Single-cell technologies in hepatology: new insights into liver biology and

disease pathogenesis

Prakash Ramachandran, Kylie P. Matchett, Ross Dobie, John R. Wilson-Kanamori, Neil C. Henderson\*.

Centre for Inflammation Research, The Queen’s Medical Research Institute, 47 Little France Crescent, Edinburgh BioQuarter, University of Edinburgh, Edinburgh, UK, EH16 4TJ.

\*Address correspondence to:

Neil Henderson,

Centre for Inflammation Research

The Queen’s Medical Research Institute

47 Little France Crescent

Edinburgh BioQuarter

University of Edinburgh

Edinburgh,

UK,

EH16 4TJ

Email: Neil.Henderson@ed.ac.uk

# Abstract

Liver disease is a major global healthcare problem, affecting an estimated 844 million people worldwide. Despite this significant burden therapeutic options remain limited, in part due to a paucity of detailed analyses defining the cellular and molecular mechanisms driving human liver disease. Single-cell transcriptomic technologies are transforming our understanding of cellular diversity and function in health and disease. In this review, we discuss how these technologies have been applied in hepatology: advancing our understanding of epithelial, immune, endothelial and mesenchymal cell heterogeneity, and providing novel insights into fundamental aspects of liver biology such as zonation and regeneration. Application of these methodologies is also uncovering critical pathophysiological changes driving hepatic fibrosis and liver cancer. In the near future, new techniques such as spatial transcriptomics and a range of single-cell multiomics approaches will help deepen our understanding of liver disease pathogenesis, allowing precise identification of novel therapeutic targets.

# Introduction

Liver disease is a global healthcare burden. Estimates suggest that 844 million people worldwide have chronic liver disease, with a mortality rate of two million deaths per year1. Repetitive liver damage, secondary to any cause, results in progressive fibrosis, disrupted hepatic architecture, vascular changes and aberrant regeneration, defining characteristics of liver cirrhosis2. Current treatment options for patients with chronic liver disease are limited to removal of the underlying cause, if possible, or liver transplantation. However demand for transplantation greatly outweighs donor organ supply, and in much of the world liver transplantation is unavailable. Importantly, the degree of liver fibrosis predicts adverse patient outcomes, including development of cirrhosis-related complications, hepatocellular carcinoma and death3. Therefore developing effective therapies for patients with chronic liver disease is likely to impact significantly on morbidity and mortality.

Single-cell genomics approaches are transforming our understanding of disease pathogenesis, allowing interrogation of homeostatic and pathogenic cell populations at unprecedented resolution and adding an illuminating dimension to transcriptomic information relative to traditional methods that profile bulk cell populations4,5. The field of single cell genomics has exploded over the last few years, chiefly because these approaches allow powerful, unbiased exploration of cell states and types at single-cell resolution, resulting in unexpected novel insights into tissue biology and disease mechanisms. Similar to many fields in biology and medicine, the liver community has embraced these new technologies, with a plethora of liver single-cell RNA sequencing (scRNASeq) studies published in the last three years (summarised in Table 1). Although the costs of have reduced somewhat with the advent of high-throughput scRNASeq workflows, it remains an expensive approach, and therefore, both to reduce experimental costs and also, importantly, to generate the most informative data possible from each experiment, a number of important considerations must be taken into account when designing single-cell sequencing experiments (Box 1). Furthermore, the amount of bioinformatics support and analysis required to maximise the information accrued from scRNASeq experiments is non-trivial, and Table 2 summarises examples of the ever-expanding range of cutting-edge analysis approaches (and types of outputs generated) which are currently used in the field.

In this review, we will discuss how single-cell technologies are delivering a step change in our understanding of liver biology and disease pathogenesis, and opening up entirely new vistas to explore in the search for effective therapies for patients with liver disease.

# Liver Epithelial Cell Biology

The two major epithelial cell types within the liver are hepatocytes and cholangiocytes. Hepatocytes comprise the bulk of the liver mass and have a myriad of functions including protein synthesis, detoxification, bile production and carbohydrate and lipid metabolism. Cholangiocytes are biliary epithelial cells, lining the bile ducts and contributing to bile secretion and drainage. ScRNAseq analysis has recently provided a number of new insights into liver epithelial cell biology and function.

## Hepatocyte zonation

Functional zonation of hepatocytes is well described, with differences in metabolism and xenobiotic processing attributed to, amongst many factors, gradients of oxygen, nutrients and Wnt signalling across the liver lobule6 (Fig. 1). However, generating comprehensive molecular definitions of individual, zonally distributed hepatocytes was not possible prior to the advent of single-cell RNA sequencing. Halpern and colleagues utilised scRNAseq in combination with single-molecule fluorescence *in situ* hybridisation (smFISH) to define mouse hepatocyte heterogeneity, enabling spatial mapping of hepatocyte subpopulations7. This elegant study dissected molecular patterns of hepatocyte zonation across the mouse liver lobule, demonstrating a greater degree of hepatocyte zonation than previously appreciated, with half of all liver genes shown to be significantly zonated7. Importantly, this profile of hepatocyte gene expression across the lobule has provided a framework for studies of zonation in other hepatic cell lineages8,9, and has also informed the study of hepatocyte zonation in human liver10. Aizarani *et al*. performed scRNAseq followed by pseudospatial trajectory analysis to order human hepatocytes across the liver lobule, showing similar patterns of zonated genes to those observed in mouse11. Pathway enrichment analysis of the zonated genes demonstrated that periportal hepatocytes were enriched in genes responsible for oxidation and fatty acid metabolism, whilst midzonal hepatocytes showed enrichment of cytochrome P450 xenobiotic metabolism genes11. Hence, scRNAseq has provided new insights into the functional attributes of hepatocytes across different zones of both the human and mouse liver lobule.

## Liver Development and Organoids

Multi-lineage organoid cell culture systems are increasingly used to model development and disease, providing a tractable platform to interrogate new therapeutic targets. ScRNAseq has been used to explore how these three-dimensional culture systems impact on cell identity and differentiation compared to traditional monolayer culture systems, and whether organoids more closely model the *in vivo* state12. Delineating the transcriptional states (at single cell level) of induced pluripotent stem cells (iPSCs) in monoculture as they progress to a mature hepatocyte-like state, and comparing this to bulk RNA-seq data from mouse embryonic day 8.5 ventral foregut and embryonic day 10 liver, demonstrated that iPSC progression to mature hepatocyte-like cells recapitulated many features of *in vivo* hepatogenesis. Culturing these iPSC hepatic endoderm (iPSC-HE) cells with endothelial and mesenchymal cells as multi-cellular organoids (termed liver buds), increased their transcriptomic complexity when compared to monoculture alone. The iPSC-HE liver buds expressed genes involved in cell-matrix adhesion, glycolysis, hypoxia and cell signalling and displayed heterogeneity, with one subset expressing an epithelial migration signature. The authors then performed scRNAseq on human fetal and adult livers to determine the degree of similarity between these and the iPSC hepatocyte-like cells. The iPSC-HE liver bud cellular transcriptome was more similar to fetal hepatocytes than iPSC-HE cells maintained as a monoculture, and endothelial and mesenchymal cells from liver bud cultures also closely resembled their fetal counterparts. Thus, the interlineage communication present within a human liver bud organoid allows a more accurate and comprehensive recapitulation of transcriptomic aspects of human hepatogenesis than iPSC monoculture alone.

## Liver Regeneration and Stem Cells

The liver has a remarkable capacity for regeneration and repair, and the sources of hepatocyte replenishment to self-renew the hepatic parenchyma are a source of ongoing debate. Proliferative subpopulations of hepatocytes, in both homeostasis and a range of different liver injury models, have been identified in the periportal region13, the pericentral region14 and stochastically distributed across the liver lobule15. In this latter study, the self-renewing hepatocyte subpopulation expressed high levels of telomerase15, a ribonucleoprotein which maintains telomere structure and integrity, prevents chromosomal fusion and degradation, and promotes correct chromosomal partitioning during cell division16. Using scRNASeq, MacParland et al. found hepatocyte heterogeneity in human liver, distinguishing alpha-fetoprotein (AFP) positive and negative hepatocytes, and observing that AFP+ hepatocytes are enriched in pathways involved in cell division and interleukin-6 signalling (a key hepatocyte mitogen)10. Of note, AFP positive and negative hepatocytes were distributed throughout the profiled hepatocytes from all three liver lobule zones10, suggesting a pan-lobular model of hepatic regenerative capacity similar to the telomerase-high hepatocytes reported in mice.

Following severe liver injury, the regenerative capacity of the liver is overwhelmed17, in part secondary to hepatocyte replicative senescence18,19. In this setting, hepatic progenitor cells (HPC) of biliary origin are thought to arise from niches within the canal of Hering, expanding as ductular structures within the hepatic parenchyma (termed ductular reactions)20. These HPC have been shown to differentiate into hepatocytes and repopulate a proportion of the liver parenchyma in murine liver injury models21–23. However, the paucity of distinct and specific human HPC markers has hindered further analysis of these cells in human liver tissue. Recent scRNAseq-based studies in human liver have confirmed that cholangiocytes express epithelial cell adhesion molecule (EpCAM); however additional heterogeneity was also observed within the EpCAM+ compartment11 . Further analysis revealed that EpCAM+ cells consist of mature cholangiocytes (KRT19highCFTRhighALBlow), a hepatocyte-biased population (ASGR1+), as well as a naïve progenitor cell population11. Fate-mapping computational algorithms (StemID2 and FateID) suggested that this naïve progenitor subpopulation has the capacity to differentiate and bifurcate into both cholangiocyte and hepatocyte progenitor subpopulations. Differential expression of TROP2 distinguished these three EpCAM+ subpopulations, with naïve bipotent progenitors showing intermediate expression of TROP211. To assess function within the TROP2int progenitor population, TROP2int cells were isolated and cultured in organoids. TROP2int progenitor cells demonstrated the highest organoid-forming capability, whilst TROP2low/- cells formed no organoids and TROP2high cells formed fewer and smaller organoids11.

ScRNAseq has also revealed functional heterogeneity within the EpCAM+ cell compartment in murine liver8. In uninjured liver, 26% of EpCAM+ cells were enriched for gene targets of YAP (Yes-associated protein), the downstream transducer of the Hippo signalling pathway8. YAP signalling was also upregulated in both cholangiocytes and a small number of peri-portal hepatocytes following biliary injury. Functionally, YAP signalling in cholangiocytes was required for the maintenance of bile duct integrity in homeostasis, whilst YAP signalling in hepatocytes was required to generate the ductular reaction following DDC injury8.

# Liver Inflammation and Immunology

The liver is a highly immunological organ, containing large numbers of innate and adaptive immune cells. In health, these populations have important roles in maintaining both local tissue homeostasis24,25 and systemic immunity25. In the context of liver diseases, hepatic immune cells have been shown to regulate key pathological processes including fibrosis26 and carcinogenesis27. However, the immune system is both complex and diverse, making it challenging to determine which specific populations mediate particular functional effects. Single-cell technologies have the potential to revolutionise our understanding of immune cell heterogeneity and function in a variety of contexts5,28. In this section, we will discuss how these approaches have shaped our understanding of the liver immune system in health and disease.

## The Mononuclear Phagocyte System

The term mononuclear phagocyte system (MPS), first established in the 1970s, encompasses monocytes, macrophages and dendritic cells (DC)29. The pervasive view at the time was that monocytes in the circulation were the precursor population of both macrophages in tissues (large vacuolar cells which excelled in the clearance of cell debris and pathogens) and DCs (stellate shaped-cells which can present antigen and activate naïve T cells)30. However, several recent studies have clearly shown that tissue-resident macrophages in a variety of organs represent an ontogenically-distinct population, derived from embryological precursors and self-renewing independent of circulating monocytes31–37. Indeed, embryological-origin and self-renewal been convincingly demonstrated for the liver-resident macrophages, (Kupffer cells, KC), using a number of lineage-tracing approaches in mice34,35,38. Similarly, DCs can also originate from distinct haematopoietic precursors, independent of circulating monocytes39. Hence, there has been a move toward reclassification of these populations on the basis of ontogeny and function30. The complexity of the MPS in the liver is increased following an inflammatory stimulus, when circulating monocytes are recruited into the inflamed tissue and differentiate into monocyte-derived macrophages (MDM)26,40–42, which are capable of adopting a phenotype which is virtually indistinguishable from KC on the basis of previously-identified marker genes43,44. This inherent heterogeneity and cellular plasticity means single-cell approaches are essential to fully understand MPS diversity and function in liver tissue.

### Kupffer Cells

Kupffer cells (KC), the predominant liver macrophage population in homeostasis, reside within the liver sinusoids (Fig. 2) and scavenge gut-derived pathogens and damaged erythrocytes45, regulate iron and lipid metabolism46, maintain immunological tolerance47 and sense tissue damage45. However, these functional characteristics of KC were largely defined using mouse models, with limited characterisation or definition of KC from human liver, largely due to the lack of known markers for human KC. Human liver scRNAseq studies from three separate groups have now defined human KC as a CD163+MARCO+CD5L+TIMD4+ macrophage population10,11,48. Importantly, the identification of distinguishing markers of human KC has, for the first time, facilitated both the spatial mapping of these cells in human liver10,48 and the demonstration of an anti-inflammatory phenotype of human KC *ex vivo*10. Unbiased cross-species comparison of scRNAseq data has demonstrated that human and murine KC demonstrate a highly-conserved transcriptional profile48. Single-cell transcriptome analysis combined with KC-specific (Clec4f-cre) transcription factor knockout mice has identified NR1H3 (LXR⍺) and ZEB2 as key transcriptional regulators of KC differentiation and maintenance49. Application of gene regulatory network reconstruction and cell state identification to scRNAseq data (SCENIC package50), has also confirmed increased NR1H3 transcription factor activity in human KC48. Whether human KC are also derived embryologically and are maintained by self-renewal remains to be determined.

### Monocyte-derived Macrophages

The liver also contains a distinct population of macrophages, derived from the recruitment and differentiation of circulating monocytes45. In homeostasis, these MDMs are responsible for erythrocyte disposal and iron recycling51. Following liver injury in mice, there is recruitment of circulating Ly-6ChiCCR2+ monocytes which differentiate into liver MDMs, resulting in a huge expansion of intrahepatic macrophages26,45. These MDMs have been shown to regulate a number of aspects of liver injury, including perpetuation of inflammation45,52 and promotion of fibrogenesis41,53,54. However, MDMs are also highly heterogenous and plastic, capable of further differentiation into functionally distinct macrophage subpopulations, fulfilling key tissue repair roles such as fibrosis regression42,55 and the restoration of normal tissue architecture56,57. This heterogeneity and plasticity has made it challenging to study the function of specific hepatic MDM subpopulations following liver injury. In particular, characterisation of human hepatic MDMs had previously been limited to the use of known markers to identify an expanded CD14+CD16+ MDM population in fibrotic human liver58,59. An unbiased scRNAseq approach has shed new light on MDM heterogeneity and function, identifying a distinct subpopulation of TREM2+CD9+MNDA+ scar-associated macrophages (SAMacs) which expand in fibrotic human livers, are spatially localised to areas of scarring (termed the fibrotic niche) and promote both hepatic stellate cell (HSC) collagen production and proliferation (Fig. 2)48.

Similarly, scRNAseq studies of macrophages from murine models of chronic liver injury including chronic carbon tetrachloride (CCl4)48, amylin/AMLN diet-induced NASH60, and Western diet-induced NASH61 demonstrated distinct injury-associated macrophage clusters. Strikingly, murine injury-associated macrophages show significant overlap in distinguishing marker genes with those observed for human SAMacs, with TREM2 and CD9 being conserved across species48,60. Indeed, unbiased cross-species mapping of scRNAseq data using canonical correlation analysis (CCA)62 confirmed that human and murine SAMacs represent corollary populations, highlighting the utility of scRNAseq approaches to define 'core' injury populations which could be therapeutically targeted. Importantly, scRNAseq data in mice also demonstrated that the Western diet induces alterations in subpopulations of bone marrow cells, suggestive of a reprogramming of systemic mononuclear phagocyte responses in response to liver injury63. Application of an analogous approach in patients with liver diseases will be helpful to assess whether similar mechanisms exist in humans.

The identification of distinct human SAMacs using scRNAseq, highlights novel computational approaches to improve our understanding of cellular function in liver disease. Trajectory inference or pseudotemporal ordering approaches place single-cells along a continuous path to help deepen our understanding of cellular differentiation pathways (Table 1). These methods can be supplemented with RNA velocity analysis which assesses the ratio of spliced to unspliced mRNA molecules within each single-cell, thereby inferring cell fate and ascribing directionality to differentiation pathways64. Application of both pseudotemporal ordering using the monocle package65 and RNA velocity assessment64 to scRNAseq data of circulating monocytes and human liver macrophages facilitated *in silico* lineage tracing of human liver mononuclear phagocytes for the first time, confirming that human SAMacs derive from recruitment and differentiation of circulating monocytes and remain distinct from KCs48.

Having used scRNAseq to define human liver macrophage subpopulations, we then generated gene expression signatures for the phenotypically different cell types48. These signatures can be applied to deconvolution algorithms, facilitating the assessment of cell type composition of whole tissue biopsies which have been subjected to bulk transcriptomic profiling66–69. Employing this approach enables an assessment of changes in cellular composition across larger cohorts of patients with different disease stages using publicly available data, applications which are not currently practical with scRNAseq. We applied a deconvolution approach to a publicly available human NAFLD microarray dataset70 to study macrophage composition across 73 patients, highlighting expansion of SAMacs in early stage NAFLD as well as advanced disease48. The use of a similar methodology for other cell types may offer further insights into the pathogenic cell populations present at different stages of human liver disease.

### Dendritic Cells

The liver also contains subpopulations of dendritic cells (DCs), the key antigen presenting innate immune population. Single-cell approaches are already transforming our understanding of human DC heterogeneity71–73. DCs can be subdivided into two separate lineages; plasmacytoid DCs (pDCs) are able to rapidly produce Type 1 interferons and regulate inflammatory responses, whilst conventional DCs (cDCs) are the most specialised antigen-presenting cells which regulate T cell responses74. In fact, cDCs can be further subdivided into type 1 cDCs (cDC1) and type 2 cDCs (cDC2). Both cDC subpopulations are capable of stimulating CD4+ T cell responses74, whilst cDC1 cells are more potent stimulators of CD8+ T cell responses75. All 3 populations of DCs have now been identified in human48 and murine48,60,61 liver tissue using scRNAseq, with multiple markers now available to distinguish them. Future work should be aimed at discerning functional differences in these populations in regulating hepatic immunity and identifying their role in liver disease pathogenesis.

## Granulocytes

The term granulocytes refers to white blood cells which contain granules in their cytoplasm. Neutrophils76,77, eosinophils78, basophils44 and mast cells79 have all been associated with liver injury and repair in different contexts. However, granulocytes often have low RNA content and contain large quantities of RNases, making scRNAseq of granulocytes challenging especially when using high-throughput droplet-based approaches. Hence, granulocyte populations are often underrepresented in scRNAseq datasets. Nonetheless, mast cells have been identified in human liver tissue48,80, and single-cell methods have shown that basophils are important in regulating lung homeostasis81. As the scRNASeq technologies improve, more data is likely to emerge on the heterogeneity and function of hepatic granulocytes.

## The Lymphoid System

### T cells

T lymphocytes co-ordinate adaptive immune responses throughout the body, and it has long been known that the liver-resident T cell compartment is distinct from that observed in the peripheral circulation82. Subpopulations of intrahepatic T cells have been identified in multiple published human liver scRNAseq datasets10,11,48, and these populations are transcriptionally distinct from circulating T cells48. Annotation of T cell subpopulations from scRNAseq data has been more challenging, due to a combination of the low transcriptional activity of T cells and the shallower read depths of droplet-based scRNAseq workflows. However, in all studies clear liver T cell heterogeneity has been demonstrated, with distinct clusters of CD4+ memory T cells, CD8+ effector T cells and ƔδT cells described10,48. A shift in T cell phenotype was observed in cirrhotic human livers, with expansion of a SELL+CCR7+CD4+ memory T cell population48. Furthermore, scRNAseq of T cells from human hepatocellular carcinoma (HCC) samples identified tumour enriched populations of exhausted CD8+ T cells and CTLA4hi regulatory T cells83, both of which are associated with impaired anti-tumour immunity. Layilin (LAYN) expression was specific to HCC-associated T cells, correlated with adverse clinical outcomes and suppressed CD8+T cell IFNƔ production, suggestive of a pro-tumour role for LAYN83. Further detail on intratumoural T cell heterogeneity has been provided by studying the cellular composition of HCC and intrahepatic cholangiocarcinoma (ICC) using scRNAseq84. Striking changes in T cell composition were observed when comparing high and low diversity tumours; low diversity tumour T cells were enriched for cytotoxic and immune checkpoint molecules whilst high diversity tumours contained increased numbers of regulatory T cells84. Importantly, patients with high diversity tumours had a much worse prognosis, suggesting that immunotherapy approaches may be effective in certain patients with HCC and ICC.

Application of scRNAseq to study T cells also enables the analysis of T cell receptor (TCR) repertoires, defining origin, clonality and potentially antigen-specificity for T cell subpopulations85. Initial application of this approach to HCC samples confirmed clonal expansion of both exhausted CD8+ T cells and regulatory T cells within the tumour microenvironment, allowing deeper analysis of T cell differentiation pathways and fates83. The advent of high throughput approaches to perform integrated single-cell T cell profiling of transcriptome, TCR and antigen specificity will provide new insights into T cell function and therapeutics in liver cancer86 and other inflammatory liver disorders.

### Innate lymphoid and NK cells

Innate lymphoid cells (ILCs) form a distinct arm of the innate immune system with diverse functions regulating immunity, inflammatory responses and tissue homeostasis. ILCs have been subclassified into natural killer (NK) cells, ILC1, ILC2, ILC3 and lymphoid tissue inducer cells on the basis of their cytokine secretion pattern and on the transcription factors driving their maturation87. Single-cell transcriptomics has been employed to study ILC heterogeneity in human tonsil, demonstrating clear transcriptional differences between each major ILC subpopulation88. In the liver, ILC subpopulations have been identified in both human10,48 and mouse89. NK cells have been implicated in the pathogenesis of liver injury and fibrosis90, with a relative loss of a subpopulation of CD56+CD16- NK cells observed in fibrotic human liver tissue using scRNAseq48. Additionally, an alteration in NK cell composition was observed in human HCC80, with potential interactions observed between NK cells and a subpopulation of tumour-enriched LAMP3+ migratory cDCs. These data highlight the utility of scRNAseq in dissecting potential molecular interactions between different cell types within a complex microenvironment. Other ILC subpopulations have also been shown to regulate liver inflammation in mouse models91,92. Future work will likely focus on performing functional analyses of human ILCs in the context of liver disease.

### B cells

B cells produce antibodies and present antigens. Liver B cells have been shown to regulate liver fibrosis93,94 and carcinogenesis93. Additionally, alterations in B cell function have been associated with increased susceptibility to infections in patients with chronic liver disease95. Unbiased scRNAseq studies of human liver identified distinct populations of both tissue resident B cells and plasma cells (terminally differentiated B cells which produce large quantities of antibodies)10,48. However, no major alterations in the transcriptional profile or composition of hepatic B cells were observed in patients with chronic liver disease48. In the future, combining scRNAseq with B cell receptor profiling96,97 could yield novel insights into B cell and antibody clonality and function in liver disease.

# Endothelial cell heterogeneity and function

The liver is a highly vascular organ, with the hepatic vasculature broadly compartmentalised into the portal vein, hepatic artery, central vein and sinusoids (Fig. 1). Nutrient-rich blood enters the liver from the gut through the portal vein and mixes with oxygen-rich blood entering via the hepatic artery. This blood then flows along the liver sinusoids, which have microscopic holes (fenestrae) allowing the blood to come into close contact with hepatocytes, Kupffer cells and hepatic stellate cells (Fig. 1). The sinusoids then drain into the central vein which directs the now nutrient and oxygen poor blood out of the liver. This complex vasculature is lined with distinct endothelial cell subpopulations, which are likely to have specialised functions in each vascular bed. Whilst some delineation of hepatic endothelial cell subpopulations has previously been possible using cell surface markers98–100, the application of an unbiased scRNAseq approach has enabled a more comprehensive annotation10,11,48. In human liver, the fenestrated sinusoidal endothelial cells (LSEC) can be distinguished by markers including CLEC4G, CLEC4M, STAB2 and CD1410,11,48. Human LSEC showed gene set enrichment for scavenging and immunoregulatory functions10,11,48 and demonstrated enhanced activity of the transcription factor GATA448, in keeping with a key role for GATA4 in maintaining murine LSEC specification and function101. Further annotation of distinct populations of RSPO3+ central venous, AIF1L+ hepatic arterial and PDPN+ lymphatic endothelial cells was also possible (Fig. 3)48, facilitating future studies directed at interrogating the functional roles of these endothelial subpopulations in liver disease.

## Liver Sinusoidal Endothelial Cell Zonation

In addition to providing new insights into liver endothelial cell heterogeneity, scRNASeq has revealed previously unknown functional zonation of LSEC across the liver lobule. Halpern *et al.* harnessed the spatial information garnered from their hepatocyte zonation work7, and coupled this with a novel approach, paired-cell sequencing (pcSeq)9. This technique sequences mRNA from pairs of attached cells, leveraging the gene expression profile of one cell lineage within the pair to allow inference of the other cell’s spatial coordinates. This allowed characterisation of endothelial cell zonation within the liver, demonstrating that LSECs are highly zonated and functionally specialised across the mouse liver lobule9. This pattern of endothelial cell zonation, summarised in Fig. 1, has been confirmed in a further recent study60.

In humans, scRNAseq analysis of LSEC also demonstrated significant zonation10,11, with 67% of LSEC genes being zonated along the portal-central axis11. Gene set enrichment analysis of LSEC zonal subsets highlighted functional pathways shared with other liver cell types located in the same zone, for example mid-zonal LSEC and hepatocytes co-express pathways related to ligand receptor binding and uptake11. This suggests that the distinct functions of each lobule zone could be regulated by conserved patterns of zonation in both epithelial and non-epithelial cells. Importantly, comparison between mouse and human scRNAseq data indicated limited cross-species conservation of zonation profiles11, emphasising the importance of studying human tissue and cells to understand the relevance of zonation to human disease pathogenesis.

## Vascular Responses to Liver Disease

Liver injury induces significant changes within the hepatic vascular landscape, including loss of LSEC fenestrae and the development of an organised basement membrane100. This LSEC capillarization is thought to play a crucial role in fibrogenesis, in part by facilitating hepatic stellate cell activation100,102,103. Furthermore, alterations in the liver vasculature are known to regulate multiple other aspects of liver disease pathogenesis including inflammation, regeneration, carcinogenesis and portal hypertension100. Comparative analysis of scRNASeq data from healthy and cirrhotic human liver tissue demonstrated the emergence of distinct populations of disease-associated CD34+PLVAP+VWA1+ and CD34+PLVAP+ACKR1+endothelial cells, which were not present in healthy livers and spatially restricted to the fibrotic niche of diseased livers48. Functionally, disease-associated endothelial cells displayed enhanced leucocyte transmigration48, suggesting a role in the regulation of hepatic inflammation.

In a murine NASH model, scRNASeq of over 10,000 endothelial cells also demonstrated an altered transcriptional profile following injury60. In response to liver injury all subpopulations of endothelial cells upregulated genes involved in lipid metabolism, antigen presentation and chemokine release with a concurrent reduction in genes regulating vascular development and homeostasis60. A number of these hepatic vascular gene expression changes were studied in human NASH biopsy samples using publicly available microarray data, defining the upregulation of endothelial factors such as CXCL9 and FABP4 in both human and murine NASH60. This type of comparative analysis provides a framework for identification and functional interrogation of specific pathogenic pathways which are conserved across species.

A distinct population of lymphatic endothelial cells (LEC) line the lymphatic vessels and play important regulatory roles in inflammatory disease104. Using scRNAseq, human LEC can be distinguished by PDPN expression48,105 (Fig. 3). PDPN immunohistochemistry demonstrated that hepatic lymphatic vessels are restricted to the periportal region in homeostasis, but expand and populate the fibrotic niche in cirrhotic livers, irrespective of liver disease aetiology48,105. LEC from diseased livers were highly proliferative and expressed high levels of the chemokine CCL21105. Interestingly, comparison of the transcriptional profile of LEC from different aetiologies of liver disease demonstrated that LEC from NASH patients showed the greatest enrichment for interleukin-13 (IL-13) signalling105, a cytokine known to regulate fibrogenesis. Functionalassays demonstrated that oxidised low-density lipoprotein (oxLDL), which has been implicated in NAFLD pathogenesis, induced IL-13 production by human LEC *in vitro* and murine LEC *in vivo*105. These data also highlight the utility of scRNAseq in defining disease-specific responses in subpopulations of rarer cell types.

# Mesenchymal Cells

Mesenchymal cells represent a heterogeneous population in the liver comprising four main subtypes; hepatic stellate cells (HSC), which are liver-specific pericytes and reside throughout the parenchyma in the perisinusoidal space, portal fibroblasts (PF) which populate the portal niche, vascular smooth muscle cells (VSMC) and mesothelial cells106–108. During homeostasis, HSC are vitamin A-storing cells involved in retinoid metabolism and immunoregulation107,109, whereas PF provide structural support to the vasculature of the hepatic artery, portal vein and bile duct in the portal niche. Following liver injury, mesenchymal cells have a crucial role in the pathogenesis of liver disease, representing the major source of pathogenic extracellular matrix deposition during hepatic fibrogenesis. Therefore, they have attracted considerable interest as a potential antifibrotic therapeutic target110–113.

## Mesenchymal cell heterogeneity and zonation

Despite their key role in the pathogenesis of liver fibrosis, characterisation of mesenchymal cell subpopulations in human liver tissue has remained limited. This may in part reflect challenges in cell isolation, with mesenchymal cells representing a very small proportion of cells in previous human liver scRNAseq atlas studies10,11. However, by using fluorescence-activated cell sorting (FACS) to enrich for non-parenchymal cell (NPC) subpopulations prior to scRNAseq, greater resolution on human mesenchymal cell heterogeneity has been obtained48. Specifically, distinct populations of human HSC, PF, VSMC and mesothelial cells have been identified48. In mice, using validated mesenchymal cell isolation techniques114,115, and a pan-mesenchymal cell reporter mouse strain (*Pdgfrb*-BAC-eGFP reporter), scRNAseq data has now also provided further robust definitions for distinct hepatic mesenchymal cell populations116.

Heterogeneity within HSC across the hepatic lobule has previously been described using immunostaining and morphological analysis117. However, the combination of scRNAseq and spatial mapping has definitively identified HSC zonation across the healthy mouse liver lobule for the first time116. HSC partition into two topographically diametric lobule regions (Fig. 1), designated portal vein-associated HSCs and central vein-associated HSCs116. Future targeted scRNAseq-based studies of the mesenchyme in human liver tissue will allow interrogation of whether mesenchymal cell zonation is conserved across species.

## Mesenchymal cells in liver fibrosis

Over the past 30 years significant increases in our understanding of the cellular and molecular mechanisms regulating liver fibrosis have largely been achieved using pre-clinical models. The origin of collagen-producing hepatic myofibroblasts has been extensively investigated in rodent models, with HSC considered major contributors to the myofibroblast pool irrespective of aetiology. However evidence also suggests a pathological matrix-contributing role for PF in biliary disease118,119.

Since their discovery as a major collagen-producing cell in the liver HSC have been generally considered a homogenous population, with the potential to transition to the activated, myofibroblast phenotype thought to be equally distributed across all HSCs. ScRNAseq has recently been utilised to interrogate the functional zonation of murine HSC, allowing high-resolution identification of the critical pathogenic collagen-producing cells following centrilobular liver injury116. Notably, pseudotemporal trajectory and RNA velocity analyses demonstrated that central vein-associated HSCs are the dominant source of pathogenic collagen-producing cells following centrilobular liver injury116. Additionally, using scRNAseq to interrogate retinol-positive myofibroblasts isolated from fibrotic mouse liver, has identified previously unknown heterogeneity and functional diversity within myofibroblasts120. Universally marked by expression of the activation marker *S100a6,* mouse liver myofibroblasts were then further subdivided into four subpopulations, including those with immunoregulatory or portal fibroblast-like characteristics120. Further studies will aim to spatially map these distinct myofibroblast populations, as well as compare present findings with other models of liver fibrosis that reflect different aetiologies of human liver disease.

In human cirrhotic liver, collagen-producing mesenchymal cells were distinguished by high levels of PDGFR expression, and were spatially located within the fibrotic niche48. Similar to murine studies, computational analysis of human scRNAseq data demonstrated that HSC are the likely source of collagen-producing scar-associated mesenchyme48 (Fig. 2). However, the overall number of mesenchymal cells currently included in these human studies remains low in comparison to other cell lineages, highlighting the need for further scRNAseq data to fully interrogate mesenchymal cell heterogeneity and function in human liver disease.

# Ligand-receptor Interactome Modelling with scRNAseq Data

ScRNAseq studies have already provided a wealth of novel insights into cellular heterogeneity and function in liver homeostasis and disease. However, disease processes such as fibrosis, regeneration and carcinogenesis are complex, with highly-orchestrated interactions between specific subpopulations of multiple different cell types likely to be important in driving disease progression. Therefore, in order to identify novel and effective therapeutic targets, it is essential that we deepen our understanding of the complex interactome present across the different forms of human liver disease.

Importantly, scRNAseq analysis not only enables the identification of distinct cell types but also defines which cell subpopulations express specific genes and to what extent. Consequently, further analysis can be performed on these data to study potential ligand-receptor interactions between different cell types, modelling the interactome and highlighting cellular and molecular mechanisms which may promote disease progression. A number of computational approaches now exist to perform interactome analysis on scRNAseq data81,121,122. In the liver, application of the unbiased CellPhoneDB algorithm122 to the scar-associated macrophages, endothelial cells and mesenchymal cells populating the fibrotic niche has identified a number of pathways which could promote mesenchymal cell activation and fibrogenesis48 (Fig. 5). Specifically, scar-associated macrophages express ligands including AREG, TGFB1, IL1B, TNFSF12 and PDGFB which could signal through their cognate receptors EGFR, IL1RA, TNFRSF12A and PDGFRA expressed on scar-associated mesenchymal cells to potentially promote mesenchymal cell activation123–125, survival54 and proliferation48,126,127. In addition, scar-associated endothelial cells express high levels of the Notch ligands JAG1, JAG2 and DLL4 which can signal through Notch receptors expressed by scar-associated mesenchymal cells48. Modelling of these interactions using *in vitro* co-culture systems confirmed a role for NOTCH signalling, specifically NOTCH3, in promoting fibrillar collagen production by activated HSCs48, highlighting the Notch pathway as a potential target for anti-fibrotic therapies128.

Modelling ligand-receptor interactions using scRNAseq data has also been performed in murine NASH and was facilitated by the manual curation of a mouse secretome60. The authors went on to identify a unique set of secreted factors exhibiting enriched expression in HSC. Termed ‘stellakines’, these factors, many of which were elevated in NASH, were predicted to act primarily on endothelial and immune cells and highlight the important role of HSC in orchestrating liver injury responses60. This approach also highlighted the overexpression of several G-protein coupled receptors (GPCRs) on HSC. Functional studies confirmed that these GPCRs can regulate cellular contractile activity, suggesting a role for HSC in controlling intrahepatic vascular tone and potentially portal pressure60.

Interactome analysis therefore represents a very powerful application of scRNAseq data, resolving key pathogenic pathways in complex multi-cellular disease processes, and defining relevant and tractable therapeutic targets which can then be functionally interrogated.

# Conclusions

It is clear that single-cell technologies have already yielded multiple new discoveries in hepatology that would previously have been unattainable. The single-cell field continues to develop at pace, with multiple emerging, powerful technologies in this area likely to drive biomedical research on to new levels of resolution and precision in the coming years.

Spatial technologies represent an exciting new approach in this field129–131. In standard scRNASeq, following generation of single cell suspensions all spatial information is lost and must be validated in tissue *post-hoc* using newly-generated markers. Conversely, spatial transcriptomics has the potential to deliver 'on-slide' transcriptome-wide information at single-cell resolution, preserving the native architecture and interactions of cells within tissue, complementing and potentially, in time, replacing information accrued via standard scRNASeq techniques. This type of spatially resolved molecular profiling is likely to have major applications and impact across the entire range of human liver disease.

A further rapidly developing area involves the integration of multi-modal single-cell omics, for example, the simultaneous analysis of transcriptome and chromatin state132, or protein and transcriptome in single cells133,134. These approaches are generating high-plex, highly-dimensional data and therefore necessitate the development of cutting-edge bioinformatics approaches to extract the maximal amount of information from these integrated datasets135. The convergence of these multi-modal single-cell technologies represent a remarkable opportunity to de-code the molecular mechanisms regulating human liver disease at single cell resolution, which should in turn help drive a new era of precision medicine in hepatology.

# Contributions

All authors wrote and edited the manuscript.

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# Competing interests

The authors declare no competing financial interests.

# Box 1: Considerations in the design of single-cell transcriptomics experiments

Optimal scRNAseq experiments involve the rapid isolation and selection of cells with high viability, whilst minimising processing time to reduce artefactual noise. The choice of approach depends on equipment availability and cost, as well as the type of data desired136.

* Depending on the research question, determine whether an unbiased approach is desirable for cell selection, or whether methods such as fluorescence-activated cell sorting (FACS) should be employed to enrich for lineages or subpopulations.
* Low-throughput cell isolation methodologies involve FACS sorting into plates (Smart-seq2137, NEBNext138) or commercial cell capture solutions (Fluidigm C1139), whereas high-throughput methodologies leverage automated workflows (MARS-seq140,141, mCEL-seq2142), bespoke cell capture arrays (seq-well143), or droplet-based microfluidics (10X Chromium144, inDrops145, Drop-seq146) to sequence thousands of cells per sample.
* Full read-length methodologies such as Smart-seq2 and NEBNext derive reads from the whole length of genes, increasing sensitivity for the detection of low abundance transcripts, reducing bias towards longer genes, and enabling additional analyses such as assessment of splice variants. Tag-based methods such as 10X Chromium use Unique Molecular Identifiers (UMIs) to quantify individual transcripts, reducing technical noise and amplification bias but introducing 3’ or 5’ bias depending on the transcript end receiving the tag.

Subsequent to generating scRNA-seq data, it is important to adequately validate findings using independent approaches, for example:

* Interrogate subpopulation markers at protein level using immunohistochemistry and flow cytometry. Protein readouts can also be incorporated in further scRNA-seq analysis, using flow cytometry index-sorting on plate-based methods147 or antibody-tagging (Cite-seq133, Reap-seq134) for droplet-based approaches. Where antibodies are unavailable, single-molecule fluorescence in situ hybridization (smFISH) can be employed, often using commercially available reagents (e.g. RNAScope®).
* Verify functional interpretation using *in vitro* biological assays or pre-clinical models, and supplement trajectory inference with formal lineage tracing studies if possible.

# Table 1: Liver Single-cell RNA-seq Studies

Liver single-cell RNA-seq studies performed to date, including the platform used and a summary of the main findings.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Paper**  | **Species**  | **Platform(s)**  | **Lineages Represented**  | **Synopsis** |
| MacParland *et al.,* 201810 | Human  | 10X Chromium | EpitheliaImmuneEndotheliaMesenchyme | Cell atlas of healthy human liver; hepatocyte zonation; hepatocyte proliferation; heterogeneity in immune and endothelial cells |
| Aizarani *et al.*, 201911 | Human, Mouse | mCEL-seq2 | EpitheliaImmuneEndotheliaMesenchyme  | Cell atlas of healthy human liver; hepatocyte zonation; defined epithelial heterogeneity and identified epithelial progenitor population. |
| Ramachandran *et al.,* 201948 | Human, Mouse | 10X Chromium  | EpitheliaImmuneEndotheliaMesenchyme | Atlas of human healthy and fibrotic liver; defined scar-associated macrophages, endothelial cells and mesenchymal cells; interactome analysis between scar-associated populations within the fibrotic niche.  |
| Zheng *et al.,* 201783 | Human | Smart-seq2 | Immune | T cells from peripheral blood, HCC and background liver tissue; defined expansion and clonality of T cell subpopulations in HCC; identified Layilin expression on HCC T cells |
| Zhang *et al*., 201980 | Human  | 10X Chromium Smart-seq2 | Immune | Immune cells from peripheral blood, HCC, background liver, lymph node and blood; identified distinct populations of migratory DCs and tumour-associated macrophages in HCC |
| Ma et *al*., 201984 | Human | 10X Chromium | EpitheliaImmuneEndotheliaMesenchyme | Cells from HCC and intrahepatic cholangiocarcinoma; tumours with higher transcriptomic diversity predict worse clinical outcomes  |
| Tamburini *et al.,* 2019105 | Human | 10X Chromium  | EpitheliaImmuneEndotheliaMesenchyme | Characterisation of lymphatic endothelial cells in healthy and chronically diseased liver.  |
| Halpern *et al.,* 20177 | Mouse  | MARS-seq | EpitheliaImmuneEndothelia  | Characterisation of hepatocyte zonation across the mouse liver lobule  |
| Halpern *et al.,* 20189 | Mouse  | MARS-seq | EpitheliaImmuneEndothelia | Characterisation of endothelial cell zonation across the mouse liver lobule using paired-cell sequencing  |
| Pepe-Mooney *et al.,* 20198 | Mouse  | inDropSeq-Well  | Epithelia  | Defined the dynamic cellular state of biliary epithelial cells during homeostasis and following injury; role of YAP signalling in biliary maintenance |
| Kenkel et al., 201963 | Mouse  | 10X Chromium  | Immune  | Defined alterations in mononuclear phagocyte heterogeneity in liver and bone marrow in a mouse model of NASH |
| Scott et al., 201849 | Mouse | 10X Chromium | Immune | Identified the role of transcription factors NR1H3 and Zeb2 in regulating Kupffer cell phenotype |
| Xiong *et al.,* 201960 | Mouse  | 10X Chromium  | EpitheliaImmuneEndotheliaMesenchyme  | Cell atlas in healthy and NASH mouse liver; modelling ligand-receptor interactions |
| Peters *et al*., 201989 | Mouse  | C1 Fluidigm | Immune | ILCs from mouse liver and extrahepatic bile duct; defined IL-33 responsive cell populations |
| Dobie *et al.,* 2019116 | Mouse  | 10X Chromium Smart-seq2 | Mesenchyme  | Interrogation of the hepatic mesenchyme and HSC zonation in healthy and fibrotic liver, and following acute injury.  |
| Krenkel *et al.,* 2019120 | Mouse  | 10X Chromium  | Mesenchyme (HSC, MFB) | Interrogation of HSC and myofibroblast heterogeneity in healthy and fibrotic liver and *in vitro* cultures. |

# Table 2: Single-cell RNA-seq Analyses

Analysis approaches applied to scRNASeq data, with example packages and a summary of the type of outputs generated.

|  |  |  |
| --- | --- | --- |
| **Analysis** | **Exemplar Tools** | **Summary** |
| **Data pre-processing** |
| From raw data to count / read matrix | Cell Ranger144 Indrops145zUMIs148 | Process raw sequencing data to obtain count or read matrices: read quality control; assignment to cellular barcodes (demultiplexing); genome alignment; quantification. |
| From count / read matrix to visualisation | Seurat62 Scater149 Scanpy150 | Barcode quality control to ensure viable cells; normalisation to obtain correct relative abundances between cells; data correction and integration to regress out unwanted biological and technical covariates such as batch effect; dimensionality reduction, feature selection, and visualisation of data. |
| **Cell-focused analyses**  |
| Clustering | Louvain151SIMLR152 | Group cells based on the similarity of their gene expression profiles, using algorithms such as multi-resolution modularity optimisation or k-means clustering with various distance metrics. |
| Cluster annotation | scmap153SingleR154 | Annotate clusters with meaningful biological labels representing cell identities, whether manually using marker genes obtained via differential expression testing, or automating the process via direct comparison of annotated reference clusters to individual cell expression profiles. |
| Trajectory inference | Monocle65 Wanderlust155Slingshot156PAGA157 | Reconstruct single-cell profiles as a snapshot of a continuous process, and find paths that minimise transcriptional change between neighbouring cells. A range of path complexities may be modelled from simple linear and bifurcating trajectories to complex graphs and trees. |
| RNA velocity  | Velocyto64scVelo158 | Infer trajectory directionality by superimposing splicing information (ratio of spliced to unspliced mRNA) and estimating the future state of single cells. |
| **Gene-focused analyses** |
| Gene set enrichment | SCRAT12  | Group genes and interpret characteristics based on gene ontology or pathway participation. |
| Gene regulatory networks | SCODE159SCENIC50 | Uncover regulatory interactions based on measurements of gene co-expression, such as correlation with expression of transcription factors and their inferred targets. |
| Ligand-receptor analysis  | CellPhoneDB48,122 NicheNet121 | Infer interaction between cell identities from expression of receptors and their cognate ligands. |

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# Figure Legends

## Figure 1: Zonation of cells across the liver sinusoid

Blood flows into the liver from the hepatic artery and portal vein and leaves the liver through the hepatic vein. The portal vein and hepatic artery are located in portal triads in close proximity to the bile ducts, which drain bile secreted by the hepatocytes. Incoming blood, which is high in oxygen and nutrients flows from the portal zone, through radial sinusoids and into the central vein. Blood oxygen and nutrients are depleted along the sinusoid creating a gradient. Concentric layers of hepatocytes (brown) are positioned between the portal triad and central vein. Non-parenchymal cells associated with the sinusoid include fenestrated sinusoidal endothelial cells (LSEC; red/purple), Kupffer cells (KC; dark blue) and hepatic stellate cells (HSC; green) which reside in the space of Disse. ScRNAseq has revealed genes which differentiate portal zone and central zone hepatocytes, LSEC and HSC. These markers such as E-Cadherin (portal zone hepatocytes), Cyp2e1 (central zone hepatocytes), NGFR (portal-associated HSC) and c-Kit (central zone LSEC) can be used to identify zonation in mouse liver using immunohistochemistry (example immunofluorescence images shown). Adapted from MacParland et al10.

## Figure 2: Macrophage dynamics in liver fibrosis

Macrophages are important regulators of liver homeostasis and injury. In healthy liver tissue, resident macrophages called Kupffer cells (KC, dark blue) predominate and reside within the sinusoidal space. These KC, which can be identified using markers such as TIMD4 and MARCO, are self-renewing and not replenished from circulating monocytes in homeostasis. In chronically damaged liver tissue, monocytes are recruited from the circulation into the liver parenchyma, differentiate and proliferate to form a population of TREM2+CD9+MNDA+ scar-associated macrophages. These scar-associated macrophages promote the activation of quiescent hepatic stellate cells into PDGFR⍺+ scar-associated mesenchymal cells, which proliferate and produce fibrillar collagens within the fibrotic niche of diseased livers.

## Figure 3: Defining endothelial cell heterogeneity

Unbiased scRNAseq studies have enabled the identification of liver endothelial cell subpopulations. Liver sinusoidal endothelial cells (LSEC) line the sinusoids and are distinguished by expression of markers including CLEC4M, CLEC4G and CD14. Distinguishing marker genes for portal vein, hepatic artery and central venous endothelial cells have also been defined. Hepatic lymphatic vessels, located in the portal zone, are lined by a distinct population of lymphatic endothelial cells which express PDPN and PROX1. Subpopulation annotation and marker gene identification adapted from Ramachandran et al48.

## Figure 4: Dissecting the cellular interactome within the fibrotic niche

Single-cell transcriptional profiles of scar-associated macrophages, endothelial cells and mesenchymal cells48 were analysed using the CellPhoneDB algorithm122 to assess potential ligand-receptor interactions. Scar-associated macrophages express ligands which can promote scar-associated mesenchyme activation (AREG, TGFβ1), proliferation (PDGFB, TNFSF12) and survival (IL-1β). Similarly, scar-associated endothelial cells can promote scar-associated mesenchyme collagen production (NOTCH3). Interactions were also predicted between scar-associated macrophages and endothelial cells, highlighting the complex interactome within the fibrotic niche.