Xu et al., 2018), and pyruvate dehydrogenase kinase 4 (*PDK4*) (Figure 3B), a key enzyme regulating energy metabolism (Ma et al., 2023). Importantly, we also identified a distinct Hep subtype (Hep9) that highly expressed the proto-oncogene *KIT* (Figure 3B), a receptor tyrosine kinase that regulates cell proliferation, survival, and migration (Sheikh et al., 2022). Hep9 (corresponding to HepIV from the developing liver) also exhibited high expression of *KDR* (kinase insert domain receptor, also known as vascular endothelial growth factor receptor 2) (Figure 3B). GO analysis revealed that DEGs of Hep9 were primarily

enriched in developmental processes and cell migration (Figure 3D), suggesting that Hep9 may represent an intermediate-type subpopulation.

Analysis of hepatocyte-specific transcription factor activity and metabolic function characteristics

To investigate key regulatory mechanisms in Heps under different physiological states, we performed single-cell regulatory network inference and clustering analysis using pySCENIC (Van de Sande et al., 2020). Based on the activity matrix of each transcription factor (TF) and its downstream targets (regulons) in Heps, we evaluated TF specificity using RSS and identified 17 Hep-specific TFs (Supplementary Figure S6A). The TFs *RUNX3* and *BCL11B* were identified in Hep6. *RUNX3* is a key regulator of tissue-resident memory CD8⁺ T cells differentiation and homeostasis (Milner et al., 2017). *BCL11B* serves as a master regulator of T cell lineage specification, and its inactivation reprograms T cells into NK-like cells (Li et al., 2025). Although *BCL11B* is typically associated with T cell differentiation, its expression in Hep6, together with *CD244* (Figure 3B), supports the classification of this subtype as immune-like Heps, suggesting that some Hep subtypes may share transcriptional programs with immune lineages. The TF *IRF5* was identified in Hep8, playing a key role in modulating immune response (Yu et al., 2025). These results further support the classification of Hep6 and Hep8 as immune-like Heps.

In addition, we identified TFs specifically activated in each Hep subtype (Figure 3E; Supplementary Table S8), including known TFs such as the circadian rhythm regulator *CLOCK*, which affects hepatic metabolic homeostasis; *HNF4A* and *HNF1A*, which regulate Hep differentiation and liver metabolism; and the important regulators of hepatic tumorigenesis *FOS*, *JUN* and *JUNB*. Furthermore, *ONECUT1* (hepatocyte nuclear factor 6, HNF6), which regulates cell cycle and glucose metabolism (Prajapati et al., 2024), was primarily identified in

Hep4 and Hep7 (Figure 3F). *ATF6* was identified as a Hep1-specific TF (Figure 3F), and it is known to promote liver fatty acid oxidation by interacting with PPAR α (Chen et al., 2016). *TCF7L2* was enriched in Hep3 (Figure 3F), and it is a key transcriptional effector of the WNT/ β -catenin signaling pathway, which regulates de novo lipogenesis and fatty acid desaturation in adipocytes (Bagchi et al., 2020; Chen et al., 2018). *BACH1* was specifically expressed in Hep5 (Figure 3F) and is known to regulate insulin signaling and glucose homeostasis in mouse liver (Jin et al., 2023). *GATA6* was enriched in Hep7 (Figure 3F) and plays a role in various stages of liver development, including hepatoblast differentiation (Zhang & He, 2018). These results reveal the specific roles of these TFs in Heps under different physiological conditions.

Given that Heps are the major metabolic hub of the human body and perform human-specific functions (Kaffe et al., 2023), and in light of the specific TFs identified in Hep subtypes, we explored the metabolic function of Heps under different physiological states (Supplementary Table S7). We observed five significant DMs between PY1 and Y1, all with higher expression levels in Y1 (Supplementary Figure S6B). Among these, phosphoenolpyruvate (PEP) and 3-phosphoglycerate (3PG), which are involved in glycolysis (Chen et al., 2019; Wu et al., 2023), showed significant differences between the two groups (Figure 3G). Interestingly, DM analysis of CY1 vs. Y1 revealed that PEP and 3PG exhibited significantly higher expression in CY1 (Supplementary Figure S6C–E). Dihydroxyacetone phosphate (DHAP), also involved in glycolysis (Sobanski et al., 2023), showed elevated expression in CY1 (Supplementary Figure S6F). This indicates that, compared with Y1, glycolytic ability is decreased in PY1 but enhanced in CY1. In addition, fumaric acid (FA), alpha-ketoglutaric acid (KGA), and oxaloacetic acid (OAA), which are intermediates of the tricarboxylic acid (TCA) cycle (Guo et al., 2013; Kuang et al., 2018; Song et al., 2013), were highly expressed in Y1 (Supplementary Figure S6G–I). NADP⁺, which is involved in the fatty acid anabolism (Katsyuba et al., 2020),

was significantly highly expressed in CY1 (Supplementary Figure S6J), suggesting that CY1 has stronger fatty acid metabolism than Y1.

Immune cell heterogeneity and state-dependent dynamics

The liver is a central immune organ harboring diverse immune cell populations that maintain immune responses and tolerance (Parlar et al., 2023; Seki & Brenner, 2008). Among these, TCs mediate adaptive immunity, while NK cells belong to the innate immune system (Parlar et al., 2023). We discovered that TC and NK contents at the Y1 stage were significantly higher than at the D30 stage (Figure 1C), consistent with previous reports (Rao et al., 2023). TC and NK contents in CY1 were markedly lower than in Y1. In contrast, TC content in PY1 was slightly higher than in Y1, whereas NK content in PY1 was lower than in Y1 (Figure 2C). These results indicate that TCs and NKs in the liver exhibit heterogeneity under different physiological states. Therefore, we re-isolated TCs and NKs and annotated them separately.

A total of 14 519 TCs from Y1, PY1, and CY1 were re-clustered into six subtypes, including four CD8⁺ T cell subtypes, one CD4⁺ T cell subtype, and one gamma delta ($\gamma\delta$) T cell subtype (Figure 4A; Supplementary Figure S7A; Supplementary Table S9). GZMH⁺CX3CR1⁺ CD8 T cells highly expressed *ZEB2* and *KLRG1* (Figure 4B), both involved in effector T cell differentiation, with *ZEB2* playing a role in TC cytotoxic function (Giles et al., 2022; Herndler-Brandstetter et al., 2018; Omilusik et al., 2015). This subtype belonged to differentiated TCs expressing the cytotoxic molecule GZMH. CCL5⁺ CD8 T cells were identified as regulatory TCs expressing chemokines. GO analysis revealed that this subtype exhibited characteristics of lymphocyte chemotaxis regulation (Supplementary Figure S7B). ITGAM⁺ CD8 T cells highly expressed *ITGAM* (integrin subunit alpha M) and *SYK* (spleen associated tyrosine kinase) (Figure 4B), which was involved in immune cell activation (Supplementary Figure S7B). Mucosa-associated invariant T cell (MAITs) and $\gamma\delta$ TCs belong to the atypical T cell family.

GO analysis indicated that MAITs were involved in antigen processing and presentation (Supplementary Figure S7B). Naive CD4⁺ T cells specifically expressed *CCR7* and *LEF1* (Figure 4B). All these TC subtypes exhibited significantly lower abundance in CY1 compared to Y1 and PY1 (Figure 4C; Supplementary Table S10), suggesting that TCs may not be the primary immune effector cells in CY1. Additionally, proliferating CD8 T cells were identified from 10 974 TCs of D30 and Y1, were associated with cell cycle process and were mainly derived from D30 (Supplementary Figure S7B–E), indicating their important role in the proliferation of young pig liver cells. CCL5⁺ CD8 T cells were primarily distributed in Y1 (Supplementary Figure S7E), indicating that during liver development, TC function shifts toward lymphocyte chemotaxis.

We re-extracted 9 883 NKs from Y1, PY1, and CY1 and re-clustered them into four distinct subpopulations, including liver-resident NK cells (LrNK), CD226⁺ NK, HP⁺ NK, and WNT5B⁺ NK (Figure 4D; Supplementary Figure S8A). LrNK belonged to NKT-like cells (Andrews et al., 2024) and specifically expressed key characteristic genes such as *EOMES*, *CMC1* and *KLRF1* (Figure 4E). GO analysis similarly indicated that this subtype is associated with T cell activation and differentiation (Supplementary Figure S8B). CD226⁺ NK cells were the least abundant of the four NK subtypes (Figure 4F), and their specific function remains unclear. HP⁺ NK cells were the most abundant subtype in CY1 (Figure 4F), highly expressing the fibrinogen genes *FGA*, *FGB*, and *FGG* (Figure 4E), which are synthesized and secreted by Heps and participate in coagulation and hemostasis (Simurda et al., 2020). WNT5B⁺ NK cells were mainly derived from Y1 and highly expressed *WNT5B* (Figure 4E). The WNT signaling pathway is closely related to NK cell development and activation (Valencia et al., 2010; Zhang et al., 2009). This subtype also highly expressed *STAT1* and *STAT2*, members of the signal transducer and activator of transcription (STAT) family (Figure 4E). The JAK/STAT pathway is involved in cellular proliferation, differentiation, organ development, immune homeostasis,

and autoimmune diseases (Hu et al., 2021). Similarly, four NK subtypes were identified from 8 674 NKs from D30 and Y1 (Supplementary Figure S8C, D). In addition to LrNK, HP⁺ NK and WNT5B⁺ NK, we also identified a conventional NK (cNK) subset with high expression of *GNLY* and *FCGR3A* (Supplementary Figure S8E). GO analysis showed that this subtype was associated with immune response activation and regulation of cell surface receptor signaling pathways (Supplementary Figure S8B). Notably, cNKs were not only the least abundant of the four subtypes but also the only subtype with higher abundance in D30 than in Y1 (Supplementary Figure S8F, G). These results indicate that the immunological specificity mediated by liver NKs during different growth stages.

Dynamic cell-cell communication networks under different states

We employed CellChat analysis to explore cell-to-cell communication between Heps and NPCs in different developmental (D30 and Y1) and physiological (Y1, PY1, and CY1) states. The number and strength of interactions among adult liver cells decrease during development (Supplementary Figure S9A). However, liver cell-cell interactions increase significantly during pregnancy (Figure 5A). Similarly, interactions between Heps and NPCs exhibited comparable patterns (Supplementary Figure S9B, C). Notably, during the PY1 stage, Heps showed significantly stronger outgoing interaction strengths compared to other states (Supplementary Figure S9D, E). Differential analysis of interactions between hepatic cell types at different states revealed that the number and strength of interactions between Heps and other cell types increased during PY1 relative to Y1 (Figure 5B), suggesting altered Hep communication patterns in the pregnancy liver.

We performed ligand-receptor analysis for D30, Y1, PY1, and CY1 and predicted numerous ligand-receptor pairs between Heps and other NPCs at each state. D30 has more interaction pairs than Y1, and PY1 had more interaction pairs than Y1 and CY1 (Supplementary Table

S11). When Heps served as signal receivers, the WNT5B-FZD5 pair was expressed between NK cells and Heps during PY1 but not in D30, Y1, or CY1 (Figure 5C; Supplementary Figure S9F). WNT5B is a non-canonical WNT ligand that often functions as an antagonist of canonical WNT signaling (Suthon et al., 2021). When Heps served as signal senders, an identical ligand-receptor pair, C3-(ITGAM+ITGB2), was expressed between Heps and NK cells during both D30 and PY1 (Figure 5D; Supplementary Figure S9G). C3 (complement component 3), mainly synthesized by Heps, promotes lipophagy to facilitate VLDL secretion, thus playing an essential role in balancing triglyceride levels in the liver (Li et al., 2021). Notably, PLG-PARD3 and F2-PARD2 were significantly expressed between Heps and HSCs, as well as between Heps and Chols (Supplementary Figure S9G). When Heps served as receptors, PLG-PARD3 was expressed only at the CY1 state between HSCs and Heps.

Further, we identified several ligand-receptor pairs that exist only between Heps and specific NPCs and are expressed exclusively during PY1 (Figure 5C, D). When Heps served as signal receivers, we observed ANGPTL1-(ITGA1+ITGB1) between BCs and Heps, ANGPT2-(ITGA5+ITGB1) between PCs and Heps, and ANGPTL4-(ITGA5+ITGB1) between ECs and Heps. The ANGPTL family plays roles in lipid metabolism, glucose metabolism, energy metabolism, and angiogenesis (Yang et al., 2022). We also observed interactions between ECs and Heps involving WNT9B-(FZD5+LRP6), WNT9B-(FZD5+LRP5), WNT2-(FZD5+LRP6), and WNT2-(FZD5+LRP5). Interactions between HSCs and Heps included BMP5-(BMPR1A+BMPR2), BMP5-(BMPR1A+ACVR2A), BMP5-(ACVR1+BMPR2), and BMP5-(ACVR1+ACVR2A). BMP5 has been shown to be a novel player in the complex regulation of hepcidin and systemic iron homeostasis (Nai, 2023). The TGFA-EGFR interaction between Chols and Heps plays a role in regulating cell proliferation and differentiation, and epidermal growth factor receptor (EGFR) signaling in Heps is essential for liver regeneration (Gonzalez-Sanchez et al., 2024; Han & Li, 2019). Furthermore, SPP1-(ITGAV+ITGB5), SPP1-

(ITGAV+ITGB1), SPP1-(ITGA9+ITGB1), and SPP1-(ITGA5+ITGB1) were also expressed between Chols and Heps. SPP1 is highly expressed in hepatocellular carcinoma tissues (Liu et al., 2022). Moreover, when Heps served as signal senders, two ligand-receptor pairs, RARRES2-CMKLR1 and HC-C5AR1, were detected between Hep and KCs. RARRES2-CMKLR1 regulates adipogenesis and metabolic homeostasis in adipocytes (Goralski et al., 2007), while C5AR1 is associated with insulin secretion (Atanes et al., 2018). These results reveal highly active cell-to-cell communication during PY1, as well as complex interactions between Heps and other NPCs, that together maintain liver function and homeostasis.

Spatial organization of liver cells and their interactions

To comprehensively analyze the spatial distribution of liver cells under different physiological states, we collected liver tissues from the Y1, PY1, and CY1 and performed spatial transcriptomics sequencing via the 10× Genomics Visium platform (Figure 1A; Supplementary Table S12). The spatial distribution of liver cells was visualized by H&E staining and unique molecular identifiers (UMIs) for each sample (Supplementary Figure S10A). Since each spatial point contains multiple cells, to determine the cellular composition of each spot, we applied the cell2location deconvolution algorithm to map the cell types defined by snRNA-seq onto spatial tissue sections (Figure 6A; Supplementary Figure S10B, C). In addition, marker genes for Hep subtypes were also mapped (Supplementary Figure S10D).

We then identified seven distinct cellular niches based on the cell2location deconvolution results for each spot (Figure 6B; Supplementary Figure S11A), Spatial niche 3 contained the most abundant cell types, including HSC, Chol, TC, BC, and DC. PCs were mainly located in spatial niche 6. Interestingly, spatial niche 7 contained a large number of Heps and NKs, suggesting a co-localized relationship and potential communication between them. Therefore, we used MISTy analysis to predict spatial co-localization relationships between hepatic cells

(Supplementary Table S13). Heps were identified as an important predictor of NK abundance (Figure 6C; Supplementary Figure S11B), and NKs were scarce in regions with a high proportion of Heps and vice versa (Supplementary Figure S11C), suggesting spatial dependency between these two cell types.

Given this spatial co-localization, we further explored the interactions between Hep subtypes and NK cell subsets. Compared with NK cells, Hep subtypes exhibited more active signal-secreting cells (Supplementary Figure S12A, B). Analysis of incoming and outgoing communication patterns revealed that HepIV and NK cells shared similar signal-receiving patterns, whereas Hep8 and NK cell subsets showed similar signal-output patterns (Supplementary Figure S12C, D). Further analysis of Hep-NK interactions identified specific upregulation of the ligand-receptor pairs FN1-CD44 and FN1-(ITGA4+ITGB1) between all Hep and NK subtypes (Supplementary Figure S12E, F). FN1, a member of the glycoprotein family, enhances insulin sensitivity via the hepatocyte receptor $\alpha 5 \beta 1$ integrin and the downstream IKK α/β -JNK1-BECN1 signaling pathway (Kuramoto et al., 2023; Li et al., 2019). Additionally, the ligand-receptor pair PDGFD-PDGFRB was also specifically upregulated between all NK and Hep subtypes (Supplementary Figure S12G, H). PDGFD is a potent angiogenic factor that can induce cell transformation and promote tumor growth (Li et al., 2003).

Using COMMOT, we explored the signal flow and abundance of specific ligand-receptor interactions in the spatial transcriptomics data. Only ligand-receptor pairs expressed in at least 5% of spots were retained, yielding 63, 41, and 31 ligand-receptor pairs for Y1, PY1, and CY1, respectively (Supplementary Table S14). The network diagram revealed complex communication between various hepatic cell types, with F2-PARD3 and PLG-PARD3 co-expressed in all three stages (Figure 6D; Supplementary Figure S13A, B). F2 encodes coagulation factor IIa, and PLG encodes plasminogen; both are serine proteases (Celkan, 2017;

Rao et al., 2023; Sato et al., 2016). PARD3 is involved in coagulation, and abnormalities in coagulation and fibrinolysis are closely associated with liver disease (Rao et al., 2023). In addition, Y1 exhibited specific ligand receptor pairs, WNT2-(FZD5+LRP6) and WNT2-(FZD5+LRP5). The combination of WNT with FZD5 receptor and LRP5/LRP6 can activate the canonical WNT signaling pathway, which regulates liver metabolism, development, and regeneration (Apte, 2018; Clerbaux et al., 2016) (Supplementary Figure S13C). These ligand-receptor pairs have been reported at the single-cell level in the liver (Rao et al., 2023), and here we elaborate on them from a spatial perspective. These findings underscore the importance of these molecules in the spatial organization and communication of the liver.

Cross-species analysis of liver cells from pigs, humans, and mice

To assess the conservation and species specificity of liver cell types, we integrated single-nucleus sequencing data from adult pig, human, and mouse livers. After quality control, normalization, and integration, 29 cell clusters were annotated as 10 major liver cell types: Hep, TC, EC, KC, NK, BC, HSC, DC, PC, and Chol (Figure 7A, B; Supplementary Figure S14A), with their specific expression genes shown in Supplementary Figure S14B. The expression patterns of classic marker genes for these 10 cell types were highly consistent across the three species (Figure 7C), indicating a conserved cellular architecture of the liver. Quantitative analysis revealed species-specific differences in cell type proportions. Compared with pigs, the proportion of Heps was higher in both humans and mice (Supplementary Figure S14C).

Given the central role of Heps in liver function, we extracted Heps from the 29 clusters for further cross-species analysis. Eight Hep clusters (clusters 0, 7, 11, 13, 14, 16, 26, and 27) exhibited distinct distribution patterns across the three species (Figure 7B; Supplementary Figure S14A). Cross-species comparison of species-specific and conserved gene sets revealed

that a substantial number of overlapping genes were shared among all three species, while Hep gene expression patterns displayed clear species-specific differences (Supplementary Figure S14D). GO enrichment analysis revealed functional divergence among Hep clusters. Cluster 11 (predominantly pig Heps) was significantly enriched in fatty acid oxidation and fatty acid beta-oxidation pathways. Cluster 13 (predominantly mouse Heps) was significantly enriched in the TCA cycle pathway. Cluster 0 (shared across pig, human, and mouse) was enriched in fatty acid metabolic process and fatty acid catabolic process pathways (Supplementary Figure S14E; Supplementary Table S15).

We next performed gene set module clustering for fatty acid metabolism and TCA cycle pathways (Figure 7D). Gene set scoring revealed that clusters 7, 11, and 16 showed high expression of genes related to fatty acid biosynthetic process (Figure 7E). Clusters 11, 13, and 14 exhibited high expression of genes related to fatty acid catabolic process (Figure 7F). Clusters 13, 14, and 27 showed high expression of genes related to the TCA cycle (Figure 7G). Together, these cross-species analyses demonstrate that while the major liver cell types and their marker gene expression patterns are highly conserved across pigs, humans, and mice, Hep subtypes exhibit species-specific transcriptional profiles and functional specializations.

DISCUSSION

In this study, we constructed a single-nuclear transcriptomic atlas of the pig liver across developmental (D30 and Y1) and physiological (Y1, PY1, and CY1) states, with particular attention to the heterogeneity of hepatocytes and immune cells and their metabolic adaptations. By integrating spatial transcriptomics, we further revealed the spatial organization and intercellular communication networks within the liver. Together, these multi-omics data provide a comprehensive resource for understanding liver development, physiological plasticity, and the spatial regulation of hepatic function.

Hepatocytes exhibit a high degree of heterogeneity during postnatal development, as revealed by single-cell transcriptomic studies in both mice (Liang et al., 2022) and pigs (Rao et al., 2023). Hepatocytes showed distinct subtype-specific gene expression patterns during development, with the HepI subtype predominating in the young liver, whereas the HepIII subtype, characterized by high expression of *FASN* and *LIPG*, predominated in the adult liver. Immune-like hepatocytes, growth-type hepatocytes, and intermediate-type hepatocytes were also identified here. Studies have shown that adult pigs require more stable energy homeostasis, and there are differences in energy demand and growth status between young and adult pigs, resulting in differences in fatty acid synthesis (Kusakabe et al., 2000; Rao et al., 2023). Here, we found that the genes up-regulated by Y1 were enriched in the fatty acid metabolism pathway, and differential metabolites highly expressed in Y1 were involved in fatty acid anabolism and oxidation. At the same time, free fatty acid levels in adult pig liver were much higher than those in young pigs. These results further illustrate the differences in fatty acid metabolism characteristics between adult and young pigs. The absence of a distinct PC cluster in the developing liver, in contrast to the gestational liver, likely reflects biological differences. Proliferation during normal growth is distributed across multiple cell types, particularly a subset of Hep (HepVI) expressing *TOP2A* and *TPX2*, whereas the PCs observed in gestation represent a transient, state-specific population. Nevertheless, technical factors such as differential capture efficiency of PCs cannot be entirely excluded. Subsequently, trajectory analysis of hepatocyte development showed that multiple catabolism- and protein-related metabolic pathways were enriched during hepatocyte maturation, and immune system regulation processes were enriched at the later end of the development trajectory, with the immune characteristics of hepatocytes gradually emerging. It should be noted that this pseudo-time analysis was based on two discrete time points (D30 and Y1). The inferred ordering represents a transcriptional continuum rather than a proven developmental trajectory, and the

intermediate states identified require validation with additional time points. Of note, all liver samples were collected from the right lateral lobe only, and given the potential functional heterogeneity across the six lobes of the pig liver, our findings may not fully represent the transcriptional and metabolic characteristics of other lobes. This finding warrants further investigation.

Pregnancy and castration affect the metabolic adaptation characteristics of the liver, and hepatocytes proliferate and enter a higher anabolic state during pregnancy (Goddard et al., 2017; Yao et al., 2024). We identified that hepatocytes, primarily derived from Y1, highly express *FASN* and the triglyceride lipase gene family member *LIPG*. The hepatocyte population of PY1 was involved in lipid catabolism and long-chain fatty acid catabolism, while CY1 specifically expressed ribosomal proteins. In addition, we identified other hepatocyte subtypes, including immune-like hepatocytes (Hep6 and Hep8), a growth-type hepatocyte (Hep10), and an intermediate-type hepatocyte (Hep9). Previous studies have shown that the liver during pregnancy exhibits the characteristics of a “pregnancy clock”, coordinated by hepatocyte proliferation and metabolism (Kusakabe et al., 2000). Here, we captured 17 hepatocyte-specific TFs and identified TFs specifically activated in each hepatocyte subtype. The hepatocyte population-specific expression in PY1 involved the TF ATF6, which interacts with PPAR α to promote liver fatty acid oxidation, and TCF7L2, a key effector of the WNT/ β -catenin signaling pathway (Bagchi et al., 2020; Chen et al., 2018; Chen et al., 2016). Hepatocytes of CY1 express the TF BACH1, which regulates insulin signaling and glucose homeostasis (Jin et al., 2023). Hepatocytes of Y1 specifically express GATA6, involved in hepatocyte differentiation, and ONECUT1, which regulates cell cycle and glucose metabolism (Prajapati et al., 2024; Zhang & He, 2018). These TFs, specifically expressed at different physiological stages, help us better understand the functional changes of hepatocytes. Furthermore, we found differences in the expression of energy metabolites in the liver under different physiological

conditions. In particular, the metabolites NADP⁺ and NAD⁺, which are involved in fatty acid synthesis and oxidation, were significantly elevated in CY1, suggesting that the CY1 stage has heightened fatty acid metabolism. These results further illustrate the metabolic adaptation characteristics of the liver under different physiological states. However, as this study was conducted exclusively on Chenghua pigs, generalization of these findings to other pig breeds (e.g., Large White or Landrace) remains to be determined.

The liver is an immune tolerance organ (Zhao et al., 2020). A study in pigs has found that from D30 onward, the liver immune system is dominated by lymphoid cells (Rao et al., 2023). Based on the identification of immune-like hepatic cell subsets and the enrichment of immune function characteristics at the later stage of the hepatocyte development trajectory, and the spatial dependence between hepatocytes and NK cells, we analyzed the subsets of two immune cell types (TC and NK). Here, we identified proliferative T cells mainly from D30 and regulatory TCs mainly derived from Y1 expressing chemokines, indicating a specific shift in liver TC function during development. At the same time, the low abundance of five TC subsets in CY1 under different physiological conditions suggests that other cell types may exert immune functions in CY1, though further exploration is needed. Studies have shown that liver-resident immune cells determine immune characteristics (Zhao et al., 2020). We found that LrNKs are highly expressed in adulthood, especially during the PY1 period, and specifically express *EOMES*, *CMC1* and *KLRF* genes, consistent with previous reports (Zhao et al., 2020). In general, these immune cell subsets collectively characterize the immune dynamics of the liver.

Beyond hepatocyte-centered interactions, we also characterized the broader cell-cell communication networks. We found that the number and strength of interactions among adult liver cells decreased during liver development but increased significantly during pregnancy. This observation aligns with the notion that liver regeneration and adaptation require intricate

coordination between parenchymal and NPCs cells (Bhatia et al., 1999). Additionally, several ligand-receptor pairs between hepatocytes and NPCs, including ANGPTL1-(ITGA1+ITGB1), ANGPT2-(ITGA5+ITGB1), and ANGPTL4-(ITGA5+ITGB1), were expressed only at the PY1 stage and regulate lipid metabolism, glucose metabolism, and energy metabolism in the liver. Notably, the ANGPTL family has been shown to mediate cell-cell communication and play critical roles in modulating triglyceride metabolism and related metabolic diseases (Yang et al., 2025). Other ligand-receptor pairs identified in our study, such as TGFA-EGFR, are related to cell proliferation, differentiation, and liver regeneration. The epidermal growth factor receptor (EGFR) and its ligand transforming growth factor-alpha (TGF- α) play critical roles in organ development, tissue homeostasis, and regeneration (Tito et al., 2025). During gestation, enhanced EGFR signaling may contribute to the increased hepatocyte proliferation and anabolic state observed in the maternal liver. These results further demonstrate the complexity of liver function and the requirement for cooperation between hepatocytes and various NPCs, consistent with previous reports that liver cell crosstalk is essential for maintaining tissue homeostasis and orchestrating adaptive responses (Kostallari et al., 2025).

Combining spatial transcriptomic data with single-cell transcriptomic data helps to understand the biological characteristics of tissues from a new perspective. Spatial transcriptomics provides spatial information that complements single-cell transcriptome data (Andersson et al., 2020; Hildebrandt et al., 2021). Here, we deconvolved the 10 liver cells identified by snRNA-seq into coordinates and found co-localization and interdependence of hepatocytes and NK cells within specific spatial niche. This may indicate potential communication between immune-like hepatocytes and NK cells. In addition, we identified three co-expressed spatial receptor pairs (F2-PARD3 and PLG-PARD3) across different physiological states, as well as Y1-specific spatial receptor pairs WNT2-(FZD5+LRP6), WNT2-(FZD5+LRP5), suggesting that complex spatial communication between liver cells

regulates liver function. However, the specific cell-cell interaction processes in space require further investigation.

Our cross-species analysis revealed that while the major liver cell types are highly conserved across pigs, humans, and mice, supporting the pig as a relevant model for human liver biology given its anatomical and metabolic similarities to humans (Ekataksin & Wake, 1991), hepatocyte subtypes exhibited pronounced species-specific functional specialization. Specifically, we found that pig-enriched hepatocyte clusters were significantly enriched in fatty acid oxidation and beta-oxidation pathways, whereas mouse-enriched clusters were biased toward the TCA cycle. These differences were independently validated by gene set scoring. This metabolic divergence is consistent with previous comparative studies showing that pig hepatocytes exhibit lower rates of ketogenesis and beta-oxidation than rodent hepatocytes, with acetate serving as a major end-product of fatty acid metabolism (Adams et al., 1997). Moreover, it has been demonstrated that functional PPAR α exists in pig liver and mediates the transcriptional response to fasting and fatty acid challenge (Cheon et al., 2005), which may underpin the enhanced fatty acid oxidation capacity observed in pig hepatocytes in our study. In contrast, the higher basal metabolic rate of mice likely drives their greater reliance on TCA cycle-mediated ATP generation. From a translational perspective, the closer alignment of pig hepatocyte metabolism with human fatty acid metabolism supports the pig as a superior model for studying human metabolic diseases such as metabolic dysfunction-associated steatotic liver disease (Hvid et al., 2024). Nevertheless, potential batch effects from publicly available datasets and species-specific differences in ages, diet, housing conditions, and physiological demands should be considered when interpreting these findings.

In summary, this study delineates the cellular and transcriptional dynamics of the pig liver across development, pregnancy, and castration, revealing hepatocyte heterogeneity, immune cell plasticity, and spatial organization. These findings provide a foundational resource for

understanding liver metabolism and intercellular communication in physiological and translational contexts.

DATA AVAILABILITY

All sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive under BioProject accession number PRJNA1419143. The snRNA-seq and stRNA-seq datasets are also available in the GEO database under accession numbers GSE326429 and GSE330889, respectively.

CODE AVAILABILITY

No custom code was generated in this study. All bioinformatic analyses were performed using publicly available software packages and tools as detailed in the Methods section.

SUPPLEMENTARY DATA

Supplementary Figure

Supplementary Figure S1 Liver tissue samples of Chenghua sows from D30, Y1, PY1, and CY1. D30, 30 days after birth; Y1, 1 year after birth; PY1, 1 year after birth for the late-pregnancy; CY1, 1 year after birth following neonatal castration.

Supplementary Figure S2 H&E and Oil Red O staining of liver tissues from D30, Y1, PY1, and CY1. Scale bars: 10× and 40× magnification.

Supplementary Figure S3 Identification of liver cells and hepatocytes in young and adult pigs. A: UMAP visualization of hepatic cell types in young and adult pigs by group. B: Dot plot displaying the expression of manually selected marker genes for liver cell types. C: Liver organ index for young and adult pigs. The liver organ index (%) was calculated as (liver weight/body weight) × 100. $n=3$. ***: $P<0.001$. D: UMAP visualization of hepatocyte subtypes

876 by group (D30 and Y1).

877 **Supplementary Figure S4 Additional plots associated with the hepatocyte developmental**

878 **trajectory and functional enrichment.** A, C: Hepatocyte developmental trajectory visualized

879 by Monocle2, colored by state (A) and by group (C). B: Pseudotime trajectory analysis

880 identifying divergent differentiation branches for hepatocyte subtypes. D, E: Density

881 distribution of hepatocytes along pseudotime by group (D) and subtype (E). F: GO_BP

882 enrichment analysis of up-regulated hepatocyte DEGs in Y1 compared to D30. GO_BP, Gene

883 Ontology Biological Process; DEGs, differentially expressed genes. G: Heatmap of

884 hepatocytes differential genes from pseudobulk analysis of Y1 and D30. Gene expression

885 levels were normalized using Z-score. Red and blue indicate up-regulation and down-

886 regulation relative to the mean, respectively; white indicates expression near the mean. Color

887 intensity corresponds to the magnitude of Z-score deviation. H: Concentration of free fatty

888 acids (FFAs) ($\mu\text{mol/g}$) and triglycerides (TGs) ($\mu\text{g/g}$) in liver tissue. Mean $\pm$ SD. $n=3$. ns:

889 $P>0.05$; ***: $P<0.001$.

890 **Supplementary Figure S5 Identification of liver cells and hepatocytes under different**

891 **physiological states and functional analysis of hepatocyte subtypes.** A: UMAP visualization

892 of hepatic cells divided by Y1, PY1, and CY1 groups. B: Heatmap of scDRS association

893 analysis between hepatic cell types and metabolic traits. Color intensity represents the strength

894 of association, with red indicating positive associations and blue indicating negative

895 associations. C: UMAP visualization of hepatocyte subtypes divided by Y1, PY1, and CY1

896 groups. D: Serum concentration of progesterone (PROG) (ng/mL) and glucocorticoid receptors

897 α (GR- α) (ng/mL). Mean $\pm$ SD. $n=3$. **: $P<0.01$. E–G: GO enrichment terms for DEGs in Hep5

898 (E), Hep1 (F), and Hep3 (G).

899 **Supplementary Figure S6 Additional plots associated with the capture of 17 hepatocyte-**

900 **specific TFs and DMs expression under different physiological states.** A: Dot plot

901 displaying 17 hepatocyte-specific TFs for hepatocyte subtypes. B: Five significantly DMs in
 902 PY1 vs. Y1. C: DMs in CY1 vs. Y1. D–F, H–J: Expression trends of phosphoenolpyruvate
 903 (PEP; D), 3-phosphoglycerate (3PG; E), dihydroxyacetone phosphate (DHAP; F), alpha-
 904 ketoglutaric acid (KGA; H), oxaloacetic acid (OAA; I), and nicotinamide adenine dinucleotide
 905 phosphate (NADP⁺; J) in CY1 and Y1. Mean±SD. *n*=6. *: *P*<0.05; **: *P*<0.01; ***: *P*<0.001.
 906 G: Expression trend of fumaric acid (FA) in PY1 vs. Y1. Mean±SD. *n*=6. *: *P*<0.05. TFs,
 907 transcription factors; DMs, differential metabolites.

908 **Supplementary Figure S7 Identification of T cell subtypes and GO enrichment analysis.**

909 A: UMAP visualization of T cells divided by Y1, PY1, and CY1 groups. B: Functional GO
 910 enrichment terms for T cell subtypes. C: UMAP visualization of six T cell subtypes identified
 911 in young and adult pigs. D: UMAP visualization of T cell subtypes in young and adult pigs by
 912 group. E: Proportion of group compositions for each T cell subtype.

913 **Supplementary Figure S8 Identification of NK cell subtypes and GO enrichment analysis.**

914 A: UMAP visualization of NK cells divided by Y1, PY1, and CY1 groups. B: Functional GO
 915 enrichment terms for NK cell subtypes. C: UMAP visualization of four NK cell subtypes
 916 identified in young and adult pigs. D: UMAP visualization of NK cell subtypes in young and
 917 adult pigs by group. E: Abundance bubble plot showing marker genes for NK cell subtypes in
 918 young and adult pigs. Bubble size indicates expression percentage, and bubble color indicates
 919 average expression level. F: Proportions of four NK cell subtypes in young and adult pigs. G:
 920 Proportions of group compositions for each NK cell subtype.

921 **Supplementary Figure S9 Additional plots associated with intercellular communication**

922 **between hepatocytes and NPCs.** A: Bar plot showing differences in number and strength of
 923 all liver cells interactions in young and adult pigs. B: Circle plots showing the number of
 924 interactions among cell types in D30 and Y1 livers. C: Circle plots showing the number of
 925 interactions among cell types in Y1, PY1, and CY1 livers. D, E: Dot plots showing incoming

and outgoing interaction strengths among cell types in young vs. adult pigs (D) and three physiological states (E). F, G: Dot plots showing ligand-receptor interactions between hepatocytes and NPCs as signal targets (F) and signal sources (G) in D30 and Y1. Arrows indicate the ligand-receptor pairs described in the text. Dot color indicates communication probability, and dot size reflects *P*-value.

Supplementary Figure S10 Additional plots associated with the spatial distribution of hepatic cells and cell type abundance in liver tissue sections. A: Spatial distribution of hepatic cells in Y1, PY1, and CY1. B, C: Spatial distribution maps showing cell type abundance in liver tissue sections from PY1 (B) and CY1 (C) using the cell2location framework. D: Spatial visualization of marker gene expression across Hep subtypes in a Y1 liver tissue section.

Supplementary Figure S11 Additional plots associated with spatial co-localization relationships. A: Spatial distribution of seven distinct cellular niches in Y1, PY1, and CY1. B: Spatial co-localization relationships between hepatic cells in PY1 and CY1 identified by MISTy analysis. C: Co-localization characteristics of hepatocytes and NK cells in spatial regions.

Supplementary Figure S12 Cell-cell communication between hepatocyte subtypes and NK cell subsets. A: Circle plots showing the number and weight of interactions between hepatocyte and NK subtypes. B: Circle plots showing the number and weight of interactions between hepatocyte subtypes and NK subsets in young and adult pigs. C: River plots illustrating the communication patterns of hepatocyte subtypes and NK subsets across different physiological states, with incoming (left) and outgoing (right) signaling patterns. D: River plots illustrating the communication patterns of hepatocyte and NK subtypes in D30 and Y1, with incoming (left) and outgoing (right) signaling patterns. E, G: Dot plots showing ligand-receptor interactions between hepatocyte and NK subtypes as signal targets (E) and signal sources (G) across different physiological states. Arrows indicate the ligand-receptor pairs described in the

text. Dot color represents communication probability, and dot size reflects *P*-value. F, H: Dot plots showing ligand-receptor interactions between hepatocyte and NK subtypes as signal targets (F) and signal sources (H) in D30 and Y1. Arrows indicate the ligand-receptor pairs described in the text. Dot color represents communication probability, and dot size reflects *P*-value.

Supplementary Figure S13 Additional plots associated with specific ligand-receptor interactions in the liver. A–C: Signaling flow and abundance of specific ligand-receptor interactions in spatial transcriptomics data of PY1 (A), CY1 (B), and Y1 (C) explored using COMMOT.

Supplementary Figure S14 Additional plots associated with hepatocytes from pigs, humans, and mice. A: UMAP visualization of hepatic cell types across three species groups. B: Heatmap of specifically expressed genes in 29 cell clusters. C: Proportions of ten hepatic cell types across pigs, humans, and mice. D: Overlaps of specifically expressed genes in hepatocytes among the three species. E: GO_BP enrichment pathways of eight hepatocyte clusters. GO_BP, Gene Ontology Biological Process.

Supplementary Table

Supplementary Table S1 Marker genes for hepatic cell types, related to Figure 2B and Supplementary Figure S3B.

Supplementary Table S2 Cell Ranger quality metrics, related to Figures 1 and 2.

Supplementary Table S3 Cell numbers and proportions of hepatic cell types and hepatocyte subtypes captured by 10× Genomics, related to Figures 1B, 1D, 2A, and 3A.

Supplementary Table S4 Differential genes in five modules clustered by pseudo-temporal expression patterns in hepatocytes, and GO and KEGG enriched pathways, related to Figure 1H.

Supplementary Table S5 GO_BP enriched pathways for hepatic cell types, related to Figures

1, 2D, and 2E.

Supplementary Table S6 Differential gene clustering and GO_BP pathway enrichment from pseudobulk analysis of Y1 vs. D30, related to Supplementary Figure S4G.

Supplementary Table S7 Differential metabolites in liver tissue under different physiological and developmental states, related to Figures 1I and 3G, and Supplementary Figures S6B–J.

Supplementary Table S8 Hepatocyte subtypes-specific regulons (126 regulons), related to Figure 3E.

Supplementary Table S9 Marker genes for liver immune cell populations, related to Figures 4A and 4D, and Supplementary Figures S7C and S8C.

Supplementary Table S10 Cell numbers and proportions of liver immune cell populations, related to Figures 4C and 4F, and Supplementary Figures S7E, S8F, and S8G.

Supplementary Table S11 Ligand-receptor interactions between hepatic cell types under different states, related to Figures 5C and 5D, and Supplementary Figures S9F and S9G.

Supplementary Table S12 Sample spatial transcriptomics sequencing data summary, related to Figure 6A.

Supplementary Table S13 Spatial co-localization relationships between hepatocytes and NPCs in Y1, PY1, and CY1, related to Figure 6C, and Supplementary Figures S11B and S11C.

Supplementary Table S14 Specific ligand-receptor interactions for spatial transcriptomics data, related to Figure 6D, and Supplementary Figure S13.

Supplementary Table S15 GO_BP enrichment pathways of eight hepatocyte clusters from humans, pigs, and mice, related to Supplementary Figure S14E.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Y.J.L. and Y.Z.J. conceived and designed the study. Y.J.L., Y.Z.J., and X.F.H. developed the methodology. Y.J.L., J.H.T., F.J.L., T.Z., X.L.X., M.J.B., and Z.Y.G. performed the experiments. R.Y. provided partial funding and resources. Y.J. L., and Y.F.W. managed and analyzed the data. Y.J.L. visualized the data. Y.J.L. wrote the original draft. Y.Z.J. reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

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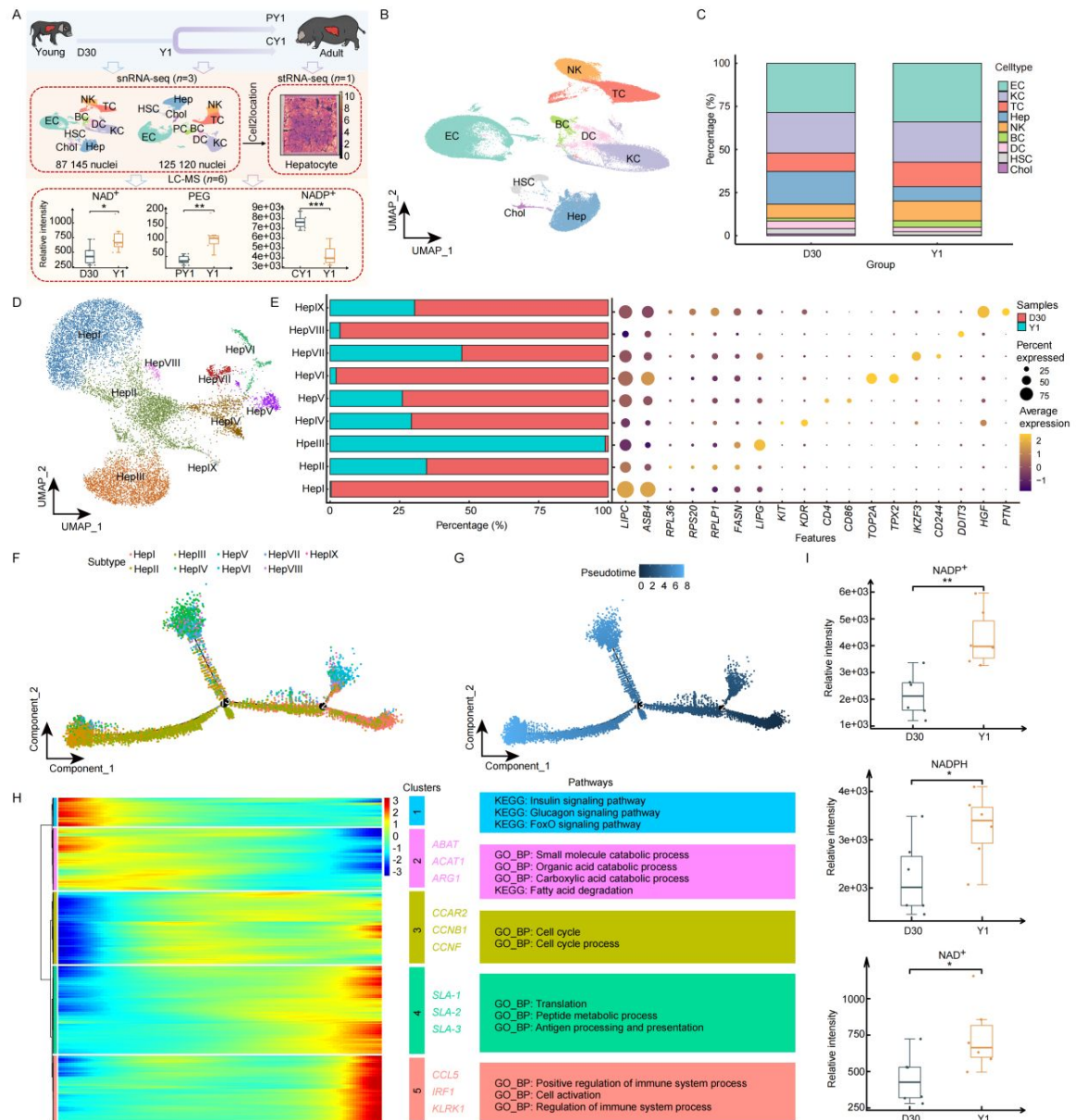


Figure 1 snRNA-seq reveals distinct transcriptomic landscapes of liver cells during postnatal development from youth to adulthood in pigs

A: Summary diagram of experimental design. **B:** UMAP visualization of nine liver cell types. Hepatocyte (Hep), Endothelial cell (EC), Kupffer cell (KC), T cell (TC), NK cell (NK), B cell (BC), Dendritic cell (DC), Hepatic stellate cell (HSC), and Cholangiocyte (Chol). The same applies to cell abbreviations hereafter. **C:** Proportions of the nine cell types in the developing liver of young (D30) and adult (Y1) pigs. **D:** UMAP visualization of nine hepatocyte subtypes.

1259 E: Bar chart showing the time point composition of each hepatocyte subtype (left) and dot plot
 1260 displaying selected genes for each subtype (right). Dot size represents the proportion of cells
 1261 expressing the gene in each cell type, and color scale represents the average expression level.
 1262 F, G: Plot of the hepatocyte development trajectory of subtypes (F) and pseudotime (G) by
 1263 Monocle2. H: Heatmap displaying expression trends of differential genes along inferred
 1264 pseudotime. Genes were clustered into five modules based on pseudotime trajectories.
 1265 Functional enrichment analyses were performed for each module (right). I: Expression trends
 1266 of nicotinamide adenine dinucleotide phosphate (NADP⁺), reduced nicotinamide adenine
 1267 dinucleotide phosphate (NADPH), and nicotinamide adenine dinucleotide (NAD⁺) in D30 and
 1268 Y1. Data are shown as mean±SD. *n*=6. *: *P*<0.05; **: *P*<0.01.

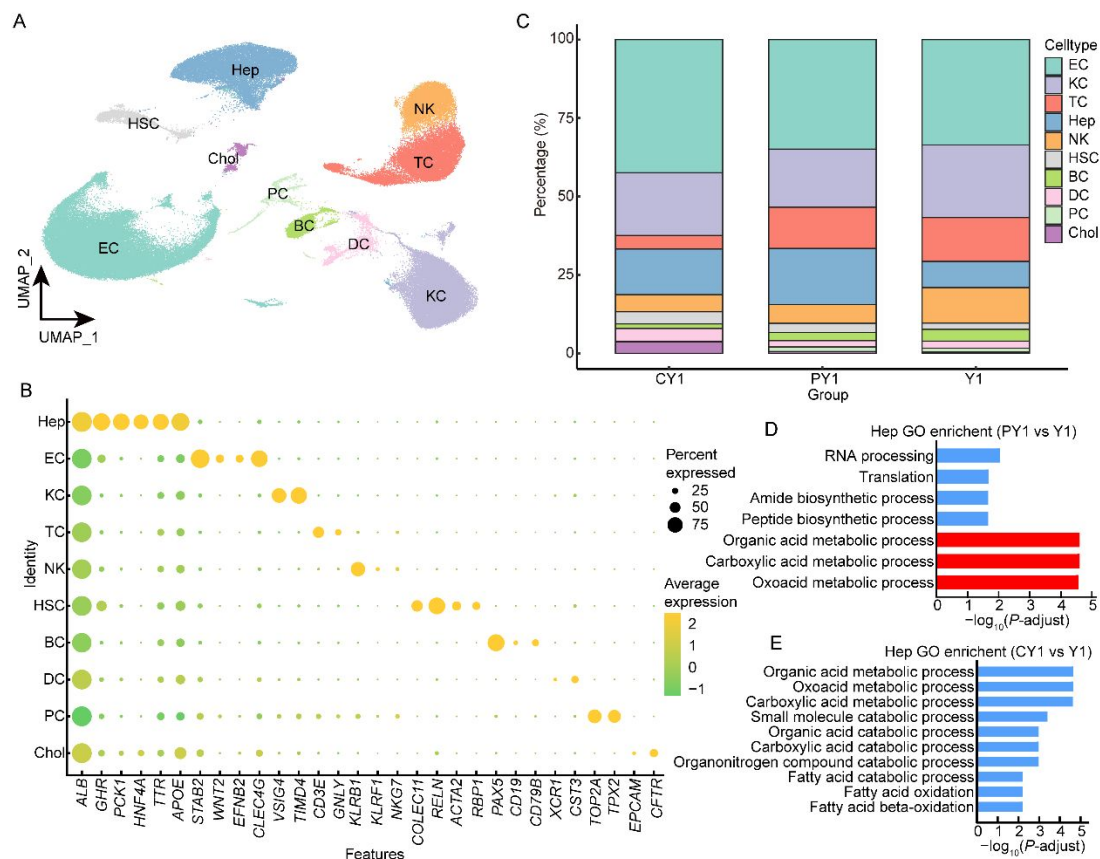
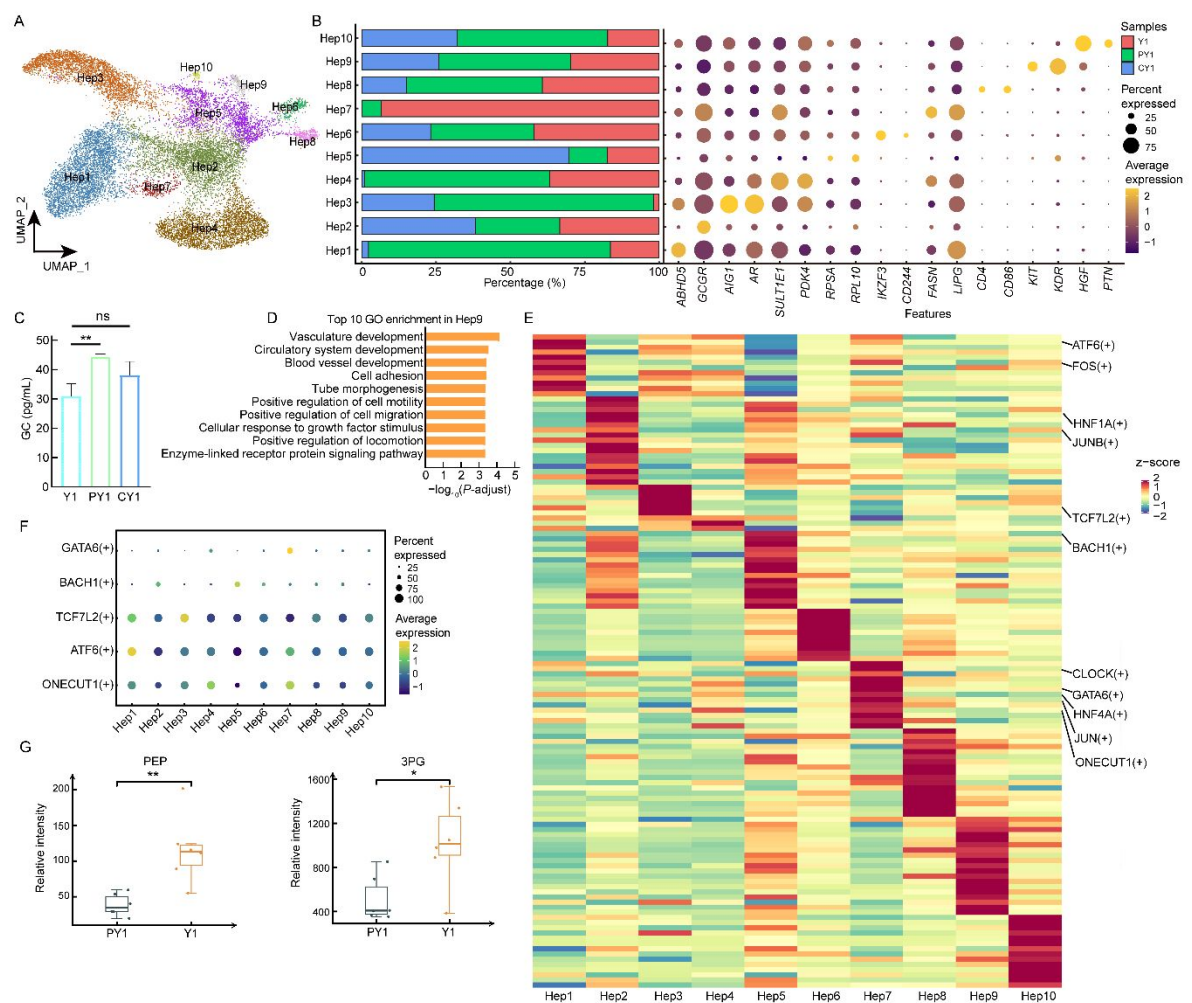


Figure 2 snRNA-seq reveals transcriptomic remodeling of the pig liver under pregnancy and castration

A: UMAP visualization of ten liver cell types. Hepatocyte (Hep), Endothelial cell (EC), Kupffer cell (KC), T cell (TC), NK cell (NK), B cell (BC), Dendritic cell (DC), Hepatic stellate cell (HSC), Cholangiocyte (Chol), and Proliferating cell (PC). B: Dot plot showing expression of manually selected marker genes for the ten liver cell types. Dot size represents the proportion of cells expressing the gene in each cell type, and color scale represents the average expression level. C: Proportions of the ten cell types across different physiological states. D, E: GO_BP enrichment analysis of up-regulated and down-regulated hepatocyte DEGs (differentially expressed genes) in PY1 (D) and CY1 (E) compared to Y1. The vertical axis shows significantly enriched GO_BP (Gene Ontology Biological Process) terms, and the horizontal axis shows $-\log_{10}(P.adjust)$. Red indicates up-regulated terms, and blue indicates down-

1282 regulated terms.

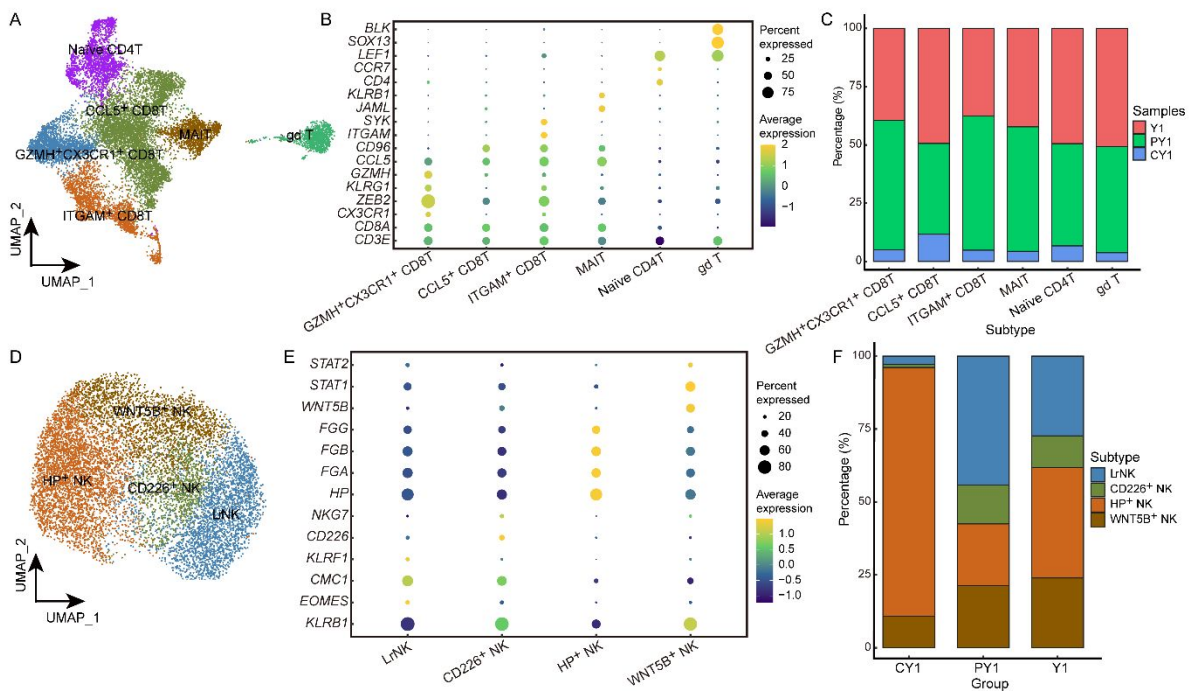


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1284 **Figure 3 SnRNA-seq identifies distinct transcriptome profiles in hepatocytes across**
1285 **different physiological states in pigs**

1286 A: UMAP visualization of ten hepatocyte (Hep) subtypes. B: Bar chart showing time point
1287 composition of each hepatocyte subtype (left) and dot plot displaying selected genes for each
1288 subtype (right). Dot size represents the proportion of cells expressing the gene in each cell type,
1289 and color scale represents the average expression level. C: Serum concentration of glucagon
1290 (GC) (pg/mL). Mean±SD. $n=3$. ns: $P>0.05$; **: $P<0.01$. D: Top ten GO_BP terms for Hep9.
1291 GO_BP, Gene Ontology Biological Process. E: Heatmap showing expression of TF-encoding
1292 genes specifically activated in each hepatocyte subtype. F: Dot plot showing the main subtype-
1293 specific transcription factors (TFs) in hepatocytes. G: Expression trends of
1294 phosphoenolpyruvate (PEP) and 3-phosphoglycerate (3PG) in PY1 vs. Y1. Mean±SD. $n=6$. *:

1295 $P<0.05$. **: $P<0.01$.



1297 **Figure 4 Dynamic changes of liver immune cells at the single-cell level**

1298 A: UMAP visualization of six T cell subtypes identified under different physiological states.

1299 B: Abundance bubble plot showing marker genes for T cell subtypes. Bubble size represents

1300 the percentage of cells expressing the gene, and bubble color represents the average expression

1301 level. C: Proportion of each T cell subtype across the three physiological states. D: UMAP

1302 visualization of four NK cell subtypes identified under different physiological states. E:

1303 Abundance bubble plot showing marker genes for NK cell subtypes. Bubble size represents the

1304 percentage of cells expressing the gene, and bubble color represents the average expression

1305 level. F: Proportion of the four NK cell subtypes across the three physiological states.

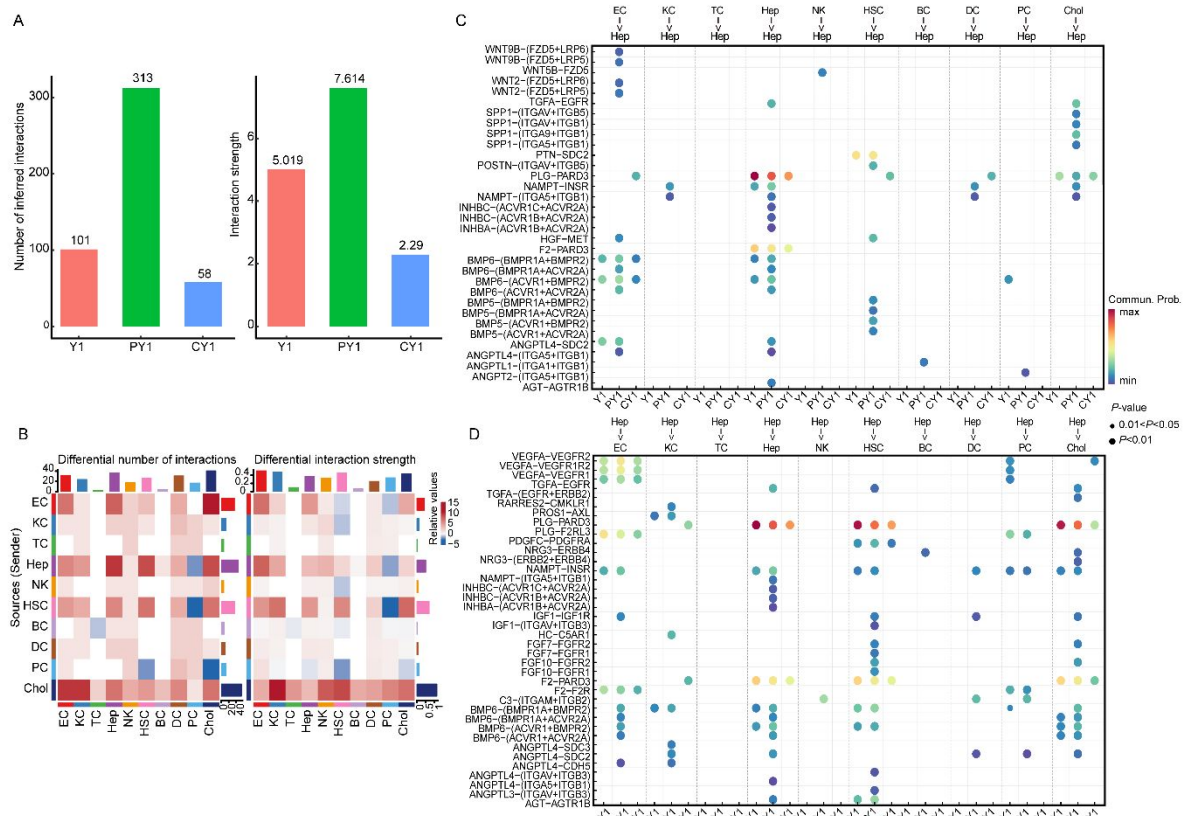


Figure 5 Dynamic cell-cell communication networks under different physiological states

A: Bar plot showing differences in the number and strength of all hepatic cell interactions under different physiological conditions. B: Heatmaps comparing differential interaction counts and strengths between the PY1 and Y1 stages. Hepatocyte (Hep), Endothelial cell (EC), Kupffer cell (KC), T cell (TC), NK cell (NK), B cell (BC), Dendritic cell (DC), Hepatic stellate cell (HSC), Cholangiocyte (Chol), and Proliferating cell (PC). C, D: Dot plots showing ligand-receptor interactions between hepatocytes and non-parenchymal cells as signal targets (C) and signal sources (D). Arrows indicate the ligand-receptor pairs described in the text. Dot color indicates communication probability, and dot size reflects *P*-value.

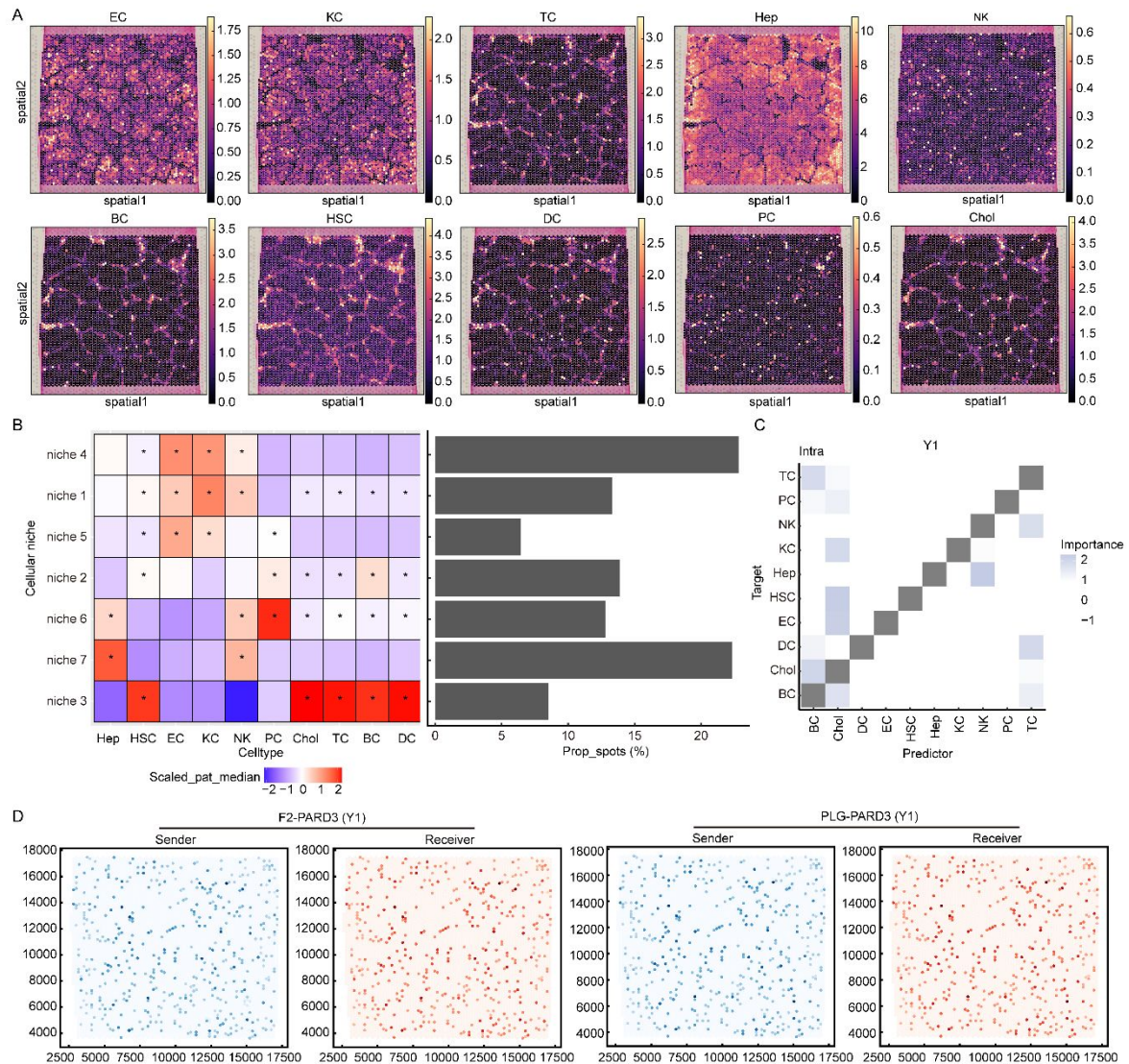


Figure 6 Spatial transcriptomic analysis of liver cells across physiological states

A: Spatial distribution map showing cell type abundance in liver tissue sections from Y1 using the cell2location framework. Hepatocyte (Hep), Endothelial cell (EC), Kupffer cell (KC), T cell (TC), NK cell (NK), B cell (BC), Dendritic cell (DC), Hepatic stellate cell (HSC), Cholangiocyte (Chol), and Proliferating cell (PC). B: Scaled median cell type compositions within each spatial niche. C: Spatial co-localization relationships between hepatic cells in Y1 identified by MISTy analysis. D: Signaling flow and abundance of specific ligand-receptor interactions in liver spatial transcriptomics data of Y1 explored using COMMOT.

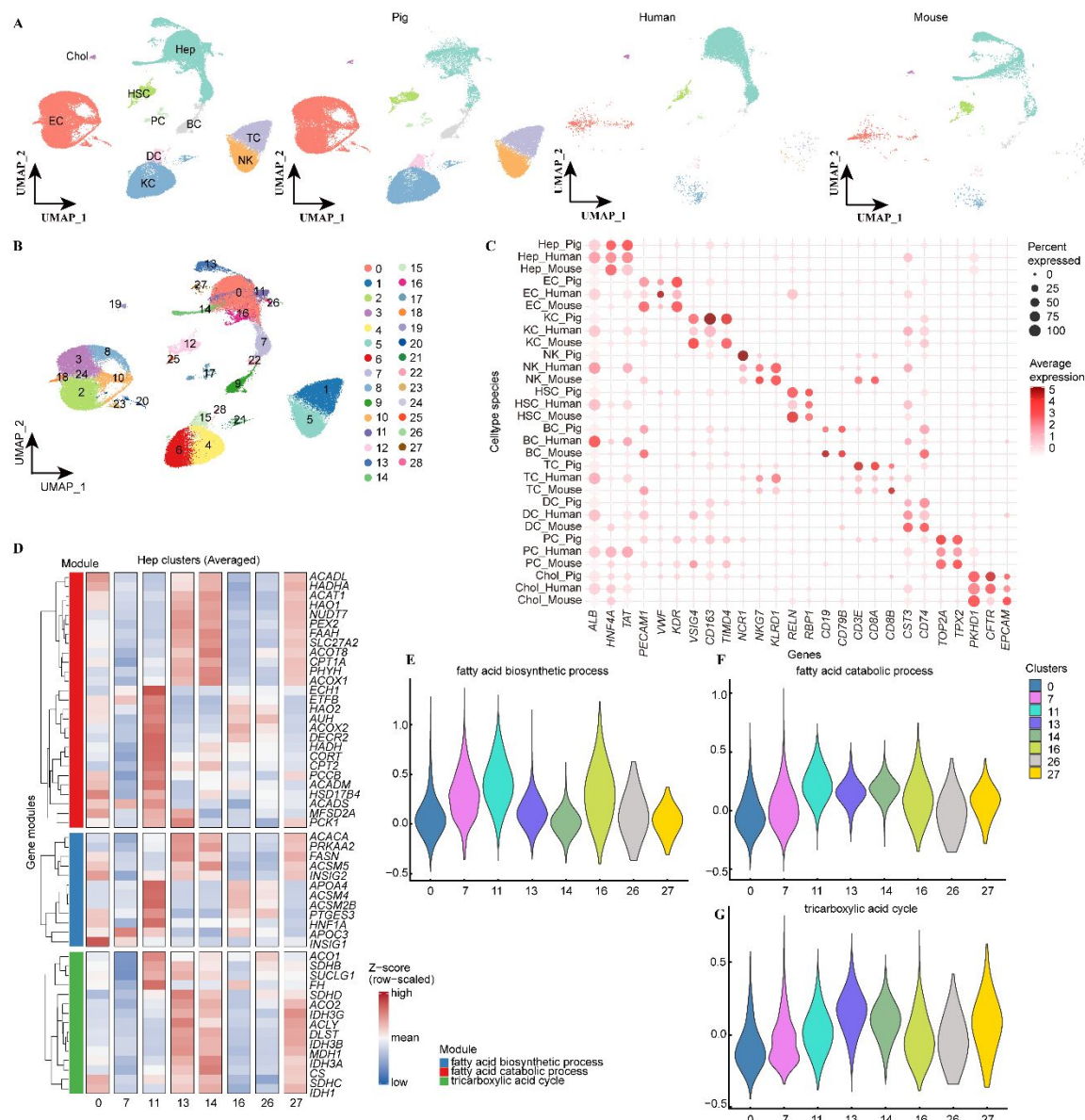


Figure 7 Cross-species analysis of hepatocytes from pigs, humans, and mice

A: UMAP visualization of integrated snRNA-seq data from pigs, humans, and mice. Hepatocyte (Hep), Endothelial cell (EC), Kupffer cell (KC), T cell (TC), NK cell (NK), B cell (BC), Dendritic cell (DC), Hepatic stellate cell (HSC), Cholangiocyte (Chol), and Proliferating cell (PC). B: UMAP visualization of hepatic cell types from pigs, humans, and mice, stratified by cluster. C: Bubble plot showing cross-species comparison of classical marker gene expression for hepatic cell types across all three species. D: Clustered heatmap of target functional gene sets in eight hepatocyte clusters. Gene expression levels were normalized using

row-scaled Z-score. Red indicates high expression above the mean, blue indicates low expression below the mean, and white or light colors indicate expression near the mean. The colored bars on the left denote gene modules: blue for fatty acid biosynthetic process, red for fatty acid catabolic process, and green for tricarboxylic acid cycle. E–G: Gene set scores for fatty acid biosynthetic process (E), fatty acid catabolic process (F), and tricarboxylic acid cycle (G) in pigs, humans, and mice.

多组学分析猪肝脏在不同生理与发育状态下的细胞动态及转录特征

李育晶¹, 唐佳豪¹, 黎富杰¹, 王逸飞¹, 郑婷¹, 徐兴龙¹, 白梦姣¹, 龚正银¹, 韩兴发¹, 袁蓉², 姜延志^{1, 3, *}

¹四川农业大学生命科学学院, 雅安, 四川 625014, 中国

²成都市畜禽遗传资源保护中心, 成都, 四川 610081, 中国

³猪禽种业全国重点实验室, 四川农业大学动物科技学院, 成都, 四川 611130, 中国

*通讯作者, 电子邮箱: jiangyz04@163.com

摘要

肝脏在发育、妊娠和去势过程中发生了巨大的代谢适应重塑，但其潜在的细胞和转录动态机制仍不清楚。本研究对不同发育阶段（幼年期，D30；成年期，Y1）和成年期不同生理状态下（妊娠晚期，PY1；去势，CY1）的成华母猪肝脏进行单细胞核测序，并结合空间转录组学和靶向能量代谢组学进行验证。从幼年期到成年期，肝细胞从增殖状态转变为脂肪酸代谢增强状态，表现为 Y1 时期 *FASN*⁺/*LIPG*⁺ 亚型（HepIII）的出现，以及 *NADP*⁺、*NADPH* 和 *NAD*⁺ 等代谢物水平的显著升高。拟时序分析揭示，肝细胞从细胞周期调控向分解代谢与免疫调节过渡的功能演化轨迹。妊娠和去势分别诱导了不同的肝细胞重塑机制：PY1 肝细胞富含脂质分解代谢相关亚型（Hep1，*ABHD5*⁺；Hep3，*AIG1*⁺、*AR*⁺），*ATF6*/*TCF7L2* 激活促进脂肪酸氧化；而 CY1 肝细胞以富含核糖体蛋白亚型（Hep5，*RPSA*⁺/*RPL10*⁺）和 *BACH1* 富集为特征，糖酵解增强且 *NADP*⁺ 水平升高，提示脂肪酸代谢活跃。除肝细胞变化外，妊娠还显著增强了细胞间通讯，PY1 特异性配体-受体对（如 *ANGPTL* 家族、*WNT* 信号）将肝细胞与非实质细胞连接起来，而免疫细胞亚群（T 细胞和 NK 细胞）表现出状态依赖性组成差异。空间转录组学进一步证实了肝细胞与 NK 细胞在特定微环境中的共定位关系。跨物种分析表明，成年期的猪、人与小鼠之间既存在保守的肝脏细胞类型，又具有物种特异性的代谢基因表达谱。本研究阐明了肝脏稳态的细胞与转录动态特征，提示猪模型用于人类肝脏研究，并提供了宝贵的多组学数据资源。

关键词：猪；肝脏；snRNA-seq；妊娠；去势