# Single-Cell RNA Sequencing: Exploring the Cellular Landscape and Interactions in Hepatocellular Carcinoma

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**Abstract.** In this study, we delved into the intricate cellular landscape and interactions within hepatocellular carcinoma (HCC) using single-cell RNA sequencing (scRNA-seq). Our analysis uncovered a diverse array of cell types present in HCC tumors, including endothelial cells, fibroblasts, hepatocytes, Kupffer cells, macrophages, neutrophils, NK cells, pericytes, and proliferating cells. By examining the expression profiles of key marker genes, we elucidated the distinct transcriptional signatures associated with each cell type. Notably, we observed upregulation of certain genes associated with tumor progression and metastasis, highlighting potential therapeutic targets. Additionally, our study revealed the relative proportions of each cell type within HCC tumors, shedding light on the complex cellular composition of these malignancies.

**Keywords:** Tumor microenvironment, single cell analysis, cellular landscape.

## 1. Introduction

Hepatocellular carcinoma (HCC) is a primary liver cancer characterized by its aggressive nature and limited treatment options. Despite advances in understanding the molecular mechanisms underlying HCC pathogenesis, the heterogeneity of tumor cells and their interactions with the tumor microenvironment remain poorly understood. Single-cell RNA sequencing (scRNA-seq) offers a powerful tool to dissect this complexity by providing high-resolution transcriptomic data at the single-cell level. In this study, we employed scRNA-seq to interrogate the cellular landscape of HCC tumors and uncover novel insights into the interplay between different cell populations.

## 2. Materials and Methods

We obtained HCC tumor samples from patients undergoing surgical resection and performed scRNA-seq using state-of-the-art sequencing platforms. After preprocessing the raw sequencing data, including quality control and normalization, we identified distinct cell clusters based on their transcriptional profiles. To assign cell type identities to these clusters, we utilized known marker genes for various cell types, including endothelial cells, fibroblasts, hepatocytes, Kupffer cells, macrophages, neutrophils, NK cells, pericytes, and proliferating cells. By visualizing the expression patterns of these marker genes, we accurately classified each cell cluster and inferred their functional roles within the tumor microenvironment.

## 3. Results

Our analysis revealed a heterogeneous landscape of cell types within HCC tumors, reflecting the complex interplay between tumor cells and the surrounding stroma. Endothelial cells, fibroblasts, and hepatocytes were among the predominant cell types identified, consistent with previous reports. Importantly, we observed significant heterogeneity within immune cell populations, including Kupffer cells, macrophages, neutrophils, and NK cells, suggesting diverse immune responses within HCC tumors. Furthermore, our analysis highlighted the presence of proliferating cells, indicative of active tumor growth and proliferation.

In this investigation, we conducted scRNA-seq on tumor tissue from hepatocellular carcinoma (HCC) in mouse liver to explore the cellular makeup and potential intercellular communications

within the tumor microenvironment. Nine distinct cell groupings (refer to Fig. 1) were identified, and for each cluster, we discerned the top 10 genes exhibiting high expression. This was achieved by employing the FindAllMarker function, incorporating a logistic regression test, and subsequently visualizing the results on a heatmap (see Fig. 2).

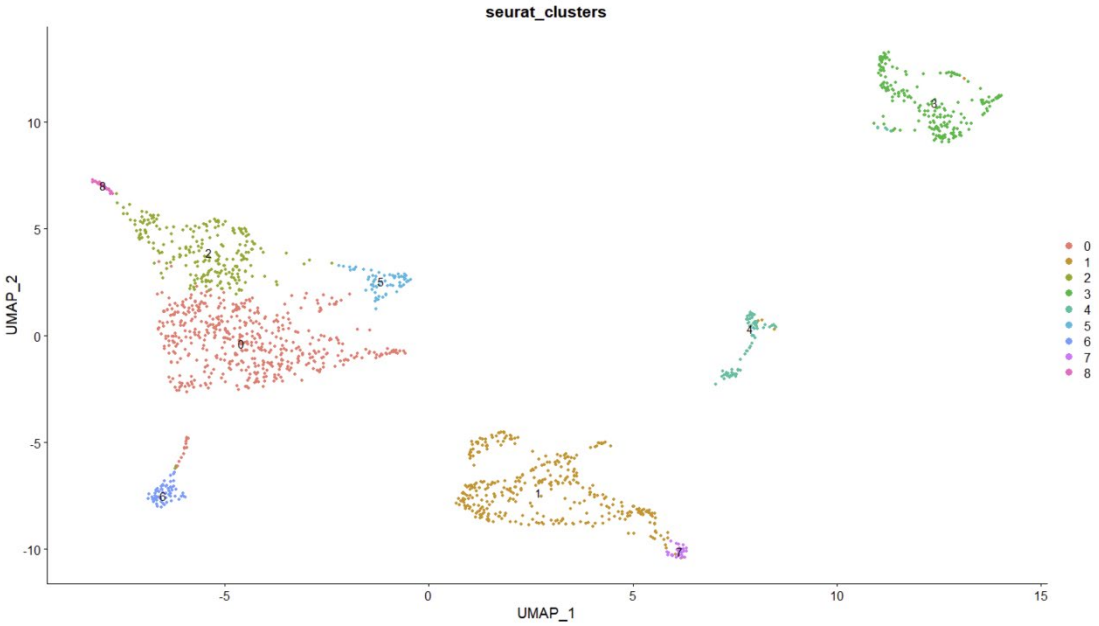


Figure 1: Identification of Nine Unique Cell Clusters

The dimensional reduction plot is created with a resolution of 0.2 and the UMAP nonlinear dimensionality reduction. This feature plot illustrates nine distinct clusters, showcasing their transcriptomic landscape visually.

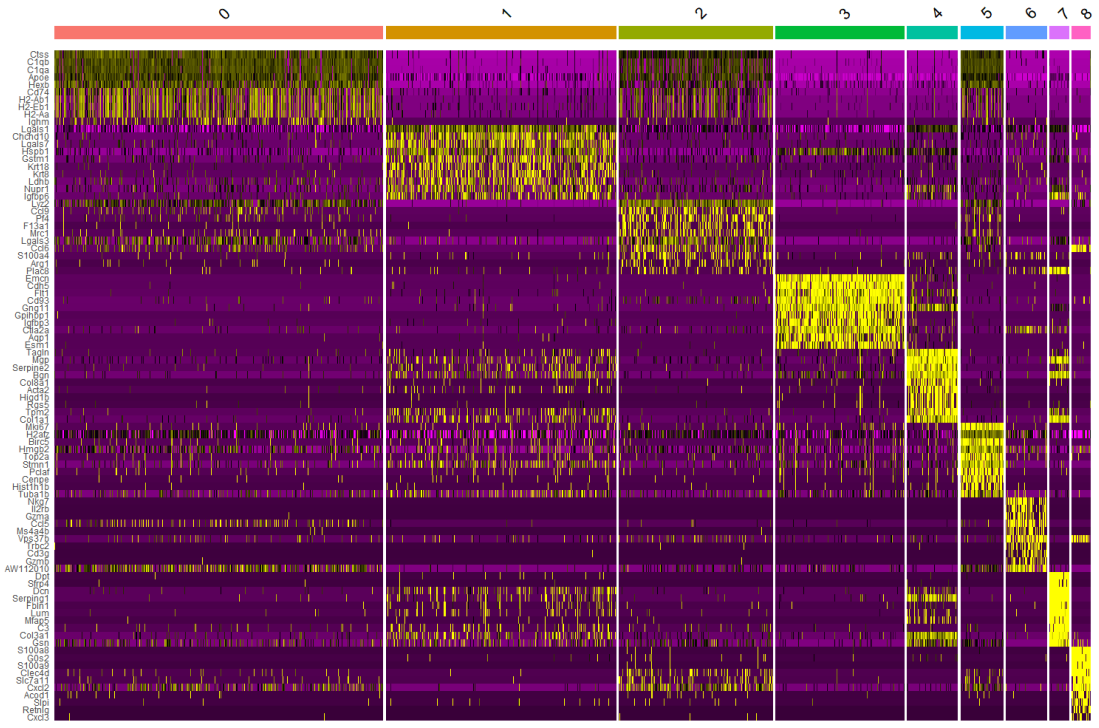


Figure 2: Heatmap of Top Ten Genes in Each Cluster

A heatmap displaying the top ten genes from each cluster, where bright yellow indicates high expression, black indicates average expression, dark purple represents low expression, and bright purple demonstrates extremely low expression. The x-axis numbers denote the order of the clusters. Genes with high expression values specific to one cluster were identified as good biomarkers.

Cellular Composition of the Tumor Microenvironment

Through analysis with the Seurat package in R, we identified nine distinct cell types in the tumor tissue, including (i) infiltrating immune cells such as macrophages, natural killer (NK) cells, and neutrophils, (ii) liver cells such as Kupffer cells and hepatocytes, (iii) endothelial and associated cells such as endothelial cells and pericytes, (iv) connective tissue cells such as fibroblasts, and (v) possible actively dividing tumor cells identified as proliferating cells. The most prevalent cell types were the liver cells, namely Kupffer cells (32.3%) and hepatocytes (22.7%), followed by macrophages (15.1%), endothelial cells (12.7%), pericytes (5%), proliferating cells (4.2%), NK cells (4%), fibroblasts (1.9%), and neutrophils (1.9%) (see Fig. 3). A cell frequency bar chart is plotted, facilitating direct comparison of the relative percentages of each cluster of cells (Fig. S21). The identification of diverse cell types in the sample reveals the tumor's heterogeneity.

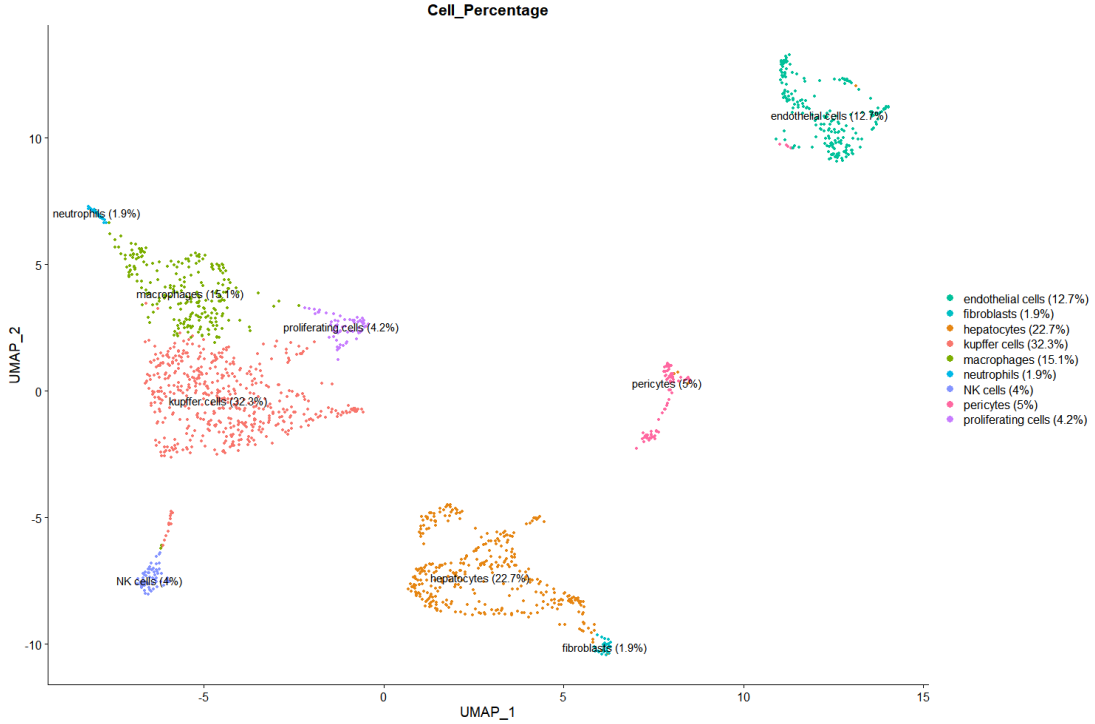


Figure 3: Diverse Cell Types Identified in The HCC Tumor Sample and Their Relative Percentages

The dimensionality reduction plot displays various cell types identified in our HCC sample, including Kupffer cells, hepatocytes, macrophages, proliferating cells, endothelial cells, NK cells, pericytes, fibroblasts, and neutrophils. The spatial distribution of these clusters illustrates similarities and differences in their gene expression profiles, with closer proximity suggesting more similar expression profiles. The relative percentages of each cluster are labeled, with Kupffer cells being the most abundant (32.3%) and neutrophils and fibroblasts both being the least (1.9%). The abundance of Kupffer cells in the tumor tissue suggests their dominant role in the HCC tumor microenvironment, further analyzed and explained in the discussion section.

Refined Identification of Subpopulations of Macrophages: Kupffer Cells, Proliferating Macrophages, and Infiltrating Macrophages

The overall macrophage cluster contains distinct macrophage subpopulations based on differential expression of biomarkers. Here, we finely identified the macrophage subpopulations.

Kupffer cells, the liver-resident macrophages, are characterized by co-expression of C1q and ApoE, high expression levels of CD68, CD72, CSF1R, Adgre1 (F4/80), Ctss, and Hexb, and relatively low expression of Lyz2 compared to the macrophage cluster (refer to Fig. 4) (Guillot & Tacke, 2019; T. Hu et al., 2019). Although typical biomarkers for Kupffer cells in normal liver tissue, Clec4f, Timd4, Marco, and Cd163, exhibit low expression levels and are rarely expressed among all clusters, Kupffer cells are characterized by high expression of Cd72, a surface protein upregulated in Kupffer cells, and C1q, a crucial protein in the classical complement cascade, along with its binding partner, ApoE,

both expressed at high levels in Kupffer cells (CD72 Protein Expression Summary - The Human Protein Atlas, n.d.; Goitsuka et al., 2001; Habenicht et al., 2022; Malaguarnera et al., 2006). Additionally, the cluster identified as Kupffer cells demonstrates a very distinctive gene expression profile from that of macrophages (refer to Fig. 5). The absence of distinctive Clec4f expression may be due to several factors, including (i) Kupffer cells in a quiescent state when sequenced, (ii) dropout events during scRNA-sequencing, (iii) heterogeneity within the Kupffer cell population, (iv) immune suppression, and altered expression in the tumor microenvironment. Macrophages, accounting for 15.1% of the total cell population, are identified based on their Lyz2, Ccl9, and Ccl6 expression.

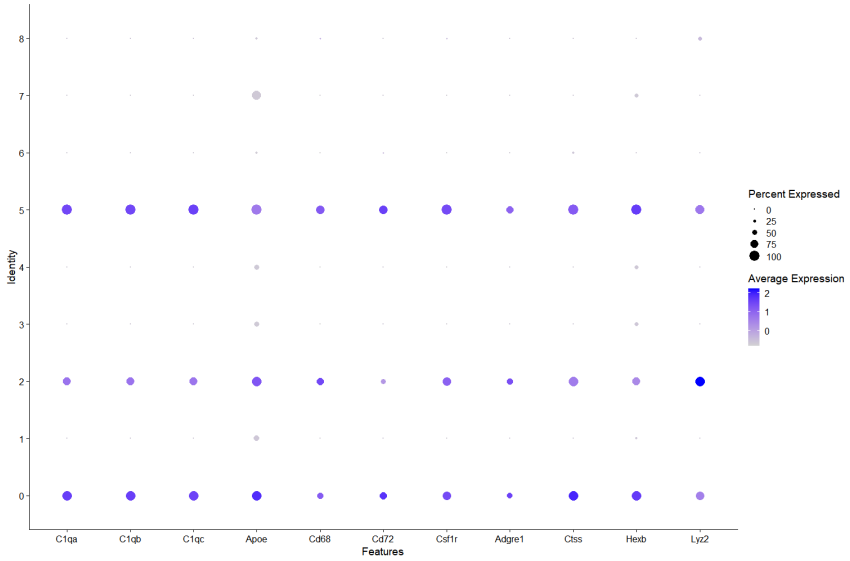


Figure 4: Identification of Kupffer Cells Through Marker Genes and Comparison between The Gene Expression Profiles of The Three Macrophage Subpopulations

Kupffer cells are identified by comparing the expression values of key marker genes with those of typical macrophages. Cluster 0 represents Kupffer cells, while clusters 2 and 5 represent infiltrating macrophages and proliferating macrophages (discussed in subsequent sections), respectively. The expression profile of Kupffer cells demonstrates characteristics of high co-expression of C1q and ApoE, high expression levels of CD68, CD72, CSF1R, Adgre1 (F4/80), Ctss, and Hexb, and relatively low expression of Lyz2 compared to the macrophage cluster. Proliferating macrophages show typical macrophage markers such as Csfr1 and Lyz2. The expression profile of proliferating macrophages is more similar to that of Kupffer cells, as their expression levels of several genes such as Hexb and Lyz2 are almost identical and differ from that of infiltrating macrophages.

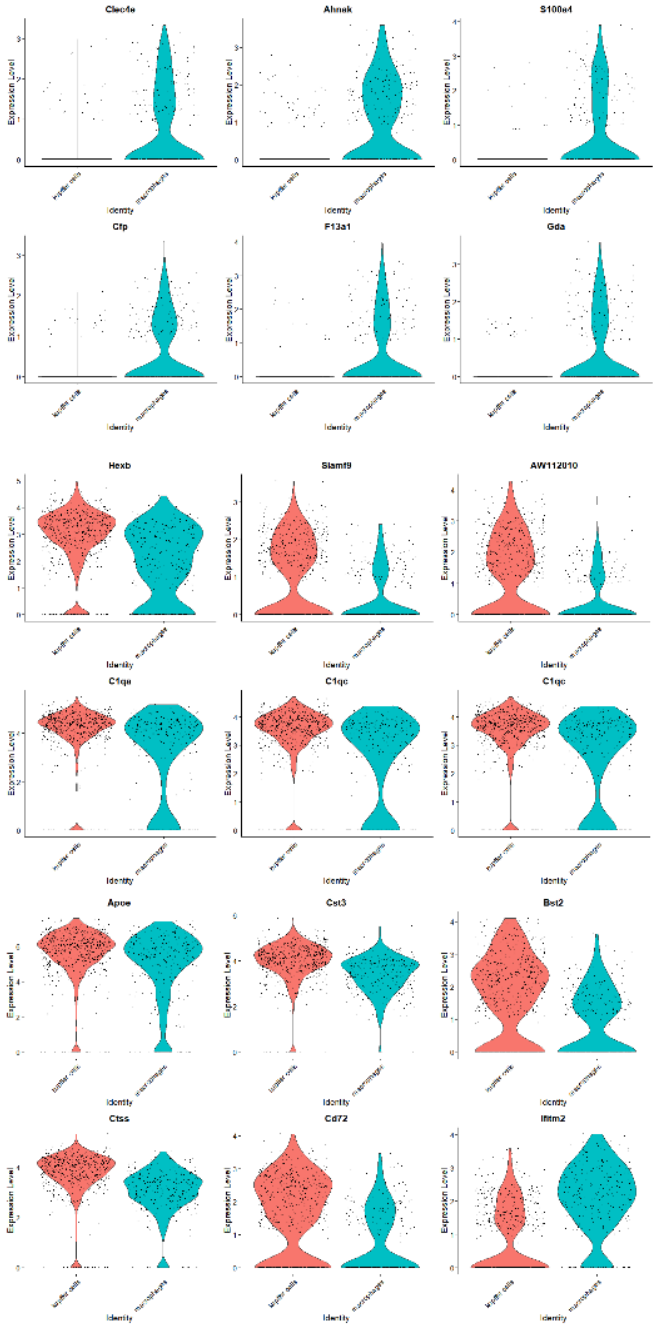


Figure 5: The Distinct Expression Profiles of Kupffer Cells and Macrophages
A violin plot showing the different gene expression profiles between Kupffer cells and macrophages.

Interestingly, a smaller subset (4.2%) of proliferating cells marked by high expression levels of Mki67, Cenpe, and Birc5 are identified as macrophages after careful inspection of their expression of macrophage biomarkers Cx3cr1, Lyz2, and Csf1r (refer to Fig. 6). The possible roles of each subpopulation of macrophages are discussed in the subsequent section.

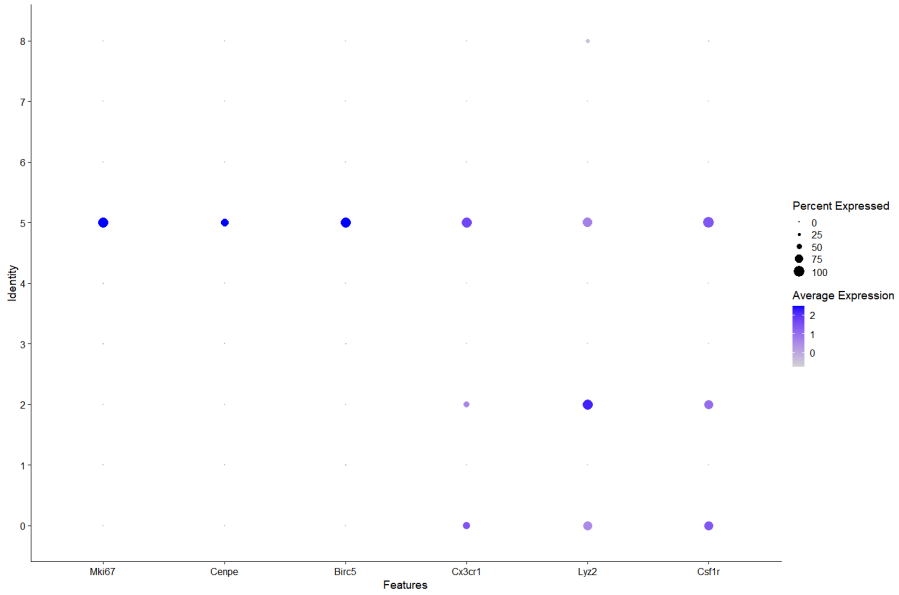


Figure 6: The Key Biomarkers of The Proliferating Macrophages

The cluster of proliferating cells expresses distinctively high values of genes related to cell proliferation (Mki67, Cenpe, and Birc5) and biomarkers of macrophages (Cx3cr1, Lyz2, and Csf1r). Thus, it is identified as a cluster of proliferating macrophages.

## 4. Discussion

The identification of distinct cell populations within HCC tumors provides valuable insights into the underlying mechanisms driving tumor progression and metastasis. By elucidating the crosstalk between tumor cells and the tumor microenvironment, our study lays the groundwork for the development of novel therapeutic strategies targeting specific cell populations. Moreover, the characterization of immune cell subsets within HCC tumors may inform the design of immunotherapy approaches aimed at enhancing anti-tumor immune responses. Overall, our findings contribute to a deeper understanding of HCC biology and have implications for the development of personalized treatment strategies for HCC patients.

## 5. Limitations and Future Prospects

While this study provides valuable insights into the cellular dynamics of the HCC tumor microenvironment, it is essential to acknowledge its inherent limitations. To begin with, the DEN mouse model does not reflect HCC tumors in humans with high accuracy as the dissimilarity between the DEN model and human HCC arises mainly from DEN mouse carrying Braf V637E mutation that is rare in humans (Dow et al., 2018). Future studies may work with the STAM mouse model that is shown to share higher molecular similarity with human HCC, or directly study human HCC samples.

It is worth noting that several types of cells common in the HCC tumor microenvironment, namely T cells, B cells, and hepatic stellate cells, are not identified in our data. This could be due to (i) technical limitations that contribute to dropout events and low sensitivity to certain biomarkers in the sequencing process, (ii) dissociation bias and low cell viability resulted from the exclusion of dead cells and difficulties in capturing transcriptomes of dead cells during sample preparation, (iii) clustering and annotation challenges in bioinformatic analysis. It is also noticeable that the hepatocytes present in the data do not or rarely express some of the key biomarkers of HCC such as AFP, Gpc3, and Axin1. The exclusion of tumor cells to avoid clumps and ensure the accuracy of scRNA-seq during sample preparation would be the likely reason for this. As the inclusion and preservation of all cell types in a tumor tissue would provide a more comprehensive panorama of the interactions between cancerous cells and stromal cells, subsequent studies should try to sequence

without leaving out any possible cell types by optimizing tissue dissociation through combining enzymatic methods and mechanical methods or preferably, employ spatial transcriptomics to avoid the need and possibility of filtering out any cell types as well as to provide a deeper understanding of the topological distribution of distinct cell types in HCC tumor.

Seurat, a popular R package, is used for the bioinformatics analysis of our single-cell RNA sequencing data. Although Seurat is renowned for its robustness in clustering, visualization, and differential expression analysis of single-cell data, it's imperative to acknowledge its inherent limitations. The sole reliance on Seurat means that only the algorithms, methodologies, and parameters incorporated in the package are utilized, which may or may not be optimal for this specific dataset. Considering our study's exclusive reliance on the Seurat package for scRNA-seq data processing, future investigators can benefit from heterogeneous analytical toolkits. A wide range of analytical methods may be used on a dataset all at once so that the optimal approach of data processing can be determined and parallel comparison can be carried out to verify the findings and eliminate biases.

## 6. Conclusion

In conclusion, our study provides a comprehensive analysis of the cellular landscape and interactions within HCC tumors using single-cell RNA sequencing. By profiling the transcriptional signatures of diverse cell populations, we have uncovered novel insights into the complexity of HCC biology. Our findings pave the way for future studies aimed at elucidating the mechanisms driving tumor progression and identifying new therapeutic targets for HCC treatment.

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