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Unraveling the Complexity of Liver Disease One Cell at a Time

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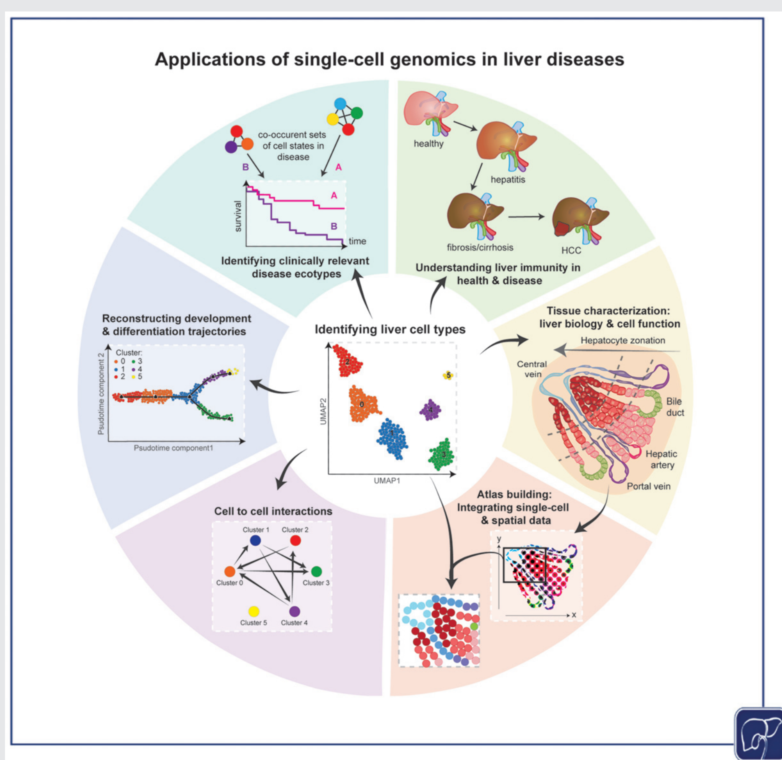
Graphical Abstract



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Abstract

The human liver is a complex organ made up of multiple specialized cell types that carry out key physiological functions. An incomplete understanding of liver biology limits our ability to develop therapeutics to prevent chronic liver diseases, liver cancers, and death as a result of organ failure. Recently, single-cell modalities have expanded our understanding of the cellular phenotypic heterogeneity and intercellular cross-talk in liver health and disease. This review summarizes these findings and looks forward to highlighting new avenues for the application of single-cell genomics to unravel unknown pathogenic pathways and disease mechanisms for the development of new therapeutics targeting liver pathology. As these technologies mature, their integration into clinical data analysis will aid in patient stratification and in developing treatment plans for patients suffering from liver disease.

Keywords

- single-cell transcriptomics
- liver cancer
- hepatitis

The liver performs a vast array of physiological functions that are key to maintaining systemic health. These functions are supported by a unique microanatomical division of labor that allows the liver to carry out various metabolic, xenobiotic, and immunological tasks.[1,2] As the liver is key to many systemic physiological processes, hepatic dysregulation is implicated in several chronic illnesses. With a mortality rate of 2 million people a year globally and few therapeutic interventions, chronic liver disease (CLD) is the 11th most leading cause of death worldwide.[3] CLD occurs on a background of several diseases including chronic hepatitis C virus (HCV) or hepatitis B virus (HBV) infections, alcoholic liver disease (ALD), and nonalcoholic fatty liver disease (NAFLD) which can progress into nonalcoholic steatohepatitis (NASH).[4] Untreated, CLD can lead to pathological changes in the liver, such as cirrhosis, and hepatic malignancies, such as hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC).[4] Beyond the liver, CLD can also promote severe and sometimes life-threatening systemic diseases, such as abnormal coagulation and hepatic encephalopathy, which could result in coma and death.[5] Current treatment options for patients with CLD and liver cancer rely on the treatment of the underlying cause of liver damage or on liver transplantation.[3] However, the demand for livers greatly outweighs the donor organ supply. Furthermore, an incomplete understanding of the biological circuits driving liver disease at the cellular level means that there is a lack of effective therapies to reduce morbidity and mortality as a result of CLD.

Our understanding of liver biology has been informed via histological and molecular biology techniques using cell lines, animal models, and human tissues. However, key questions, such as the role of rare-cell populations in the development and severity of liver disease, have been limited by a lack of appropriate animal models and molecular biology tools. Recent advances in single-cell technologies limit the cellular examination of solid organs only by tissue resources and our imagination. The purpose of this review is to (1) examine single-cell atlasing through the lens of liver disease and (2) revisit open questions that might benefit from such approaches. Particular attention will be paid to the unique features of liver disease pathology and progression that might profit from multimodal examinations.

The functional unit of multicellular organisms is the cell. Single-cell genomics and transcriptomics allow us to unbiasedly capture the functional diversity and molecular features present in the liver at a single-cell resolution. Multiple omics features (e.g., genome, epigenome, transcriptome, and proteins) can also be mapped for the same cells using a variety of experimental methods (►**Fig. 1**; ►**Tables 1** and **2**).[6] These approaches enable a deep characterization of rare human tissue samples and have been applied to many major organs across multiple disease contexts.[7–12] Within the liver, an explosion of single-cell research has provided insight into cellular heterogeneity, cell-to-cell communication and disease-associated reprogramming. While single-cell RNA sequencing (scRNA-seq) has been heavily relied on to map the transcriptomic cellular landscape in liver development, health, CLD, and cancer, recent publications have adopted multimodal single-cell approaches to generate more comprehensive atlases.[13–17] Previous reviews have described the recent efforts toward generating a liver cell atlas using single-cell genomics in detail.[18] This review highlights the unique challenges in studying the liver at a single-cell level, discusses new insights revealed by such approaches, and future opportunities for the application of single-cell technologies to pave the way for effective disease interventions.

Transcriptomic Profiling Reveals Liver-Cell Heterogeneity and Zonation

Identities and Functions of the Cells in the Liver

The liver is the largest solid organ in the human body. It is composed of hepatocytes, cholangiocytes, and nonparenchymal cells including liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs), and recruited and tissue resident immune cells.[19] ScRNA-seq has allowed the examination of parenchymal and nonparenchymal cells in the healthy liver, revealed new insights into hepatocyte phenotypic zonation, and refined our understanding of LSECs, cholangiocytes, and hepatic progenitor cells (►**Table 3**).[2,17,20–22] These studies have elucidated zonated functional pathways

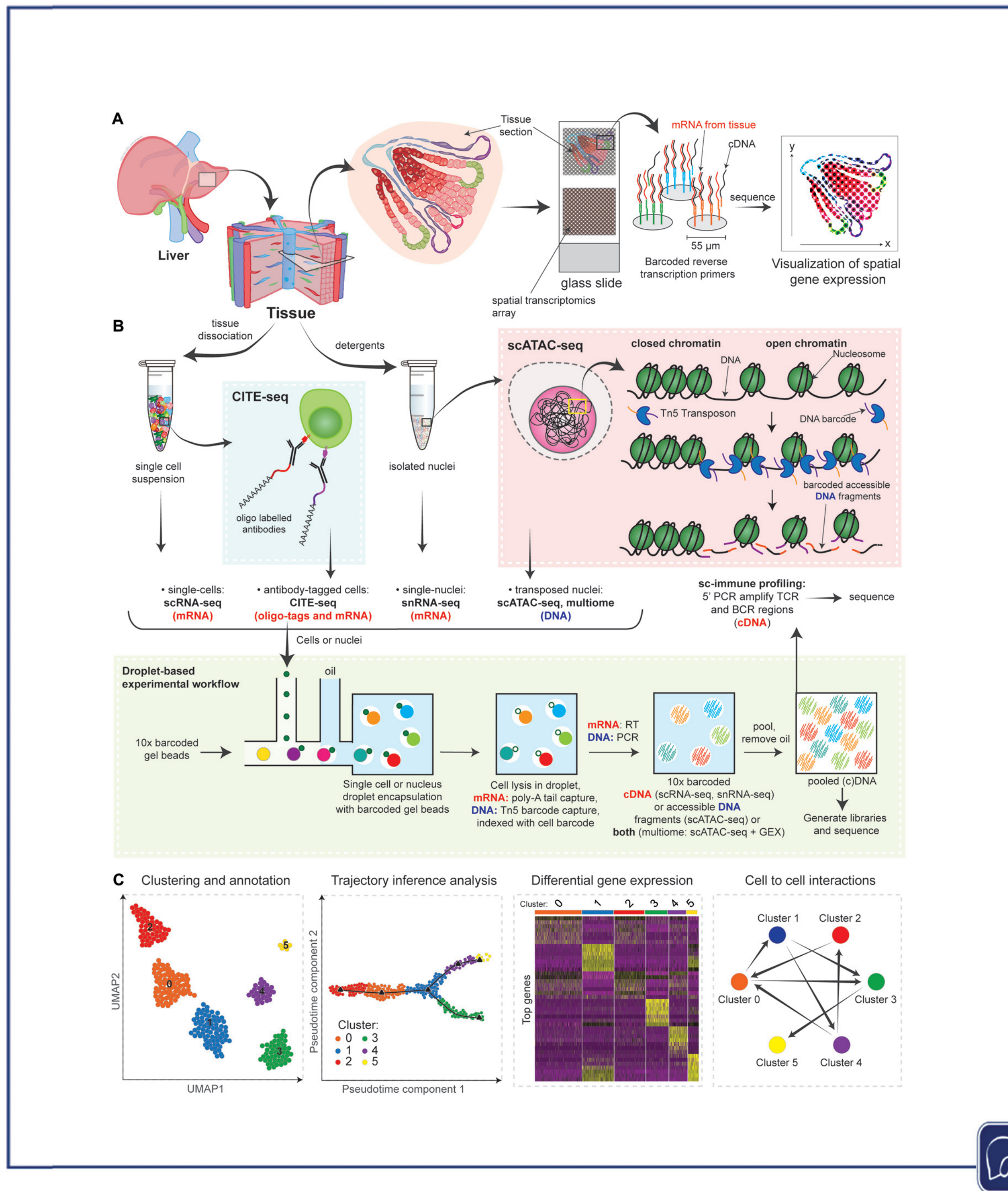

Fig. 1 Single-cell experimental and analysis workflow. **(A)** Spatial transcriptomics: liver tissue samples are sectioned, and transcripts are barcoded according to their location based on a matrix of spots. These barcodes are then used to spatially resolve gene signatures across the tissue section. **(B)** Droplet-based experimental workflow: dissected tissues are dissociated into either single-cell or single-nucleus suspensions. CITE-seq (cellular indexing of transcriptomes and epitopes by sequencing): cells can be tagged using oligo-labeled antibodies to link protein to RNA expression. ScATAC-seq: (single-cell assay for transposase-accessible chromatin with sequencing) is an unbiased, epigenetic regulation discovery tool that determines regions of open chromatin genomic DNA that are accessible to transcriptional machinery. Tn5 is used to sequentially cleave accessible DNA regions and to attach PCR amplification primers to generated barcoded accessible DNA fragments. RNA from single cells, DNA-oligomer labeled antibody-tagged cells, and single-nuclei or DNA from transposed nuclei are used to generate gene expression and accessible DNA libraries at a single-cell resolution through droplet-based experimental workflows such as the 10× genomics platform. Amplification of T and B cell receptor regions is used to link adaptive lymphocyte transcriptomes to their receptor sequences and determines clonal expansion. **(C)** Downstream analysis of these data relies on clustering to group cells together based on similarity of transcriptomic, proteomic, or epigenetic features. Trajectory inference analysis orders cells along a smooth continuous path of transcriptomic changes and can help deepen our understanding of cellular differentiation pathways and how cell states change with conditions. Differential gene expression analysis helps determine the genes directing these differences in cell type and or state and intracellular interaction analysis can be used to infer the pathways that cells use to communicate with each other in health and disease. GEX, gene expression; PCR, polymerase chain reaction; RT, reverse transcription; scRNA-seq, single-cell RNA-sequencing; snRNA-seq, single-nucleus RNA-sequencing; Tn5, Transposon Tn5.

Table 1 Single-cell modalities and their applications

Single-cell modalities	Molecular layer	Molecular features	Applications and challenges
ScRNA-seq	Transcriptomic	Whole cell: mature mRNA gene expression, captured via poly-A tail	Best suited for analysis of highly expressed genes. Can be coupled with protein quantification using CITE-seq. Application to fresh tissue, cell types can be enriched using fluorescence-activated cell sorting if necessary.
SnRNA-seq	Transcriptomic	Nuclear mRNA fraction: primary, unspliced mRNA	Application to fresh and frozen samples, particularly those that are difficult to dissociate into single-cell suspensions. Can provide data on difficult to isolate cells with some loss of transcriptional depth and the cytoplasmic RNA fraction.
ScATAc-seq	Epigenetic	Captures open chromatin, transcriptional machinery accessible genomic DNA regions with single-cell resolution	Unbiased detection of epigenetic landscape across the human genome. Capture of early lineage-determining epigenetic features may allow for a higher resolution when identifying cell subsets than with transcriptomic data.
CITE-seq	Multiomic: transcriptomic, proteomic	DNA-oligomer tagged antibodies are used to label proteins on the cell surface and protein and mRNA are simultaneously quantified in the same cell at a single-cell resolution	CITE-seq provides important immunophenotyping information for each cell that can help determine cell sorting and isolation strategies and to reconstruct signaling networks. Protein characterization is limited to specific molecules with validated antibodies.
Spatial transcriptomics (e.g., ×10 genomics Visium spatial gene expression)	Transcriptomic	Spatially barcoded spots are used to capture tissue-derived mRNA and reverse transcribed to generate a spatially resolved cDNA library	Current technologies are not yet at single-cell resolution. In the future, spatial transcriptomics has the potential to deliver on-slide transcriptome wide information at single-cell resolution. Preserves the native architecture and interactions of cells and algorithms can be used to deconvolve the constituent cells.
Single-cell immune profiling	Transcriptomic, TCR sequencing	Targeted amplification of TCR and B-cell receptor sequences enables the matching of adaptive immune receptors with gene expression patterns in source cells.	Enables annotation of invariant T-cells, tracking the expansion of T- and B-cells and the linking of antigen receptor sequences to lymphocyte transcriptome. Challenges in computationally predicting antigen-specificity using T- and B-cell receptor sequences remain.
Single-cell immune receptor mapping (e.g., barcoded dCODE Dextramer [×10]) [127]	Multi-omic: transcriptomic, antigen specificity, TCR sequences	Multimeric MHC—peptide complexes are used to integrate TCR data, preselected epitope specificity and RNA gene expression analysis	Provides insights into how T-cell phenotype is linked to antigen specificity. Expanded TCR and epitope pair information will be helpful for generating machine learning algorithms for the prediction of TCR antigen specificity. These data may also be used to identify new TCRs for engineering a CAR T-cell therapy.
Single-cell whole genome sequencing[128]	Genomic	Physical isolation of cell types is followed by single-cell whole genome amplification technology and next generation sequencing	Characterizing mutations, copy number variants and genetics is applicable to the study of cancer genetics and in revealing rare genetic variants associated with disease.
ScNMT-seq	Multiomic: epigenetic and transcriptomic	mRNA, DNA methylation and nucleosome sequencing simultaneously in the same single cell	Critical for understanding the association between epigenetic regulation and transcription in different cell types.
scATAC-seq + GEX	Multiomic: epigenetic and transcriptomic	Simultaneous characterization of DNA chromatin accessibility and mRNA gene expression in the same cell	Critical for understanding the association between the epigenome and transcriptome. Increased molecular features leads to increased resolution of cellular heterogeneity.
Single-cell CRISPR screens	Transcriptomic	Perturbations using feature barcoded CRISPR guide RNAs at a single-cell resolution and downstream combined capture of gene expression and guide RNAs	Enables exploration of mammalian gene function and genetic regulatory networks resulting from perturbations to gene expression using guide RNAs. Determines which perturbations result in similar gene expression signatures.

Abbreviations: CAR T-cell, chimeric antigen receptor T-cell; CITE-seq, cellular indexing of transcriptomes and epitopes by sequencing; CRISPR, clustered regularly interspaced short palindromic repeats; GEX, gene expression; IPSC, induced pluripotent stem cell; miRNA, microRNA; ScATAc-seq, single-cell assay for transposase-accessible chromatin with sequencing; ScNMT-seq, single-cell nucleosome, methylation and transcription sequencing; scRNA-seq, single-cell RNA-seq; SnRNA-seq, single-nucleus RNA-seq; TCR, T-cell receptor.

Table 2 Key steps in single-cell analysis

Analysis	Exemplar tools	Summary
Raw databased analyses		
Detection of viral genes	Viral-Track[129]	Detection of viral RNA gene expression and viral-human gene fusion events in single-cell RNA sequencing data using a database of viral genomes and sequence alignment technology.
HLA-typing	ArcasHLA[130]	Human HLA genotyping of single-cell data using genome alignment files.
CNV analysis	inferCNV[131]	Identification of large genomic deletions, and duplication events between normal and tumor samples to trace tumor-cell lineages and to identify sets of genes that may be responsible for the aberrant gene expression in tumor cells.
RNA velocity	scVelo[132], Velocyto[133]	Inferreing cell fate, and assigning directionality to cell differentiation dynamics by assessing the ratio of spliced to unspliced mRNA molecules within each single cell.
Data preprocessing		
Genome alignment and molecular counting	Cell Ranger[134]	Raw reads are aligned to a reference genome, filtered based on quality and alignment score, and then assigned to cells. mRNA molecules per cell are counted. The output is a feature-barcode matrix and preliminary clustering and gene expression analysis results.
Doublet removal	Scrublet[135], DoubletFinder[136]	Removal of heterotypic doublets based on additive gene expression signatures of the other clusters present in the data.
Ambient RNA correction	SoupX[137]	Estimation of ambient RNA using sequencing data from empty droplets and correcting the gene expression matrix of cells for the ambient RNA.
Removal of low quality cells	Cell Ranger[134], Seurat[138]	Distinguishing between empty droplets with ambient RNA and droplets containing cellular material (cellranger) is used to generate a filtered cell x gene matrix. Downstream removal of cells with high mitochondrial content as a signature of cell death, and outlier cells with very small or large library sizes is carried out to retain high quality data for biological analysis.
Data integration	Harmony[139], Seurat[138]	Correcting for technical effects across samples to resolve the shared biological signals present across samples.
Cellular level analysis		
Dimensionality reduction and visualization	Seurat[138], scanpy [140]	High dimensional gene expression and epigenetic data is reduced into a few dimensions (2D with UMAP) while maintaining as much variation or biological signal present in the original dataset as possible. This is useful for data visualization, reducing the noise in the data, and reduces the computational resources required for analysis.
Clustering	Seurat[138], scanpy [140]	Grouping cell types based on similarity of transcriptomic or epigenetic features.
Cluster annotation	Scmap[141], singleR[142]	Annotations of cell types present in single-cell data using automated annotation methods, literature based landmark genes and differential gene expression analysis as we recently summarized in Nature Protocols.[143]
Trajectory inference	Slingshot[144], Monocle[145]	Orders cells along a smooth continuous path of transcriptomic changes to help deepen our understanding of cellular differentiation pathways and changes in cell state as a result of stimulation.
TCR clonality	Gliph[146], scRepertoire[147]	Algorithms to query, cluster and visualize TCR sequences and their distribution and clonality across different clusters in scRNA-seq gene expression data.
Gene and pathway level analysis		
Gene set enrichment analysis	GSEA[148,149]	Identify phenotype or function associated gene sets that are overrepresented in cell clusters by using custom or curated gene set databases like gene ontology.
Gene regulatory network analysis	SCENIC[150]	Reconstruction of cell identity determining gene regulatory networks in transcriptomic and epigenetic data by assessing the coexpression of transcription factors and downstream target genes.
Ligand-receptor analysis	CellPhoneDB[151], NicheNet[152]	Identifying potential ligand-receptor interactions and mechanisms between different cell types in a complex molecular environment.
Differential gene expression analysis	Seurat[138], EdgeR[153]	Determining genes unique to cell types or those that show perturbed expression with a change in conditions.
Spatial transcriptomics deconvolution	MuSiC[154], Giotto [155]	Deconvolution of spots in spatial transcriptomics into constituent cell types based on reference gene signatures.
Downstream data usage		
Deconvolution of bulk data: clinical outcome	CIBERSORT [155,156]	Use of gene signature determined by scRNA-seq in deconvolution algorithms, to assess the cell type composition of whole-tissue biopsy samples that have been subjected to bulk transcriptomic profiling. These analyses can be run on RNA-seq data from large cohorts of patients at different disease stages.
Cross species analysis	–	Querying the physiological relevance of animal models to human disease by comparing the transcriptomic signatures present across species.

Abbreviations: 2D, two-dimensional; CNV, copy number variant; HLA, human leukocyte antigen; scRNA-seq, single-cell RNA-seq; TCR, T-cell receptor; UMAP, uniform manifold approximation and projection.

Table 3 Liver single-cell genomics studies

Study	Species	Platform	Disease context and tissue sites	Cell lineages characterized	Major findings	Data availability
MacParland et al (2018)[2]	Human	10× Chromium	Healthy, perfused liver samples	Epithelia, immune, endothelium, mesenchyme	Atlas of the healthy human liver. Major liver cell subtype transcriptional signature, immune cell heterogeneity and hepatocyte zonation	GEO GSE115469
Aizarani et al (2019)[20]	Human, mouse	mCEL-seq2	Healthy	Hepatocyte, cholangiocyte, immune, endothelium, mesenchyme	Atlas of the healthy human liver establishing hepatocyte zonation, and epithelial heterogeneity and the identification of epithelial bipotent progenitor cells	GEO GSE124395
Ramachandran et al (2019)[21]	Human, mouse	10× Chromium	Healthy, fibrosis	Epithelium, immune, endothelium, mesenchyme	Scar-associated macrophage, mesenchymal and endothelial cell populations interact within the fibrotic niche to reprogram the cellular landscape	GEO GSE136103
Halpern et al (2017)[31]	Mouse	MARS-seq	Healthy	Epithelium, immune, endothelium	Characterization of hepatocyte signatures across the mouse liver lobule	GEO GSE84498
Halpern et al (2018)[22]	Mouse	MARS-seq	Healthy	Epithelium, immune, endothelium	Paired cell sequencing enables the characterization of endothelial cell zonation across the mouse liver lobule using hepatocyte zonation signatures as a reference	GEO GSE108561
Zheng et al (2017)[28]	Human	Smart-seq2	HCC, PBMC, tumor, normal-adjacent tissue	T-cells	T-cell clonal expansion, phenotypic changes and identification of *LAYN* expression on T-cells as a potential therapeutic target	EGA EGAS00001002072, GEO GSE98638
Zhang et al (2019)[83]	Human	10× Chromium Smart-seq2	HCC, PBMC, ascites, hepatic lymph node, tumor, normal-adjacent tissue	Immune	Identification of HCC associated migratory DCs that interact with T-cells in the lymph nodes and the characterization of heterogeneity in tumor associated macrophage populations	GSA HRA000069, EGA EGAS00001003449
Ma et al (2019)[84]	Human	10× Chromium	HCC, ICC	Epithelia, immune, endothelium, mesenchyme	Higher transcriptomic diversity in tumors is associated with a worse patient prognosis	GEO GSE125449
Tamburini et al (2019)[52]	Human	10× Chromium	Healthy, chronic liver disease	Epithelia, immune, endothelium, mesenchyme	Characterization of *PDPN*$^{+}$ lymphatic endothelial cells in the healthy liver and expansion in fibrosis	GEO GSE129933
Pepe-Mooney et al (2019)[98]	Mouse	inDrop Seq-Well	Healthy, deoxycholic acid feeding	Hepatocyte, cholangiocyte	Determine the role of YAP signaling in maintaining intrahepatic biliary cells and determining alterations in cell state after injury	GEO GSE125688
Planas-Paz et al (2019)[99]	Mouse	10× Chromium	Healthy, 3,5-Dicarbethoxy-1,4-dihydrocollidine feeding	Hepatocyte, cholangiocyte	Heterogeneity in *EPCAM*$^{+}$ biliary epithelial cells identified and YAP signaling shown to promote biliary epithelial cell expansion during liver injury	SRA PRJNA384008
Krenkel et al (2020)[48]	Mouse	10× Chromium	Diet-induced NASH, acetaminophen poisoning model	Myeloid cells	The inflammatory state of myeloid cells derived from mouse bone marrow and liver samples is altered in NASH and acetaminophen poisoning	GEO GSE131834
Scott et al (2018)[25]	Mouse	10× Chromium	*Clec4f-cre*, *Itgax-cre*, and *Fcgr1-cre*	Myeloid cells	Identified key transcription factors (LXRα and *ZEB2*) in determining Kupffer cell identity	GEO GSE117081
Xiong et al (2019)[51]	Mouse	10× Chromium	Diet induced NASH	Epithelia, immune, endothelium, mesenchyme	Modeling of intracellular ligand-receptor interactions in NASH	GEO GSE119340, GSE129516
Ægidius et al (2020)[14]	Mouse	10× Chromium	Diet induced NASH	Immune, endothelial	Increased lipid metabolism in hepatocytes, stellate cell activation and accumulation of *Trem2*$^{+}$ *Cd9*$^{+}$ macrophages drive microenvironmental changes	–

(*Continued*)

Table 3 (*Continued*)

Study	Species	Platform	Disease context and tissue sites	Cell lineages characterized	Major findings	Data availability
Dobie et al (2019)[35]	Mouse	10× Chromium, Smart-seq2	Healthy, acute and chronic CCl_4 treatment model	Mesenchyme	Characterization of mesenchymal cell dynamics (HSCs, VSMcs, and portal fibroblasts) in healthy and fibrotic livers	GEO GSE137720
Krenkel et al (2019)[54]	Mouse	10× Chromium	Healthy, 3-week CCl_4 treatment model	Mesenchyme	Characterization of healthy, fibrotic and in vitro cultivated HSCs and myofibroblasts	GEO GSE132662
Andrews et al (2022)[17]	Human	10× Chromium, single-nucleus RNA sequencing, 10X Visium Spatial Gene expression	Healthy	Epithelia, immune, endothelium, mesenchyme	Establishment of the transcriptional signatures of putative epithelial bipotent progenitor cells. Presence of multiple subtypes of mesenchymal states in quiescent and activated states (HSCs, VSMCs, portal fibroblasts) is noted	GEO GSE185477
Guilliams et al (2022)[16]	Mouse, Human, Pig, Macaque, Chicken, Hamster, Zebrafish	10× Visium Spatial Gene expression, 10X Chromium	Healthy adjacent tissue removed during liver resection due to colorectal cancer metastasis	Epithelia, immune, endothelium, mesenchyme	Reliable markers for the localization of all major liver cell types were established. Identification of an evolutionarily conserved Kupffer cell signature and the microenvironmental signatures required to maintain liver macrophages	–
Sharma et al (2020)[85]	Human, Mouse	10× Chromium	HCC: tissue: adjacent-normal and tumor. Human fetal liver samples	Epithelia, immune, endothelium, mesenchyme	Parallels between features of HCC microenvironment and fetal development are noted using scRNA-seq and spatial transcriptomics. Fetal-associated endothelial cells (*PLVAP/VEGFR2*) and fetal-like (*FOLR2*) tumor-associated macrophages co-occupy this niche. VEGF and NOTCH signaling implicated in maintaining endothelial cell and tumor-associated macrophage phenotype	GEO GSE156337
Losic et al (2020)[77]	Human	10× Chromium	Multifocal HCC	Tumor cells, immune, endothelium	Intra-patient tumor cell transcriptomic heterogeneity across multiple HCC biopsy sites	GEO GSE112271
Sun et al (2021)[33]	Mouse	10× Visium Spatial Gene expression,	Healthy, R-spondin1 blockade, RNF43 or/and ZNRF3 knockout mice	Hepatocytes	Mechanisms regulating metabolic gene expression as a result of Wnt/β-catenin signaling without proliferation in hepatocytes	SRA PRJNA705085
Genshaft et al (2021)[36]	Human	Seq-Well, 10X Chromium	Chronic HBV infection, fine-needle aspirates of the liver and PBMC	Hepatocyte, immune, endothelium	Neutrophil, $CD8^+$ T-cell, monocyte and macrophage heterogeneity signatures in chronic HBV hepatitis	–
Hensel et al (2021)[39]	Human	mCEL-Seq2	Chronic HCV infection, before and after direct acting antiviral therapy	$CD8^+$ HCV-specific T-cells	Memory-like exhausted HCV specific $CD8^+$ T-cells are maintained while terminally exhausted $CD8^+$ T-cells are lost after direct acting antiviral therapy. A molecular signature of T-cell exhaustion is maintained in HCV specific $CD8^+$ T-cells after antigen clearance from the liver.	GEO GSE150305
Seidman et al (2020)[49]	Mouse	10× Chromium	Diet induced NASH	Macrophages	NASH diet induced a partial loss of Kupffer cell identity, and induced *TREM2* and *CD9* expression as a result of reprogrammed LXRα transcription factor activity	GEO GSE128338

Table 3 (*Continued*)

Study	Species	Platform	Disease context and tissue sites	Cell lineages characterized	Major findings	Data availability
Poch et al (2021)[27]	Human	10× Chromium	PSC and healthy donors, liver and PBMC samples	Intrahepatic and peripheral $CD4^+$ T-cells	Naïve $CD4^+$ T-cells expand in PSC and are primed to acquire a Th17 polarization state	EBI E-MTAB-10143
Zhang et al (2020)[75]	Human	10× Chromium	ICC, tumor, matched normal adjacent	Hepatocytes, cholangiocytes, immune, endothelium, mesenchyme	Markers of intratumoral heterogeneity in tumor cells and immunosuppressive Treg signatures. Cancer-associated fibroblast heterogeneity and interactions with malignant cells through the IL6/IL6R signaling axis. Tumor cells interact with cancer associated fibroblasts using exosomal miRNAs	GEO GSE138709, GSE142784
Su et al (2021)[78]	Human	C1 Fluidigm Single-Cell DNA seq, C1 Fluidigm whole cell and target gene sequencing,	HCC, tumor samples	Tumor cells	Key genetic events in HCC tumorigenesis and metastasis occur early and are carried down into subsequent tumor cell clones	GEO GSE146115, SRA PRJNA606993
Ma et al (2021)[41]	Human	10× Chromium	HCC, ICC	Hepatocytes, cholangiocytes, immune, endothelium, mesenchyme	Tumor cell heterogeneity is tightly linked to patient prognosis in response to therapy. *SPP1* identified as a key factor driving reprogramming of the tumor microenvironment after treatment	GEO GSE151530
Segal et al (2019)[91]	Human	Smart-seq2	Human fetal and adult liver tissue	Hepatocyte, cholangiocyte	Identify hepatobiliary hybrid progenitor populations that reside in the ductal plate of the human fetal liver. These cells are distinct from fetal hepatocytes, and adult hepatocytes and biliary epithelial cells	GEO GSE130473
Massalha et al (2020)[86]	Human	MARS-seq	HCC, ICC	Hepatocytes, cholangiocytes, immune, endothelium, mesenchyme	Patient independent stroma-tumor interactions in the tumor microenvironment and RNA-sequencing of microdissected tissues establish zonation patterns in malignant and non-malignant regions of the tumor-bearing liver	GEO GSE146409
Popescu et al (2019)[90]	Human	10× Chromium	Fetal tissue	Hepatocytes, cholangiocytes, immune, endothelium, mesenchyme	Modeling definitive fetal hematopoiesis and erythropoiesis during various points in gestation	EBI E-MTAB-7407
Kolodziejczyk et al., (2020)[106]	Mouse	10× Chromium	Acetaminophen and thioacetamide acute liver failure model	Hepatocytes, cholangiocytes, immune, endothelium, mesenchyme	Toll-like receptor and MYC-mediated activation of Kupffer cells, neutrophils, monocytes, and stellate cells and their intercellular interactions drive acute liver failure. Depletion of the microbiota, pharmacological MYC inhibition and toll-like receptor signaling ameliorate these effects	EBI E-MTAB-8263

Abbreviations: EBI, European bioinformatics institute; GEO, gene expression omnibus; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; HSC, hepatic stellate cell; ICC, cholangiocarcinoma; MARS-Seq, massively parallel single-cell RNA sequencing; NASH, nonalcoholic steatohepatitis; miRNA, microRNA; PBMC, peripheral blood mononuclear cells; PSC, primary sclerosing cholangitis; SRA, sequence read archive; VEGF, vascular endothelial growth factor.

present in LSECs that mirror hepatocyte heterogeneity.[2,20,22] Furthermore, distinct transcriptomes of vascular endothelial cells that line the central vein and the hepatic artery and *PDPN*$^{+}$ hepatic lymphatic vessels that expand in fibrosis have been noted.[2,21]

A meta-analysis of immune cells from these studies established stable features of the liver at steady state and together, these signatures are reference points for comparison to disease states.[23,24] There has been particular interest in the roles of resident and recruited myeloid cells in the liver microenvironment. Kupffer cells (KC), tissue resident macrophages residing within the liver sinusoid, are key to supporting organ function and immunological tolerance by scavenging gut-derived pathogens and damaged erythrocytes, and regulating iron and lipid metabolism.[25] In addition to highlighting hepatocyte, macrophage, and endothelial cell zonation, scRNA-seq has provided evidence for the presence of distinct inflammatory (*LYZ*$^{+}$, *HLA*$-$, and *DPB1*$^{+}$) and immunoregulatory (*CD163*$^{+}$, *MARCO*$^{+}$, and *CD5L*$^{+}$) myeloid subsets in the healthy human liver.[2,20,21] Guilliams et al recently developed a workflow integrating cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), single-nucleus RNA-sequencing (snRNA-seq), spatial transcriptomics (see ►**Fig. 1**; ►**Tables 1** and **2** for an explanation of these terms), and spatial proteomics to build atlases of the human and murine liver and determine spatial distributions and distinguishable markers for all hepatic cell types.[16] These data were then leveraged for a cross-species analysis to determine an evolutionarily conserved KC and bile-duct macrophage signature and to propose the microenvironmental signals necessary for imprinting their cell identity. Additionally, conventional and plasmacytoid dendritic cells (DC), cells that link the innate and adaptive arms of the immune system, were profiled in healthy and diseased mouse and human livers.[20,21,26] Hepatic lymphocyte populations that are transcriptionally distinct from circulating cells have also been characterized. These include resident αβ CD4^{+} and CD8^{+} T-cells, γδ T-cells, natural killer (NK)-like cells, mature and antibody secreting B-cells, and innate lymphoid cells.[2,23] In the future, the incorporation of CITE-

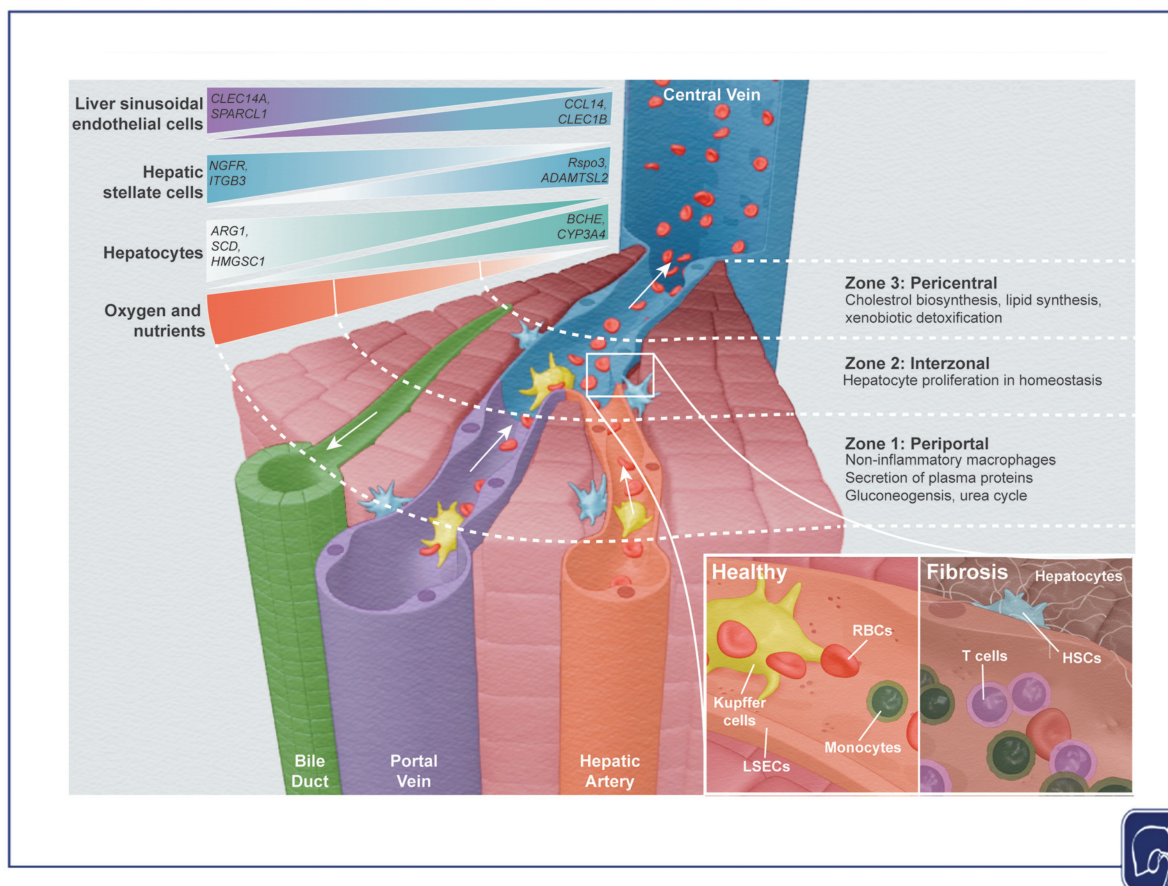

Fig. 2 Single-cell technologies allow for a characterization of the molecular signals involved in spatial zonation across the hepatic lobule. As blood, oxygen and nutrients flow from the portal triad (made up of the portal vein, bile duct, and hepatic artery) to the central vein, functional specialization of major liver cells are mediated by key signaling pathways as indicated and intracellular crosstalk. Gene set enrichment analyses have revealed the biological pathways present in each zone of the liver, lending an insight into the changing functional specialization of hepatocytes with the gradient of oxygen and nutrients. In the healthy liver, fenestrations in the LSECs allow for the communication between Kupffer cells (*yellow*), hepatic stellate cells (*blue*), and hepatocytes. Panel: With fibrosis, there is a loss of fenestrations in the LSEC layer that prevents communication of hepatocytes and macrophages. Hepatic stellate cells secrete extracellular matrix proteins leading to a buildup of collagens in the tissue microenvironment. As a response to liver injury, chemokine release by LSECs and stellate cells results in increased monocyte (*green*) and T-cell infiltration (*purple*) to respond to and clear pathogens. HSCs: hepatic stellate cells; LSEC: liver sinusoidal endothelial cells; RBS: red blood cells.

seq and the analysis of the T-cell receptor (TCR) repertoire will allow a detailed annotation of helper and innate T-cell subsets and enable the concurrent mapping of T-cell clonality and antigen specificity.[27–29]

What is the impact of tissue dissociation on the cellular atlases generated? One of the challenges of scRNA-seq is the requirement of fresh tissue and the relative loss of enzymatic dissociation-sensitive cells. As an alternative, snRNA-seq does not include an enzymatic dissociation step and relies on nuclei extracted using detergents from either fresh or flash frozen tissue samples to map the nuclear transcriptome present in each cell.[30] To address these issues, we performed a systematic comparison of scRNA-seq and snRNA-seq in profiling the healthy human liver.[17] We found that cell frequencies were less impacted by dissociation in snRNA-seq enabling us to identify previously unprofiled subsets of cholangiocytes, hepatic progenitor cells, and mesenchymal cells such as HSCs.[17] Further, scRNA-seq was determined to be crucial for the characterization of immune cells, particularly lymphocytes present in the liver.

Spatial Organization of the Cells of the Liver

The liver receives a dual blood supply from the portal vein and the hepatic artery. Blood flows from the portal triad to the central vein, creating a gradient of nutrients, oxygen, gut-derived microbial products, and hormones.[1] These gradients shape the molecular and functional heterogeneity of hepatocytes and establish a division of labor across three zones to carry out a variety of functions (►**Fig. 2**). The functional units of the liver are the hepatic lobules wherein sinusoids radiate from the central vein towards the portal triads between plates of hepatocytes.[19] Our understanding of the organization of hepatic cells within these zones was supported by histological studies and is now informed by spatially resolved transcriptomic assays through the application of unbiased bulk RNA-seq or scRNA-seq.[31] Halpern et al measured the mRNA expression of zonated landmark genes using single-molecule fluorescent in situ hybridization (smFISH) and applied these patterns through a probability inference method to predict the likelihood of expression of each gene within a murine liver scRNA-seq dataset across the portocentral lobule axis.[31] This analysis determined key zonation signaling pathways and revealed that 50% of liver-expressed genes are spatially zonated. Wnt and hypoxia signaling pathways were identified in the pericentral region, whereas Ras signaling was enriched in periportal zonation profiles. Notably, the majority of spatially zonated genes were not predicted downstream targets of these signaling pathways, indicating that many molecular pathways, key to liver zonation, remain to be elucidated. Intriguingly, this work identified mouse interzonal hepatocyte marker genes, however, these genes are not applicable to human liver data.[2,17]

Building off of this work, Ben-Moshe et al used zone-specific markers, CD73 and E-cadherin, to isolate distinct mouse hepatocyte subgroups and generate a complementary spatial map of micro-RNA (miRNA) expression patterns and proteomic profiles.[32] As expected, in most cases, protein zonation that closely mirrors mRNA levels was observed, while miRNA expression patterns were inversely zonated to their target genes in hepatocytes. However, the hepatocyte zonation signatures inferred from spatially zonated gene, miRNA, and protein expression data may be less predictive in disease and in knockout mice, especially when Wnt signaling is perturbed. Spatial transcriptomic approaches, while not yet at single-cell resolution, have the capacity to unbiasedly reconstruct metabolic liver zonation as recently shown by Sun et al.[33] Moreover, scRNA-seq has been employed at different time points across the diurnal circadian cycle to highlight the spatiotemporal metabolic organization of the liver.[34] These approaches have reinforced our understanding of functional specialization of hepatocytes in human livers and could be used to determine how this division of labor may be restored after perturbations due to liver injury.[2,20]

Just like hepatocytes, LSECs are exposed to varying concentrations of nutrients and hormones from the blood. However, zone-specific markers for LSECs were unknown before the application of scRNA-seq. Using their previously identified zone-specific hepatocyte signatures, Halpern et al inferred the transcriptomic signature of spatially distinct LSEC subsets by examining mouse hepatocyte–LSEC doublets in paired-cell sequencing.[22] Pseudospatial trajectory bioinformatic analysis (►**Fig. 1**; ►**Table 2**) confirmed these zonated gene expression patterns in human hepatocytes and LSEC scRNA-seq data.[20] Human periportal and pericentral LSECs and portal endothelial transcriptomic signatures were further spatially validated using spatial transcriptomics.[17] Similarly, macrophage zonation has been examined in healthy human liver as measured by MARCO expression through immunohistochemistry.[2] A closer examination of macrophage signatures using spatial transcriptomics revealed that inflammatory/recently recruited monocytes/macrophages and noninflammatory macrophages are distributed within the pericentral and periportal regions, respectively.[17] Additionally, through the application of a murine reporter system and different mesenchymal-cell isolation techniques, two HSC phenotypes were revealed that are distinctly associated with the pericentral and periportal regions of the hepatic sinusoid.[35] Zonation in LSEC, HSC, and KCs are perturbed in CLD and understanding the mechanisms will reveal new targets for therapeutic interventions.

Microenvironmental Reprogramming in Chronic Liver Disease

Transcriptomic Profiling of Hepatic Inflammation

What are the experimental challenges to profiling the inflamed liver with single-cell resolution? Liver inflammation as a result of HBV or HCV infection, ALD or NAFLD leads to hepatic inflammation as a response to necrotic hepatocyte death.[4] Repetitive liver damage results in progressive fibrosis, a disrupted hepatic architecture, and the loss of the regenerative capacity of the liver resulting in cirrhosis and eventually HCC. The characterization of cellular reprogramming through the course of CLD is impeded by insufficient

access to human liver tissue longitudinally over time and, until recently, limited options for examining rare, and, potentially, disease-driving cell populations. However, single-cell genomics has shed new light on the mechanisms of liver disease.

Determining the impact of viral infections and liver cancer on immune diversity and virus-specific immunity is a potential avenue for the application of single-cell technologies.[37–43] ScRNA-seq combined with serial sampling of the liver via fine-needle aspiration biopsies has been recently employed to longitudinally study hepatic immune diversity during the course of HBV infection.[36] Several studies in humans and mice have also employed transcriptional and epigenetic approaches to examine the cellular programs and effector functions of virus-specific $CD8^{+}$ T-cells before and after viral antigen clearance.[37–40,42] Furthermore, the impact of direct acting antiviral therapy for chronic HCV infection has been mapped transcriptionally, revealing treatment-induced alterations in innate immune and interferon signalling.[42] Yates and colleagues employed assay for transposase-accessible chromatin with sequencing (ATAC-seq) to examine the presence of exhausted HCV-specific T-cells during longitudinal follow-up of HCV treatment and found that virus-specific T-cell exhaustion was irreversible after treatment-induced clearance, a phenomenon labeled epigenetic scarring.[38] This finding was also reflected in parallel studies examining transcriptional and phenotypic changes in HCV-specific T-cells using scRNA-seq with TCR sequencing and mirrors T-cell dynamics in chronic LCMV infection in mice.[37–40,42]

How CLD, particularly steatosis, impacts systemic immunity and metabolism remains to be characterized and presents an opportunity for the application of multiomic modalities. Hepatic steatosis is observed in ALD and in diet and obesity-induced NAFLD.[44] Alcohol stimulates hepatic lipogenesis and inflammation by its toxicity to hepatocytes, and by causing increased leakage of gut-derived microbial byproducts such as LPS to the liver.[45,46] Using scRNA-seq, it was shown that individuals with ALD had a higher proportion of circulating nonclassical monocytes, and blood-derived monocytes displayed a higher inflammatory response to LPS stimulation compared with healthy control donors.[47] However, scRNA-seq data comparing hepatic myeloid populations present during NAFLD in mice described a decrease in inflammatory gene expression across both macrophages and dendritic cells.[48] This signature was also present in the bone marrow myeloid compartment and could be recapitulated in vitro after lipid treatment and in vivo using a model of acute liver injury as a result of acetaminophen poisoning. These studies raise the question of the systemic impact of CLD and fatty liver disease on global immune-cell phenotype and metabolism as the organ is responsible for carrying out key metabolic tasks to maintain overall bodily function.

Macrophage, Hepatic Stellate Cells, and Liver Sinusoidal Endothelial Cells Heterogeneity in Liver Fibrosis and Cirrhosis

Fibrosis results in major alterations to tissue architecture, intercellular interaction networks, and cellular transcriptional programs in the liver. Capillarization of LSEC fenestrations results in the interruption of KC contact with HSCs and hepatocytes leading to the loss of KC identity and the activation of resident HSCs.[49,50] Ramachandran et al described the emergence of disease-specific *PLVAP*+ LSEC populations, found in the fibrotic niche that enhance leukocyte migration to fibrotic lesions.[21] Furthermore, in NASH, LSEC vascular signaling is downregulated in favor of lipid metabolism relative to healthy LSEC cells, contributing to disease pathogenesis.[51] An expansion of lymphatic vessels has been noted in NASH patients and single-cell examination of associated lymphatic endothelial cells indicate expression of potent leukocyte attracting chemokines (CCL21) and fibrogenic cytokines (interleukin [IL]-13).[52] While rare, these cells likely play a key role in regulating in situ immune activity but, their role in the development of tertiary lymphoid structures in CLD remains to be explored. As the scale of scRNA-seq datasets increases, the roles of rare subpopulations of liver cells in health and disease, like specialized endothelial cells and components of the hepatic nervous system, will be revealed.

In multiple murine models of liver fibrosis, resident KC frequencies are lower and the recruitment of bone marrow–derived macrophages is observed.[21,49,51] These macrophages are extremely plastic and are imprinted by features of the hepatic microenvironment to occupy the KC niche in homeostasis.[50] Murine and human transcriptomic studies have identified the emergence of *TREM2*$^{+}$ *CD9*$^{+}$ macrophages in CLD of various etiologies.[14,21,49,51] Diet-induced NASH has been shown to lead to changes in KC enhancers and gene expression resulting in the partial loss of KC identity and cell death.[49] A study which paired scRNA-seq with lineage tracing in mice showed that the transcription factor gene *ZEB2* and downstream expression of the transcription factor LXRα was key in maintaining the tissue specific identity of liver macrophages.[25] In diet-induced NASH, aberrations in KC gene expression and enhancers as measured by scRNA-seq and ATAC-seq identified that a reprogramming of LXRα functions drive a *TREM2*$^{+}$ *CD9*$^{+}$ phenotype.[49] Pseudotime analysis suggests that NASH and scar-associated macrophages are derived from monocytes and are distinct from embryonic KCs.[21,49] In NASH-associated mouse fibrosis and in human fibrosis, these macrophages express genes for lysosomal degradation, phagocytosis, and antigen presentation, suggesting that they may play a role in the clearance of apoptotic hepatocytes.[21,51] These cells colocalize to the fibrotic niche, display a profibrogenic phenotype, promote scar deposition by activating mesenchymal cells and are good candidates for future targeted cell therapeutics in CLD.

Activation of quiescent HSCs into proliferative, fibrogenic myofibroblasts has been identified as a central driver of liver fibrosis through the deposition of extracellular matrix proteins.[53] Myofibroblasts represent an effective target for antifibrotic therapies but have been difficult to characterize due to challenges in cell isolation. Mesenchymal cells can be captured using scRNA-seq in healthy and fibrotic human liver, and collagen-producing myofibroblasts are enriched for and localize to the fibrotic niche.[2,21] In carbon

tetrachloride (CCl_4) murine liver fibrosis, retinol-positive HSCs transition into a heterogeneous population, with variably increased expression of α-smooth muscle actin, collagens, and immunological effector proteins.[54] In centrilobular CCl_4 injury, RNA velocity and pseudotemporal trajectory analysis of scRNA-seq data suggested that HSCs, and no other mesenchymal populations like vascular smooth muscle cells (VSMCs) or portal fibroblasts, are the dominant source of pathogenic collagen producing cells.[35] Activated pericentral HSCs specifically express *LPAR1*, a receptor for lipid-signaling molecules which could be a potential antifibrotic therapeutic target. In murine amylin diet-induced NASH, interactome mapping has been employed to characterize the local regulatory role of myofibroblasts in fibrosis.[51] This mapping suggested that HSCs act as a central regulator of immune cells and endothelial cells by secreting cytokines, growth factors, and chemokines during liver injury, impacting vascular signaling and lymphocyte recruitment, survival, and activation.[51] Mesenchymal populations have been previously masked by hepatocytes in tissue profiled by bulk RNA-seq, and application of snRNA-seq in the healthy liver has revealed previously unprofiled portal fibroblasts and VSMCs.[17] Application of snRNA-seq to fibrotic liver samples may uncover targetable pathways in myofibroblasts to treat CLD.

Potential Application of Transcriptomics to Map Cholestatic Liver Disease

Can single-cell genomics help us characterize disease mechanisms in cholestatic liver disease? Primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) are examples of autoimmune liver diseases in which necroinflammatory injury of the bile ducts lead to bile leakage and cholestatic liver disease.[55] Autoimmune-mediated injury of bile ducts, remodeling of the ductal network, periductal fibrosis, molecular alterations in ATP-binding cassette transporters at the bile canaliculi membranes of hepatocytes, and activation of KCs and HSCs have all been implicated in the pathophysiology of cholestatic liver disease.[55] In PBC, the small and intrahepatic bile ducts are the principal targets of disease. By contrast, in classical PSC, the large bile ducts are predominantly injured. In both diseases, immune infiltrates are featured and an association with typical immune-related pathways, encoding human leukocyte antigens, cytokines, interferon, and immune-regulatory genes, have been identified.[56,57] Recent scRNA-seq studies described transcriptomic signatures present in PBC and PSC. Interrogation of PBC susceptibility-associated gene variants in an scRNA-seq dataset revealed the immune-modulatory role of *ORMDL3* expression in cholangiocytes.[58] *ORMDL3*$^{+}$ cholangiocytes were highly metabolically active and interacted with macrophages and monocytes via the vascular endothelial growth factor (VEGF) pathway. In PSC, scRNA-seq studies identified the expansion of a unique population of naive-like CD4^{+} T-cells with the potential to develop into Th17 cells.[27] More comprehensive atlases of patient-derived samples from various stages of disease are required to understand the pathogenic dynamics at play in these diseases

Cholestatic liver diseases have been challenging to profile due to patchy liver pathology, as well as a high degree of fibrosis that limits access to larger vessels and release of cells during dissociation. Recent advances in single-cell genomics, like snRNA-seq and spatial transcriptomics, are making it possible to overcome dissociation-related challenges and characterize the underlying pathology in PSC and PBC. In these contexts, tissue sampling will need to be preselected based on pathological presentations in histology to overcome biases associated with localized, patchy disease.

Many open questions in cholestatic liver disease remain to be addressed. Pathway analysis from RNA-seq data of formalin-fixed liver tissue from PSC patients have identified the downregulation of fibrosis-independent disease pathways, such as transcription and protein folding, in response to endoplasmic-reticulum stress.[59] Furthermore, genetic risk factors in both PSC and PBC overlap with other autoimmune disorders such as ulcerative colitis, psoriasis, and rheumatoid arthritis.[60] PBC is associated with vitiligo and Sjörgen's syndrome, while ulcerative colitis has been hypothesized to be involved within the disease pathogenesis of PSC through increased gut permeability.[61] Previous studies have shown that immune-competent gut-homing T-cell lymphocytes may traffic from the gut to the bile ducts and cause an immune-mediated injury to large and intermediate ducts.[62] Application of single-cell genomics can uncover the link between cholestatic liver diseases and other autoimmune disorders and the fibrosis-independent and gut-axis related etiological pathways in PSC. Moreover, distinct lipid associated and decreased immunoregulatory macrophage phenotypes were identified in pediatric cholestasis due to extrahepatic (biliary atresia) and genetic (Alagille's syndrome) clinical etiologies.[63] A comparison of these populations to those in PSC and PBC using single-cell genomics may reveal cholestatic liver macrophage pathways that can be targeted mechanisms for treatment.

Transcriptomic Profiling in Liver Cancer

The Current State of Liver Cancer Therapy

Liver cancer is the second most common cause of cancer-associated mortality worldwide.[64] Unchecked, cycles of progressive injury and activation of repair pathways in CLD result in the accumulation of DNA damage in hepatocytes which is a major risk factor to develop HCC. The most effective treatment options for HCC are surgical resection, localized ablation, or liver transplant which are only curative with early diagnosis.[65] However, 70 to 80% of resection and ablation patients and 10 to 20% of liver transplant patients relapse within 5 years after surgery, and HCC is most frequently diagnosed at advanced stages.[64] In general, systemic, targeted therapy remains essential. Advanced HCC is usually treated by transarterial chemoembolization, radiation, and protein kinase inhibitors such as sorafenib.[66] Targeting the tumor immune microenvironment is a current area of research with encouraging results from phase-III clinical trials of treatment of advanced HCC with anti PD-1 antibodies (nivolumab and pembrolizumab), in combination

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with anti-VEGF antibodies.[67] This combination had additive effects on limiting tumor growth, and led to an immunostimulatory microenvironment.[67] The application of single-cell genomics will support new prognostic algorithms and lead to the development of more effective immunotherapy strategies for cancer patients.

ICC are rare and aggressive malignancies, and the 5-year survival rate for metastatic disease is at best 2%. Surgery is the only curative treatment but is again only possible in early-stage disease. Unfortunately, the majority of patients with cholangiocarcinoma (60–70%) present with advanced or metastatic disease, and in these patients palliative, locoregional, and systemic therapy are the only options for treatment.[68,69] Patients harboring mutations within *FGFR2*, *NTRK*, or mismatch repair genes are suitable for targeted therapies such as infigratinib, an FGFR2-specific tyrosine kinase inhibitor, entrectinib, a tropomyosin kinase receptor inhibitor, ivosidenib, an IDH1 inhibitor and pembrolizumab, and an immune checkpoint inhibitor.[70–73] Therefore, immune checkpoint inhibition may be common pathways to target the tumor microenvironment (TME) in both HCC and ICC.

Heterogeneity and Phenotype of Malignant Cells in Hepatocellular Carcinoma and Intrahepatic Cholangiocarcinoma

Using strategies that provide single-cell resolution, tumor heterogeneity and development can be deeply characterized. The HCC tumor is composed of aberrant hepatocyte lineage cells that evolve at various stages of hepatic maturity. Comparing nonmalignant tissue to the tumor core can reveal cancerous clonal development and changes in biological function with oncogenesis. Healthy hepatocytes are largely well differentiated, while HCC tumor cells exhibit a range of undifferentiated and stem cell-like features. These cells are highly heterogeneous at the transcriptomic level, both across different regions of the tumor, and between patients as shown by scRNA-seq.[74,75] For example, an examination of the tumor stem-cell heterogeneity in HCC revealed a heterogeneous population of *CD24*, *CD133*, and *EPCAM* expressing cells with distinct molecular signatures.[74,76] These gene signatures were enriched within the tumor relative to the uninvolved nonmalignant liver and were independently associated with HCC outcomes.[74,76]

Recently, an integration of bulk RNA-, DNA-, TCR-sequencing data and single nucleotide polymorphism data across multiple regions of HCC patient samples was employed to map the spatial and temporal interactions between cancer and immune cells.[77] Spatially restricted subclone mutations were associated with distinct adaptive immune responses as opposed to early somatic driver mutations in HCC driver genes like *TP53*, *CTNNB1*, and *NFE2L2*. Gene regulatory network analysis of scRNA-seq showed that distant regions within the same tumor display wildly different transcription factor networks and even different molecular subclasses of HCC.[77] To reconstruct the clonal evolution of single-cell mutational profiles in HCC samples, custom primers for somatic mutation sites, and mutational signatures were used for targeted gene scRNA-seq.[78] This analysis indicated that mutations in early clones were carried down the lineage and that other clones were derived from additional subclonal mutations. In parallel, scRNA-seq analysis showed that the phenotypic heterogeneity of patient specific tumor cells was mirrored by their genetic heterogeneity. It is becoming clear that tumor cells across patients do not necessarily share transcriptomic signatures but have patient-specific transcriptomic profiles.[15,75,78] These studies raise questions regarding the link between HCC driver mutations and the tumor immune signature that may be queried using single-cell genomics.

HBV integration into the human genome leads to genomic instability and is a key mechanism of HCC development in the absence of cirrhosis.[79] An analysis of the HBV integration sites in single-nodule HCC using single-cell whole genome sequencing found that identical integration sites were present across all tumor cells, indicating that HBV integration is likely a key, early step in tumor development.[80] Questions regarding the proportion of virally infected cells in HBV and HCV positive livers and the impact of HBV and HCV infection on individual cell transcription remain unanswered. A small study quantifying viral reads in HBV-induced HCC determined that viral read expression was correlated with the degree of hepatocyte differentiation and activation of downstream pathways associated with adipogenesis and cell stemness.[81] A more comprehensive analysis, in a large cohort of patient-derived tumor and matched adjacent tissue, is needed to further elucidate the impact of HBV infection on hepatocyte function and oncogenesis.

An analysis of tumor cell evolution in response to immunotherapy in HCC and ICC using core needle biopsies, and using transcriptomic clusters as a measure of functionally similar tumor clusters determined that the degree of tumor cell state heterogeneity was tightly linked to patient prognosis.[41] Analyzing evolutionary trajectories from RNA velocity within one patient-identified osteopontin (*SPP1*) as a major factor driving tumor evolution. In the future, studies with these approaches could be used to model the prognosis of patients with different classes of cancer stem cell molecular signatures and to further stratify patients based on expected treatment response.

Targeting the Tumor Microenvironment in Hepatocellular Carcinoma and Intrahepatic Cholangiocarcinoma

Evolution of tumor cell genetics is linked to the temporal and spatial evolution of the TME, which consists of the extracellular matrix, and infiltrating mesenchymal and immune cells. The TME in multiple types of cancer shares the accumulation of regulatory T-cells, $CD8^+$ T-cell exhaustion, and tumor-associated macrophage (TAM) expansion with an immunoregulatory phenotype.[12,82] These cells interact with tumor-derived antigens and drive tumor cell evolution and are in turn reprogrammed by tumor cells. Single-cell technologies enable us to take a snapshot of the TME and how the cellular compositions and intercellular communication differ from the healthy and the adjacent, nontransformed liver.

TCR- and scRNA-seq of sorted T-cells from HCC patient-derived peripheral blood, uninvolved normal and tumor tissue from 6 HCC patients characterized the lymphocyte response in HCC.[28] Cytotoxic T-cells displayed increased exhaustion and TCR clonal expansion in the tumor site and there was an accumulation of regulatory T-cells in the tumor region. This suggests an immunosuppressive environment within the TME. Both exhausted $CD8^+$ T-cells and tumor-infiltrating regulatory T-cells expressed *LAYN* which inhibited activated $CD8^+$ T-cell function in vitro and may be a future clinical target for HCC.[28] A follow-up study of all sorted immune cells in HCC from the tumor region, the adjacent uninvolved liver, blood, ascites, and the hepatic lymph node determined that these lymphocyte populations interacted with antigen presenting DCs through immunosuppressive pathways.[83] Using evolutionary modeling through RNA velocity, *LAMP3*-expressing DCs were shown to be activated within the tumor region and to migrate to hepatic lymph nodes where they can regulate lymphocyte activation via immunosuppressive ligand-receptor signaling.[83] Additionally, genes that are associated with worse outcomes, such as *GPNMB* and *SLC401*, were identified in TAM clusters.[83]

Tumor cell signatures and diversity clearly impact the reprogramming of the TME in liver cancer. For example, single-cell genomics studies have revealed that there are distinct T-cell signatures present in a dataset of HCC and ICC tumors with high or low biodiversity.[84] Low-biodiversity tumors tend to be associated with cytotoxic T-cells and immune checkpoint molecules, whereas high-biodiversity tumors are associated with an increased number of regulatory T-cells and worse patient prognosis. This finding suggests that tumors of high diversity adapt aggressive characteristics through the alteration in T-cell composition and reprogramming. Large, multiomic surveys of HCC relying on mass cytometry, single-cell transcriptomics, and mass spectrometry based proteomics and metabolomics of HCC samples allowed the identification of HCC TME subtypes.[15] A detailed analysis of these datasets may identify new therapeutic targets within the TME of each subtype with regard to immune cells, cytokines, and metabolism. For example, markers of immune infiltration and T-cell tolerance are of value as therapeutic targets for future prognostic prediction and guidance of decision-making for therapeutic interventions.

A comparison of signatures present across the fetal mouse and human liver, and normal and HCC tumor tissues by Sharma et al demonstrated a reprogramming of the TME that mimics those of early organ development.[85] Spatial colocalization of $PLVAP^+$ endothelial cells, $FOLR2^+$ TAMs, and $TIGIT^+$ Treg cells, as revealed by nanostring spatial transcriptomics, suggest a shared immunosuppressive transcriptional signature between fetal liver and liver cancer. While spatial analyses of liver cancer are not yet at a single-cell resolution, the application of identified cell state signatures in spatial transcriptomics should identify pathogenic, disease-driving cellular niches within liver disease, even if the liver architecture is massively reorganized.

To address the issue of patchy liver pathology, Massalha et al used bulk RNA sequencing of laser capture microdissected tissue regions from the tumor core, margin, and fibrotic liver regions of patients with ICC or liver metastases to determine the gene signatures associated with these niches.[86] Using scRNA-seq, the proportions of individual cell types were then deconvoluted and showed an enrichment of T-cells at the tumor border and highly overlapping spatial abundance of tumor-derived LSECs and pericytes like cancer-associated fibroblasts (CAFs). A closer examination at fibroblast heterogeneity in ICC using scRNA-seq determined that CAFs are reprogrammed by tumor cells through effectors like VEGF to form various distinct subpopulations enriched for functions such as vascular development, extracellular matrix protein deposition, antigen presentation, and immune modulation. In response to tumor exosomal miRNAs, $CD146^+$ CAFs secrete IL-6 to promote tumor stemness and epigenetic alterations via the IL-6/IL-6R axis in vitro.[75]

The value of these studies in determining disease mechanisms and developing prognostic signatures in ICC is high. A recent multicenter study identified a uniform molecular and histological signature similar to extrahepatic cholangiocarcinoma in PSC patients with bile duct carcinoma.[87] Genomic analysis identified high frequency of genomic alterations typical of extrahepatic cholangiocarcinoma, such as *TP53*, *KRAS*, *CDKN2A*, *SMAD4*, as well as potentially druggable mutations. An evaluation of the prognostic value of differentially expressed genes in ICC using scRNA-seq described a nine-gene prognostic signature with enrichment of immune response-activating signal transduction, immune response–regulating cell surface receptor signaling pathway, and lymphocyte activation that predicted longer survival.[88] Application of single-cell genomics will lead to the development of more accurate prognostic signatures and uncover targetable disease pathways in HCC and ICC leading to the development of effective cell-specific anticancer therapies.

Liver Development and Regeneration

How can we leverage rich transcriptomic datasets to define and promote liver regeneration? The liver possesses an impressive regenerative capacity which translates to the ability to recover its complete weight 8 to 15 days after losing up to 66% of the liver mass (through two-thirds partial hepatectomy).[89] However, the regenerative potential of the liver is impaired during CLD and is an avenue for therapeutic targeting. Transcriptomic profiling has broadened our understanding of cell circuits involved in fetal liver hematopoiesis, development, and regeneration.[90] Within the fetal liver, Segal et al, employed scRNA-seq to identify a hybrid hepatic progenitor with the potential to become either a hepatocyte or a cholangiocyte and future studies characterizing sequential specification of hepatobiliary progenitors may lead to the development of cell-based regenerative therapies.[91] ScRNA-seq can be employed to explore the similarities in cell identity and differentiation in developing adoptive cell therapies to the native liver tissue. These comparisons have been applied to induced pluripotent stem cell (IPSC)-derived hepatic endothelial cells, cholangiocytes, and hepatocytes.[89,92–94]

For example, a comparison of IPSC-derived two-dimensional (2D) monocultures to three-dimensional (3D) multicellular organoids containing mesenchymal, endothelial, and IPSC-derived hepatic parenchymal cells using scRNA-seq determined that while monocultures are able to recapitulate many features of in vivo hepatogenesis, organoid systems are better able to reconstruct liver cell intercellular cross-talk during development.[95] Within the organoid system, an epithelial migratory signature reminiscent of the liver bud during development is seen, potentially coordinated by intracellular cross-talk, such as VEGF signaling, that promotes endothelial cell network formation and hepatoblast differentiation. Another interesting observation is that IPSC-derived hepatocytes are more similar to fetal liver hepatocytes than adult hepatocytes.[95]

The source of hepatocyte replenishment in the injured liver is an ongoing debate. Using sophisticated imaging and lineage tracing technology in mice, it has been demonstrated that interzonal hepatocytes are capable of proliferating and maintaining hepatocyte numbers at baseline and during regeneration in mice.[96,97] To examine the dynamics of cell proliferation after injury, Pepe-Mooney et al performed scRNA-seq before and after chemical injury to the mouse liver and, using smFISH, found that yes-associated protein 1 (YAP) expression is enriched in *EPCAM*+ cells at steady-state, and that YAP signaling is upregulated in cholangiocytes and periportal hepatocytes after injury.[98,99] Hepatocyte-specific YAP inactivation revealed the role of YAP signaling in hepatocytes for the generation of a ductular reaction and in the reprogramming of hepatocytes toward a biliary-like cell fate.[99] In humans, scRNA-seq analysis of the *EPCAM*+ compartment in the liver revealed bipotent progenitor cells that reside in proximity to the bile ducts and are able to differentiate into either hepatocyte or cholangiocytes in vitro.[20] These cells are able to repopulate mouse hepatic parenchyma in NASH, thioacetamide injury, and in models of chemical injury to the bile ducts.[20] Bipotent progenitor cells are better captured by snRNA-seq, and validation using spatial transcriptomics and histology data from the Human Protein Atlas indicate that they are present in the portal region.[17] Uncovering the mechanisms of hepatocyte replenishment after injury could be harnessed to stimulate liver regeneration.

Future Perspectives

Understudied Aspects of Liver Biology

There remain many aspects of our understanding of the liver that could benefit from the application of single-cell omics (►**Fig. 3**). For example, genome-wide association studies (GWAS) have clarified the role of the host genetic

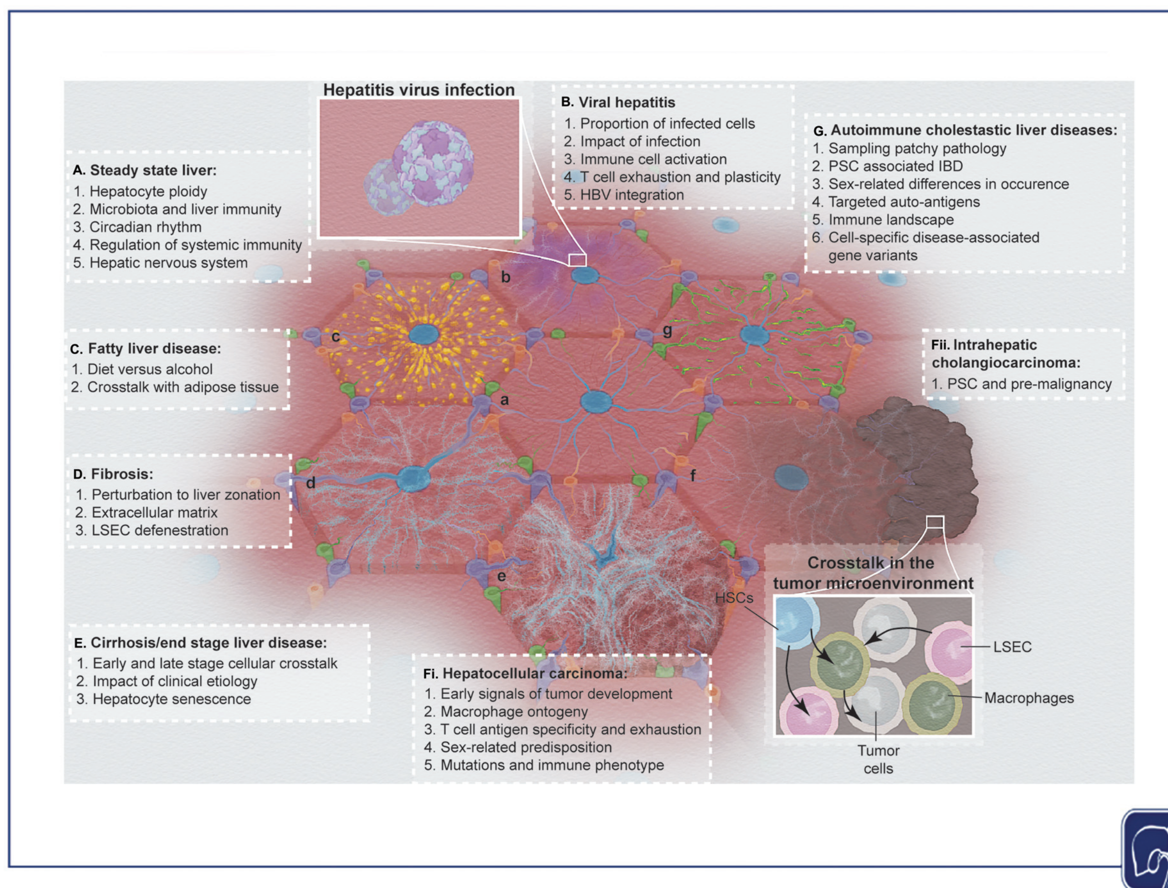

Fig. 3 Future perspective in liver diseases: themes with potential applications of single-cell genomics. Hepatic lobules indicate different liver states in **(A)** steady state, **(B)** viral hepatitis, **(C)** fatty liver disease, **(D)** fibrosis, **(E)** cirrhosis, **(Fi)** hepatocellular carcinoma, and **(Fii)** intrahepatic cholangiocarcinoma and **(G)** autoimmune cholestatic liver diseases like primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC). HBV, hepatitis B Virus; HSC: hepatic stellate cells; IBD, Inflammatory bowel disease; LSEC, liver sinusoidal endothelial cells.

background in many disease states, like ulcerative colitis, PSC, PBC, and CLD.[58,100–103] In the future, a unique application of scRNA-seq in combination with such GWAS analyses could delineate the precise cellular source of such disease-associated gene variants.[104,105] While these combined analyses are still being refined, they have previously clarified the cellular origins for genetic alterations of interest in ulcerative colitis[105] and could be applied to autoimmune liver diseases.

A comprehensive characterization of the events leading up to acute liver failure in humans remains to be performed. ScRNA-seq applied to murine acute liver failure identified a MYC-dependent activation signature in KCs, HSCs, LSECs, and recruited monocytes and neutrophils that were regulated by the gut microbiome.[106] A detailed report on the cellular landscape in human acute liver failure may reveal potential targets for interventions to ultimately forgo the need for liver transplantation. However, much remains to be learned about host and donor cell interactions in liver transplantation. Recently, in an orthotopic murine liver transplant model, scRNA-seq was used to track the perioperative recipient and donor macrophage dynamics. It was noted that recipient derived macrophages, DCs, and granulocytes took over the transplanted liver by day 30 ($\sim$ 99%), but that graft-derived macrophages still contributed to the immunoregulatory KC niche.[107] These dynamics remain unexplored in humans and single-cell modalities can be used to monitor hepatocyte proliferation, graft, and recipient immune cell seeding and to determine the genetic and cellular features associated with transplant rejection.

Future Clinical and Therapeutic Applications

In addition to atlasing the diseased liver to elucidate cellular drivers of liver pathogenesis, single-cell transcriptomics offer an avenue to improve the efficacy of and track the response to treatment modalities that target the liver. In a similar manner to what has been performed in the context of breast cancer, multiomic machine learning could be employed to predict treatment response to immune oncology therapies.[108] For example, prognostic modeling of tumor cell stemness and intratumoral heterogeneity signatures in liver cancer have future relevance in patient stratification for treatments.[43,76,88,109] Moreover, these unbiased analyses enable the identification of immune markers for molecular classification of HCC patients through immunophenotyping.[15,110] Deep characterization of diseased liver tissue using single-cell omics has highlighted diagnostic markers and gene signatures, pathogenic cellular subsets, and potential targetable pathways.[28,59] However, in order to bring this potential to reality, additional interrogation in preclinical lineage-tracing and gene knockout studies with clinical trials are required to identify druggable mechanisms for future therapeutic applications.

Single-cell genomics are already being employed to track disease trajectories before and after HCV treatments and cancer immunotherapy signifying future clinical applications of single-cell technologies as a precision medicine approach.[39,41,42] Specifically, these studies aim to determine whether antiviral interventions might lead to the restoration of T-cell responsiveness and to identify the potential drivers of tumor evolution in response to immunotherapy. Furthermore, an examination of the tumor ecosystem in early-relapse HCC identified new mechanisms of immune evasion as a step toward developing more effective immunotherapies against HCC.[43] Single-cell genomics can be used to evaluate treatment efficacy and disease progression in clinical trials of a variety of liver diseases to generate frameworks for genetic, transcriptional, epigenetic, and cellular landscape-based treatment regimes.[111] For example, application of scRNA-seq in clinical trials of multiple myeloma patient cohorts have identified novel mechanisms and biomarkers of drug resistance to stratify patients and guide personalized therapeutic decisions.[112,113] In the future, case-specific single-cell analysis of patient liver biopsies have the capacity to reveal opportunities for personalized oncoimmunology by aiding in the prediction of patient outcomes and treatment effects.[87] Widespread clinical application of single-cell genomics will revolutionize our ability to track the efficacy and tailor treatment regimens for understudied liver pathologies.

Current Challenges in Single-Cell Genomics

Challenges in the implementation of single-cell processing platforms and analysis pipelines make a complete analysis a nontrivial task. However, with new methods of development, alternative approaches are becoming available. In addition to the problem of limited access to human liver tissue in various diseases, the dissociation and handling of liver tissue, particularly in the case of difficult to dissociate fibrotic tissue remains a challenge. SnRNA-seq can be employed as an approach to capture hard to dissociate and fragile cell types like cholangiocytes and mesenchymal cells.[17] As well, capturing neutrophils in droplet-based single-cell datasets has proved challenging. A full length, well-based and deep sequencing approach is required to gain insight into the role of these cells in various liver pathologies.[36] Patchy liver pathology can lead to sampling bias and allow certain gene signatures to be overrepresented and other features to be entirely overlooked. This problem arises specifically in locoregional liver diseases, like liver cancer and scarring, where the regular lobular structure of the healthy liver is disrupted. However, an integrated approach utilizing a multifocal sampling strategy with validation of cell signatures using spatial transcriptomics can enable a more complete and unbiased characterization of the liver in disease.[16,86]

Current single-cell genomics studies have relied heavily on end-stage disease tissues. However, the cellular landscape in these contexts may not be a good representative of early disease dynamics and early disease time points should be considered. In addition, integrative approaches using single-cell multimodal omics such as mass cytometry, spatial transcriptomics, CITE-seq, TCR and BCR-seq, scDNA-seq, and scATAC-seq enable the layering of multiple molecular features on cells derived from the same sample to comprehensively profile tissue samples.[14] This data may be used to build models for future deconvolution of large-scale bulk RNA and ATAC-seq patient databases.[86,114] Furthermore, data that bridges the transcriptome, proteome, and spatial localization

of cells in understudied liver pathologies would aid in the discovery of reliable surface markers to identify, purify, and locate these cells using flow cytometry, immunohistochemistry, and confocal microscopy. Defined disease-associated markers would further help differentiate specific liver diseases, like PSC and PBC, during diagnosis.

While these technologies remain expensive, the costliest step remains sequencing and hosting the infrastructure to carry out the more computationally taxing steps in the analysis. In addition, the bioinformatics expertise required to carry out these analyses is not trivial. However, standardization of single-cell analyses and the establishment of sequencing and analysis cores at major research institutes enable the clinical biologist to bypass these obstacles. Further, efforts toward generating a single-cell atlas of the whole body with the aim to provide a reference map of all human cells in every organ across various contexts will greatly increase our understanding of human health and disease.[115]

Developing New Methods to Map the Cellular Etiology of Liver Disease

The human body is composed of trillions of cells that work together to execute many high-level functions. Most biomedical research has focused on how individual cells or anatomical and physiological systems work, leaving a gap of knowledge in between about how cells coordinate to give rise to tissue-level functions and how these fail and cause disease. To address this to better understand the liver, we need to map the activities of dozens of cell types and subtypes, learned from millions of single-cell measurements. Large, international projects, like the Human Cell Atlas project, have the goal to map all cell types in the healthy human body, including the liver.[116] The main take home message of the analysis and interpretation of hundreds of thousands of single-cell transcriptomes thus far is that human tissues are dramatically more heterogeneous than previously understood. This is also seen at the epigenome, protein expression, and somatic mutation levels.[117–119] Much of what we have learned from bulk genomics technologies that average signals across tens of thousands to millions of cells must now be reevaluated using single-cell genomics.

A major challenge with using single-cell genomic technology to profile human tissues is in interpreting the massive data produced. For example, one scRNA-seq experiment frequently profiles more than 5,000 cells, conceptually matching the complexity of 5,000 traditional RNA-seq experiments. Interpreting this data will need new analysis methods that consider the function of many cells cooperating within an ecosystem of cells to implement physiological functions. This ecosystem includes a variety of rare and common cell types, differentiated cells, pathways, somatic mutations, gene regulatory networks, epigenomes and a network of cell-to-cell interactions that together define the tissue and its functional potential over its lifespan. The cellular ecosystem approach, combined with the power of single-cell genomics and state-of-the-art computational analysis, brings heterogeneity to the forefront of our thinking, and enables us to better model the biology of human body systems in contrast to previous approaches based on bulk genomics. This approach will also be useful to identify disease ecosystem properties that are predictive of outcome (e.g., biomarkers composed of groups of different cell types interacting), as well as novel therapeutic targets.

New analysis methods are starting to be developed to address the challenge of mapping the function of cellular ecosystems. For example, EcoTyper was recently developed to automatically define cellular ecosystems.[120] Using it to analyze a large cancer genomics database uncovered 10 ecosystems across 16 types of carcinoma, including two proinflammatory communities with canonical T-cell state that correlated with favorable overall survival. Another example is the covarying neighborhood analysis method which identifies sets of covarying cells that correlate with specific sample level information such as disease outcome.[121] Spatial transcriptomics data provides an excellent opportunity to study cellular communities and histological patterns associated with disease. Genomics analysis recently identified a PSC-associated ICC-specific genomic signature and histological subtypes which were not prevalent in ICC with non-PSC etiology.[87] Therefore it would be of interest to merge spatial transcriptomic data in nonneoplastic CLD to identify specific histological patterns and spatially resolved disease-specific transcriptional signatures. Current tools, such as ImageCCA, may be adaptable for this purpose.[122] Additionally, it is becoming evident that mapping parenchymal and nonparenchymal cells in the diseased liver may require paired single-cell approaches selected based on the capacity of each approach to capture populations of interest. For example, scRNA-seq appears to better capture intrahepatic immune cells while snRNA-seq better captures dissociation-sensitive cells such as hepatocytes.[17] With that in mind, new algorithms, supporting the examination of cell-to-cell interactions between populations identified by different techniques in the same liver sample, would be highly valuable. Current cell-to-cell interaction inference tools, such as CellChat, could likely be adapted for this purpose.[123]

Current technologies using oligo-labeled major histocompatibility complex (MHC)-peptide complexes and scRNA-seq enable mapping of T-cell phenotype to TCR antigen specificity.[29] However, these technologies rely on the prior knowledge of peptides of interest, and there is a need for new algorithms that reliably model TCR and predict neoantigen peptide interactions in cancer samples. Recent machine learning algorithms using TCR-α and -β sequenced data have begun the development of these methods but their effectiveness remains to be evaluated.[29,124–126] Not only will these algorithms provide insight into T-cell function and therapeutics in viral CLD, liver cancer, and other inflammatory disorders, they would also help identify new TCRs that could be adapted for use in chimeric antigen receptor T-cell therapy.

Conclusion

In conclusion, single-cell genomics and transcriptomics are revolutionizing our understanding of liver biology, at steady state and during disease. These ever-expanding approaches

have rapidly accelerated our ability to examine rare and disease-driving cell populations within the hepatic microenvironment. As we continue to master this technology, expand efforts to map early stages of CLD in human tissue and to longitudinally sample liver tissue through disease development, we will uncover new therapeutic targets and treatment options for patients suffering from liver disease.

Abbreviations

ALD, alcoholic liver disease; CLD, chronic liver disease; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSC, hepatic stellate cells; ICC, intrahepatic cholangiocarcinoma; IPSC, induced pluripotent stem cell; KC, Kupffer cells; LSECs, liver sinusoidal endothelial cells; miRNA, microRNA; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; scRNA-seq, single-cell RNA sequencing; snRNA-seq, single-nucleus RNA sequencing; TAM, tumor-associated macrophages.

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Conflict of Interest

None declared.

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