

# Single-Cell RNA Sequencing: A Deep Dive into The Cellular Landscape and Interactions in Hepatocellular Carcinoma

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**Abstract.** Hepatocellular carcinoma (HCC) is a leading cause of cancer-related deaths worldwide, with its progression highly influenced by the cellular interplay within the tumor microenvironment that is underexplored. Aiming to bridge this gap, our study utilizes single-cell RNA sequencing (scRNA-seq) to examine the cellular heterogeneity of HCC and investigate the roles of distinct cell populations. scRNA-seq was performed on eight DEN mice HCC samples, followed by bioinformatic analysis with Seurat package. Nine distinct cell populations were identified, with three unique macrophage populations suggestive of their role as tumor-associated macrophages (TAMs). The detected endothelial cells and pericytes hint at ongoing neoangiogenesis, with implications that endothelial cells might function as tumor-associated endothelial cells (TECs) and pericytes as carcinoma-associated fibroblasts (CAFs). Our findings provide insights into the potential roles of various cell populations in the HCC tumor microenvironment, which paves the way for developing novel therapies. These postulations, while offering a deeper understanding of HCC's cellular landscape, necessitate experimental validation for confirmation.

**Keywords:** hepatocellular carcinoma (HCC), single-cell RNA sequencing (scRNA-seq), tumor microenvironment, intratumor heterogeneity, cellular composition.

## 1. Introduction

Hepatocellular carcinoma (HCC), ranking fifth among all cancers, is the second leading contributor to cancer-related mortality (Wen et al., 2022). Even with modern technology, therapeutic response and prognosis of HCC are still poor due to therapeutic resistance, variable drug sensitivity in individual patients, and metastasis, all of which arise from the heterogeneity of cancer.

Besides the widely-studied intertumor heterogeneity, recent studies have revealed that neoplastic subclones of considerable genotypic and phenotypic variations are found within the same tumor (intratumor heterogeneity), which accounts for treatment failure, metastasis, and cancer evolution (Ramón Y Cajal et al., 2020). The tumor microenvironment, a significant contributing factor to intratumor heterogeneity and tumorigenesis, encompasses primarily the proliferating tumor cells, along with the tumor stroma, vascular networks facilitating nutrient supply to neoplastic cells, infiltrating immune inflammatory cells, microbiota, and a broad spectrum of associated tissue cells (Whiteside, 2008). The wide variety of cells interacts within the tumor microenvironment, generating an incredibly complex signaling network that shapes tumor pathogenesis (Hanahan & Weinberg, 2011). Therefore, understanding intratumor heterogeneity and the cellular composition of the tumor microenvironment plays a crucial role in formulating novel therapies and realizing the ambition of precision medicine.

As the intricate cellular interplay within the HCC tumor microenvironment remains a challenge to decipher, this study aims to bridge this knowledge gap by uncovering distinct cell populations and shedding light on their potential roles and interactions in the tumor landscape, paving the way for the development of novel therapies.

## 2. Methods

### 2.1 Animals and DEN Treatment

Chemical HCC was induced by the combination of DEN (25 mg/kg i.p.) given at week 2 postpartum, followed by weekly injections of CCl<sub>4</sub> (0.5 mL/kg i.p., dissolved in corn oil). Mice were sacrificed at the age of 5 months for further studies. NASH–HCC was induced in male mice by a single subcutaneous injection of 200 µg STZ 2 days after birth and feeding high-fat diet (HFD) after 4 weeks of age. Mice were sacrificed at the age of 20 weeks. All animal experimental protocols were approved by the Animal Ethics Committee of The Affiliated Drum Tower Hospital of Nanjing University Medical School and the Animal Ethics Committee of Nanjing Medical University.

### 2.2 Single-Cell RNA Sequencing

Single-cell RNA sequencing was performed using the Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (10x Genomics). Tumor samples were dissociated, washed, and resuspended into single-cell suspensions following the manufacturer's protocol (Cell Preparation for Single Cell Protocols - Official 10x Genomics Support, n.d.). The cell suspension was loaded onto a Chromium Single Cell A Chip along with the reverse transcription master mix and Single Cell 3' Gel Beads in order to generate Gel Beads in Emulsion (GEMs).

### 2.3 RNA Sequencing and Bioinformatics Analysis

Sequencing was performed on an Illumina Nextseq 2000 according to the sequencing protocol provided by 10x Genomics. The resulting data were processed using the Cell Ranger Single Cell Software Suite (10x Genomics) to perform sample demultiplexing, barcode processing, and single-cell 3' gene counting. Further downstream analysis was performed using the Seurat R package. With the help of a histogram showing the number of nFeature\_RNA and its frequency, quality control parameters were set to between 200 and 7000 nFeature\_RNA and less than 5% mitochondrial gene. Principle component analysis (PCA) was performed after normalization and scaling of the top 2000 highly variable genes. Based on the principle component (PC) elbow plot, the first 13 PCs were considered for the subsequent analysis. Two-dimensional data visualization was achieved by performing a nonlinear dimensionality reduction with a uniform manifold approximation and projection (UMAP) algorithm and a resolution of 0.2. A total of 9 clusters were generated, with the spatial relationship between single-cell clusters denoting the similarities/differences between their transcriptomic landscape. Up-regulated marker genes were identified by the FindAllMarkers function, with a log fold change threshold of 0.25, minimum percentage of 0.2, and a logistic regression test only finding the positive markers. A heatmap showcasing the top 10 biomarker genes across each cluster was plotted. Cluster identities were labeled manually by using reference datasets such as Mouse Cell Atlas (MCA), PanglaoDB, National Center for Biotechnology Information, and other published research (Choose Gene Expression Markers | PanglaoDB, n.d.; Genes & Expression - Site Guide - NCBI, n.d.; MCA | Mouse Cell Atlas, n.d.). Specifically, the top ten genes from each cluster were identified and served as key markers. The primary identification work is carried out with MCA, with the marker genes input into the MCA adult liver gallery and searched for the corresponding cell types. The marker genes not presented in MCA were researched otherwise using PanglaoDB, etc. Certain markers that were poorly recognized by the FindAllMarkers function were manually identified and validated through dot plots and feature plots. Cell type-specific marker genes were identified for each of the nine cell types based on literature review and validation by dot plots and dim plots. All feature plots in this study were plotted using a minimum cutoff value of 0.2, unless otherwise stated.

### 3. Results

In this study, single-cell RNA sequencing (scRNA-seq) was performed on mouse liver hepatocellular carcinoma (HCC) tumor tissue to investigate the cellular composition and potential intercellular interactions within the tumor microenvironment. Nine distinct clusters of cells (Fig. 1) were identified, and the top 10 highly expressed genes of each clusters were determined using the FindAllMarker function with a logistic regression test and plotted onto a heatmap (Fig. 2).

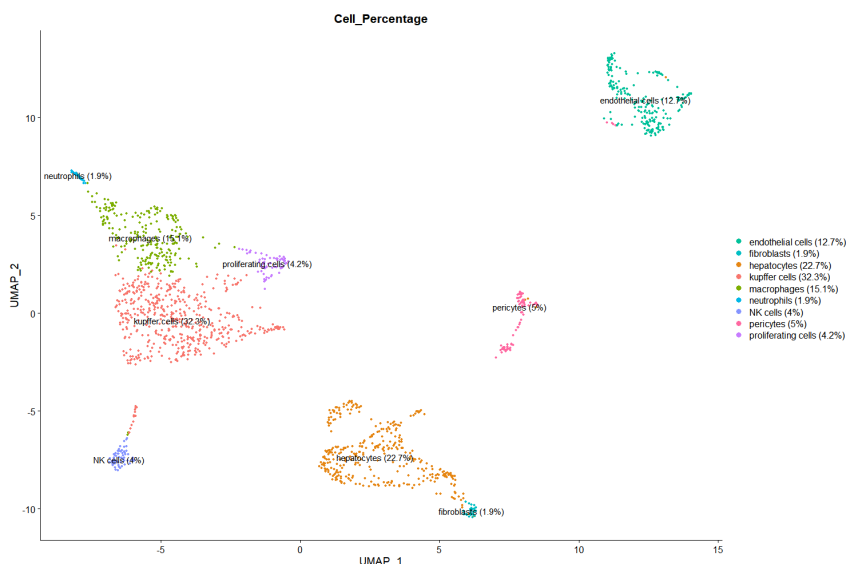


Figure 1: Diverse Cell Types Identified in The HCC Tumor Sample and Their Relative Percentages

The dimensional reduction plot is plotted with a 0.2 resolution and the UMAP nonlinear dimensionality reduction. This dimensionality reduction plot shows a wide range of cell types identified in our HCC sample, including Kupffer cells, hepatocytes, macrophages, proliferating cells, endothelial cells, NK cells, pericytes, fibroblasts, and neutrophils. The similarities and differences in their gene expression are shown in the spatial distribution of these clusters, with closer proximity suggesting more similar expression profile, and vice versa. The relative percentages of each cluster are labeled, with the Kupffer cells being the most abundant (32.3%) and the neutrophils and fibroblasts both being the least (1.9%). The large number of Kupffer cells in the tumor tissue suggests the dominant role they play in HCC tumor microenvironment, which is further analyzed and explained in the discussion section.

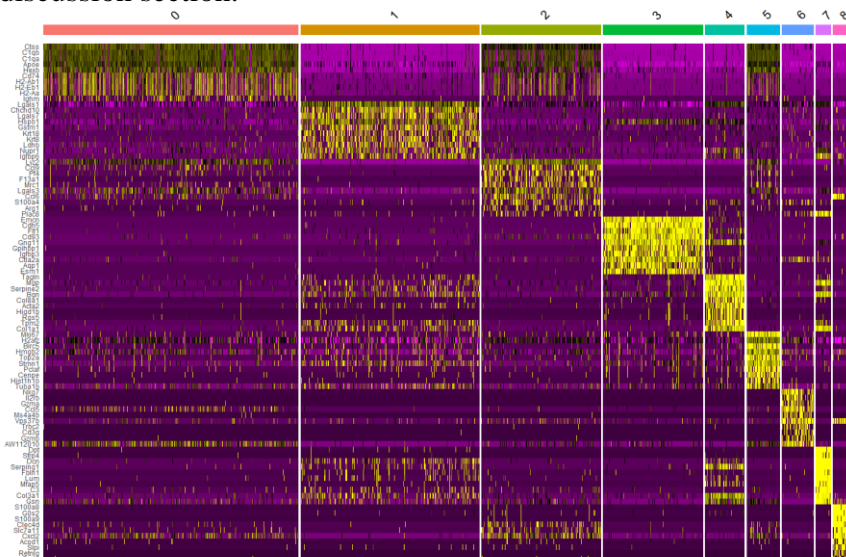


Figure 2: Heatmap of Top Ten Genes in Each Cluster

The top ten genes from each cluster were plotted on a heatmap, with the bright, yellow color indicating high expression, black indicating average expression, dark purple representing low expression, and bright purple color demonstrating extremely low expression. The numbers on the x-axis represent the order of the clusters. The genes displaying high expression values only particular to one cluster were considered good biomarkers.

### 3.1 Cellular Composition of the Tumor Microenvironment

Through analysis by Seurat package in R, nine distinct cell types present in the tumor tissue were identified, 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 abundant 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%) (Fig. 1). A cell frequency bar chart is plotted, allowing more direct comparison between the relative percentages of each cluster of cells. The diverse cell types identified in the sample revealed the heterogeneity of the tumor.

### 3.2 Refined Identification of Subpopulation of Macrophages: Kupffer Cells, Proliferating Macrophages, and Infiltrating Macrophages

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

Kupffer cells, the liver-resident macrophages, are marked by the 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 (Fig. 3) (Guillot & Tacke, 2019; T. Hu et al., 2019). Although the typical biomarkers for Kupffer cells in normal liver tissue, Clec4f, Timd4, Marco, and Cd163, have low expression levels and are rarely expressed among all clusters, the Kupffer cells are characterized by their high expression of Cd72, a surface protein that is upregulated in Kupffer cells, C1q, a crucial protein in the classical complement cascade, along with its binding partner, ApoE, both of which are expressed at a high level in Kupffer cells in particular (CD72 Protein Expression Summary - The Human Protein Atlas, n.d.; Goitsuka et al., 2001; Habenicht et al., 2022; Malaguarnera et al., 2006). Besides, the cluster identified as Kupffer cells has a very distinctive gene expression profile from that of macrophages (Fig. 4). 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) and immune suppression and altered expression in tumor microenvironment. The macrophages, accounting for 15.1% of the total cell population, are identified based on their Lyz2, Ccl9, and Ccl6 expression.

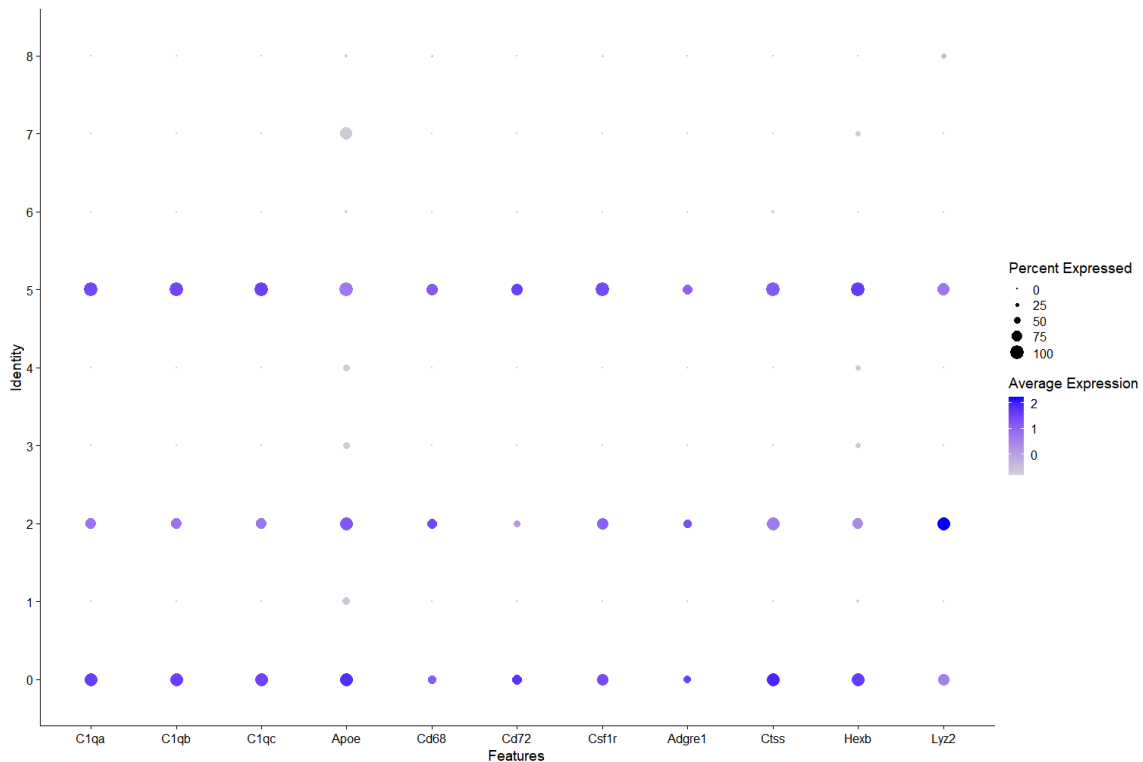


Figure 3: Identification of Kupffer Cells Through Marker Genes and Comparison between The Gene Expression Profiles of The Three Macrophage Subpopulations

The Kupffer cells are identified by comparing the expression values of key marker genes with that of typical macrophages. Cluster 0 is the Kupffer cells, while cluster 2 and 5 are infiltrating macrophages and proliferating macrophages (which are talked about in the subsequent sections), respectively. The expression profile of Kupffer cells demonstrates the characteristics of high co-expression of C1q and ApoE, high expression level of CD68, CD72, CSF1R, Adgre1 (F4/80), Ctss, and Hexb, and relatively low expression of Lyz2 compared to the macrophage cluster. The proliferating macrophages show typical macrophage markers such as Csf1r and Lyz2. The expression profile of the 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.

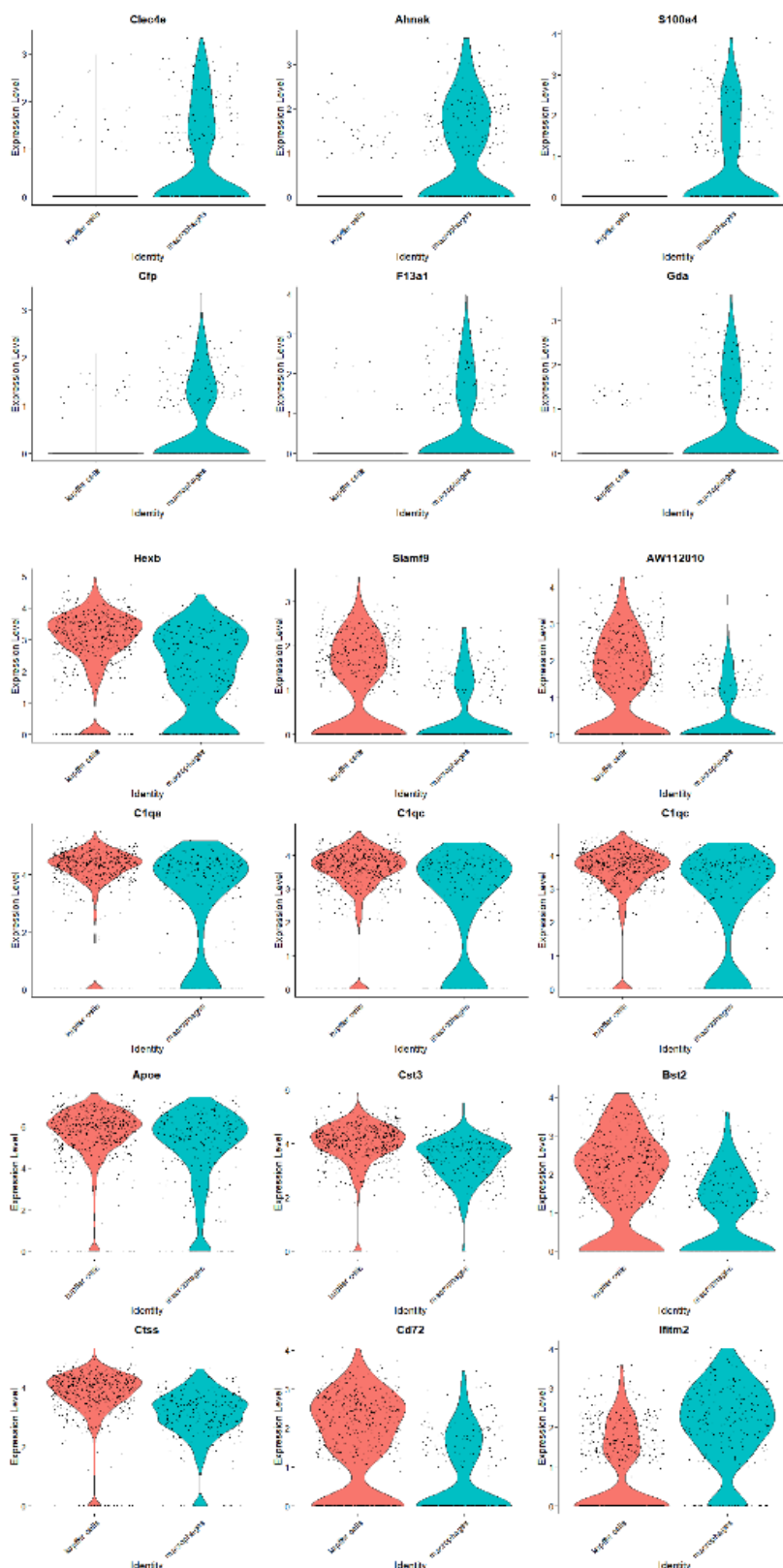


Figure 4: The Distinct Expression Profiles of Kupffer Cells and Macrophages

Violin plot showing the different gene expression profile 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 (Fig. 5). The possible roles of each subpopulation of macrophages are discussed in the subsequent section.

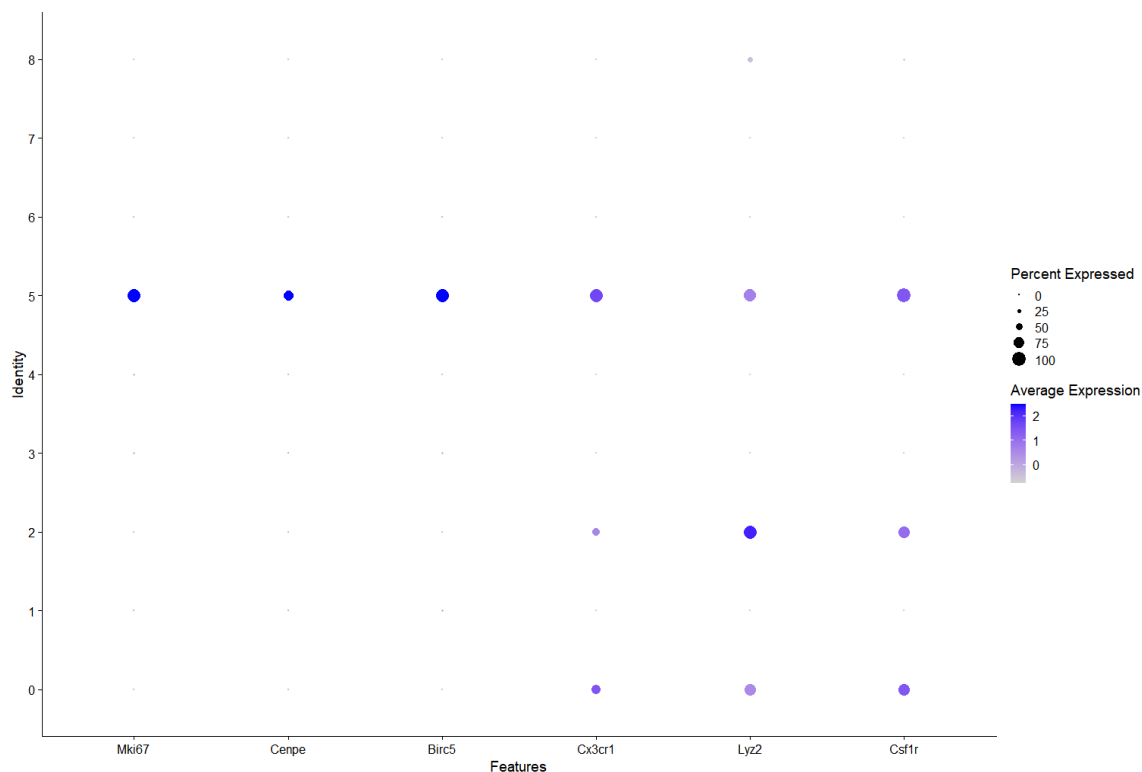


Figure 5: The Key Biomarkers of The Proliferating Macrophages

The cluster of proliferating cells express 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

### 4.1 Macrophages in the HCC Landscape: Potential Role as Tumor-Associated Macrophages (TAMs) and Beyond

In our study, an unusually high percentage of macrophages and distinct macrophage subpopulations in the tumor microenvironment are identified, which indicates their special roles in the HCC tumor microenvironment.

To begin with, the potential reasons accounting for the abundance of macrophages and the possible relationship between the three macrophage subpopulations are discussed. The Kupffer cells account for the majority of the cells found in the HCC tumor tissue (32.3%), which is more than twice the percentage they usually make up of the total liver cell population (15%) (Kolios et al., 2006). The high Kupffer cell percentage suggests chronic inflammation in the tumor microenvironment and possible tumor-associated macrophage (TAM) recruitment. These Kupffer cells are very likely to originate from monocytic macrophages infiltrating the tumor tissue due to the high expression level of Cd68, an indicator of monocytic macrophages, in the infiltrating macrophages and the prevalence of canonical macrophage differentiation Kupffer cell replenishment scheme under pathological conditions (Ding et al., 2009; IJMS | Free Full-Text | An Eye on Kupffer Cells: Development, Phenotype and the Macrophage Niche, n.d.). In addition, the proliferating macrophages potentially demonstrate the process of monocyte-derived Kupffer cell replenishment as they show expression profiles that are similar to that of both infiltrating macrophages and Kupffer cells but with higher similarity to that of Kupffer cells, showing possible differentiation that allows them to adapt to their niche in the HCC tumor microenvironment (Fig. 3). Noticeably, the proliferating macrophages show upregulation in Ccl2 and Cxcl10, which are chemokines responsible for macrophage recruitment and T-cell recruitment, respectively. In particular, Ccl2 is usually secreted by hepatocytes at the early

stage of HCC, and a high Ccl2 level is associated with poor prognosis as they attract pro-tumoral TAMs (Capece et al., 2012). However, in this case, the macrophage population, especially the proliferating macrophages, express high levels of Ccl2, indicating a positive feedback loop that leads to the cumulation of TAMs, which may contribute to the quick progression and metastasis of HCC. (Fig. 8)

To further explore the role of these macrophages in HCC cancer progression, more detailed gene expression profiles of the macrophages are investigated. Through close examination of biomarkers, the overall macrophage population, including the Kupffer cells, infiltrating macrophages, and proliferating macrophages, displays strong M2 traits by expressing the biomarker genes for M2 macrophages, Cd14 and Cd68, at a high level (Hepatic Macrophages in Homeostasis and Liver Diseases: From Pathogenesis to Novel Therapeutic Strategies | Cellular & Molecular Immunology, n.d.). In particular, the infiltrating macrophage subpopulation demonstrates even more prominent M2 characteristics as the expression levels of other M2 markers, Arg1, Il-10, and Chil3, are higher in the macrophage cluster than the rest (Fig. 8) (Liu et al., 2013; Oxytocin System Alleviates Intestinal Inflammation by Regulating Macrophages Polarization in Experimental Colitis | Clinical Science | Portland Press, n.d.). The macrophages displaying M2 polarization have an anti-inflammatory and pro-repair role. However, in the context of HCC, M2-like macrophages are TAMs that usually promote tumor growth and metastasis. The identification of upregulated expression of ferritin light chain 1 (Ftl1) (Fig. 6 and Fig. 7), vascular endothelial growth factor a (Vegfa), cathepsin S (Ctss), cathepsin B (Ctss), and matrix metalloproteinases 14 (Fig. 8) verify that these M2-like macrophages are pro-tumoral TAMs since growth factors such as Vegfa, Tgfb1, and Pdgfb enhance tumor cell proliferation, while MMPs result in remodeling of the extracellular matrix, which promotes metastasis (Z.-W. Hu et al., 2021; Tang, 2013; Targeting Tumor-associated Macrophages in the Tumor Microenvironment (Review), n.d.).

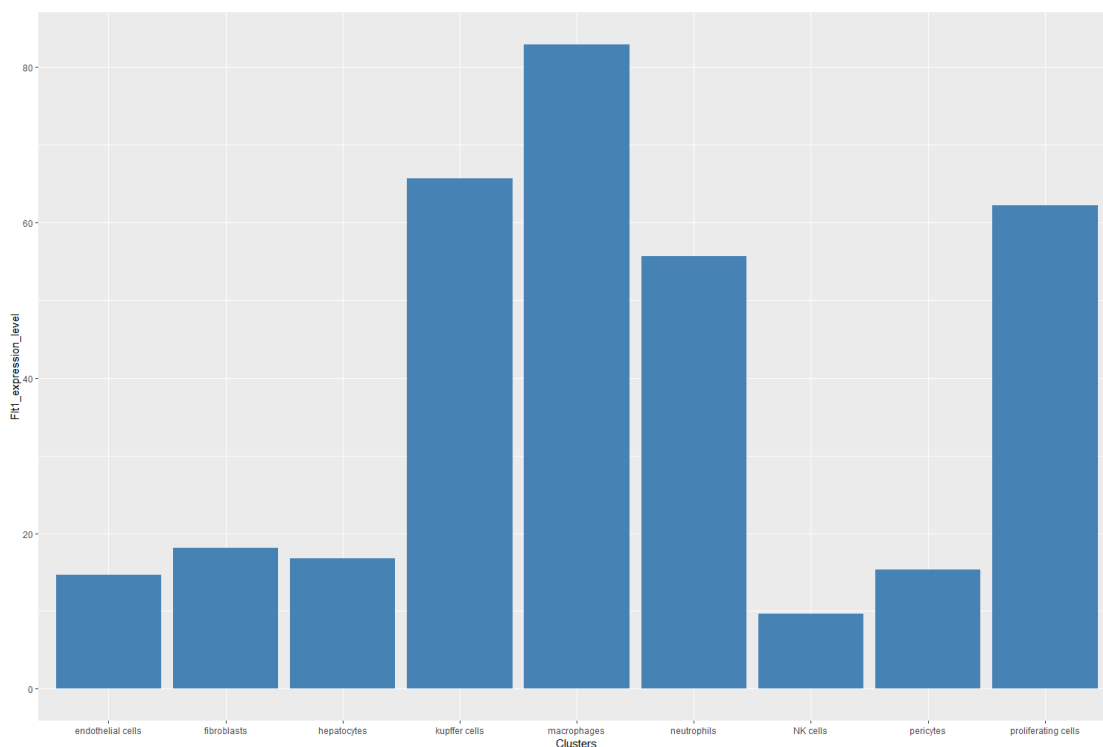


Figure 6: The Comparison of Ftl1 Expression Across All Clusters

Ftl1 has particularly high expressions among the immune cells, especially in the macrophages, Kupffer cells, and proliferating cells (proliferating macrophages).

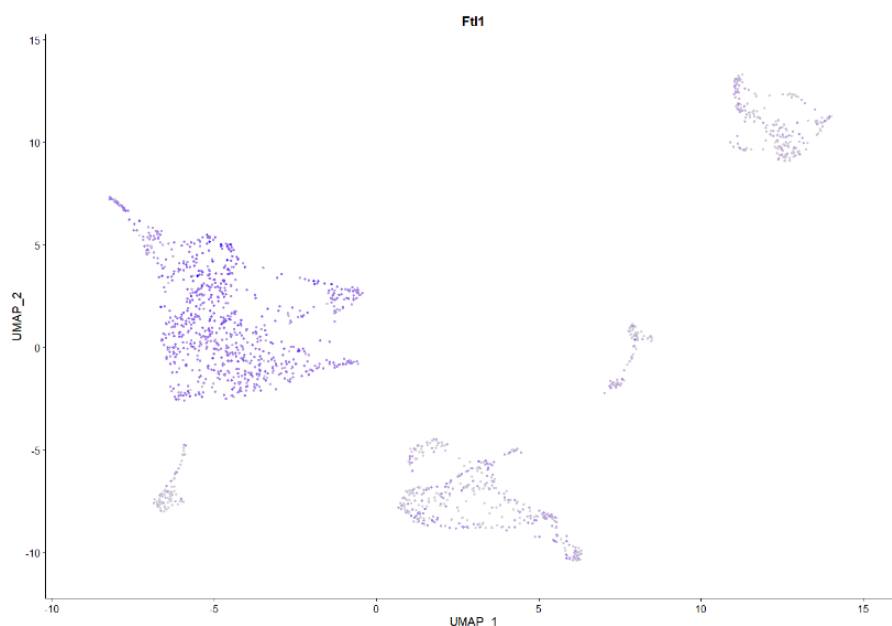


Figure 7: Ftl1 Expression in The Macrophages

In the feature plot, cells with a Ftl1 expression above a cutoff of 2.2 were highlighted to emphasize clusters with particularly high expression. The overall macrophage clusters show extremely high Ftl1 expression, suggesting their pro-tumoral TAM nature.

In summary, the relative abundance of Kupffer cells and their M2 phenotypes suggest their dominant role as tumor-promoting TAMs in the HCC environment. The presence of infiltrating macrophages and their proliferative counterparts underscores the dynamic interplay of immune cells in HCC, with the high expression of Ccl2 by the macrophages potentially establishing a positive feedback loop of TAM recruitment. Whether the proliferating macrophages derive from Kupffer cell self-perpetuation or monocytic macrophage differentiation is still unclear, and further studies investigating the origin of these proliferating macrophages may elucidate the functions of these cells and provide a panorama of the macrophage landscape in HCC tumor microenvironment. The lack of widely studied Kupffer cell biomarkers in our sample also poses great challenges to efficiently identify Kupffer cells. Immunohistochemistry with Kupffer cell-specific antibodies could be used to verify the presence of Kupffer cells. Besides, as Kupffer cells have a unique distribution in the liver tissue, with the majority found in the periportal area of the lobule (43%), 28% exist in the midzonal area and 29% in the central area, spatial transcriptomics and spatial enhanced resolution omics sequencing may be carried out in further studies to investigate Kupffer cell identities with higher accuracies and cell lineage tracing may be performed to visualize the infiltration of monocytic macrophages and movements of distinct cell types during tumor progression (Bouwens et al., 1986). Our finding of Ccl2 being highly expressed by macrophages and possibly leading to a positive feedback loop of TAM recruitment suggests that Ccr2 blockade may be a valid therapeutic measure to tune down TAM recruitment so that a more antitumoral immune environment can be established (Guillot & Tacke, 2019). Since M1 Kupffer cells are shown to display antitumoral activities by phagocytosing tumor cells, producing nitric oxide, and inducing NK-cell cytotoxicity, the repolarization of Kupffer cells into their pro-inflammatory, antitumoral phenotype has been a hopeful idea (Kolios et al., 2006; Nguyen-Lefebvre & Horuzsko, 2015). Our observation of the M2-like phenotype in the TAMs further illustrates that Kupffer cell repolarization into the M1 phenotype is a promising potential therapeutic target to inhibit angiogenesis, prevent metastasis, and re-establish the antitumoral role of Kupffer cells (van der Heide et al., 2019).

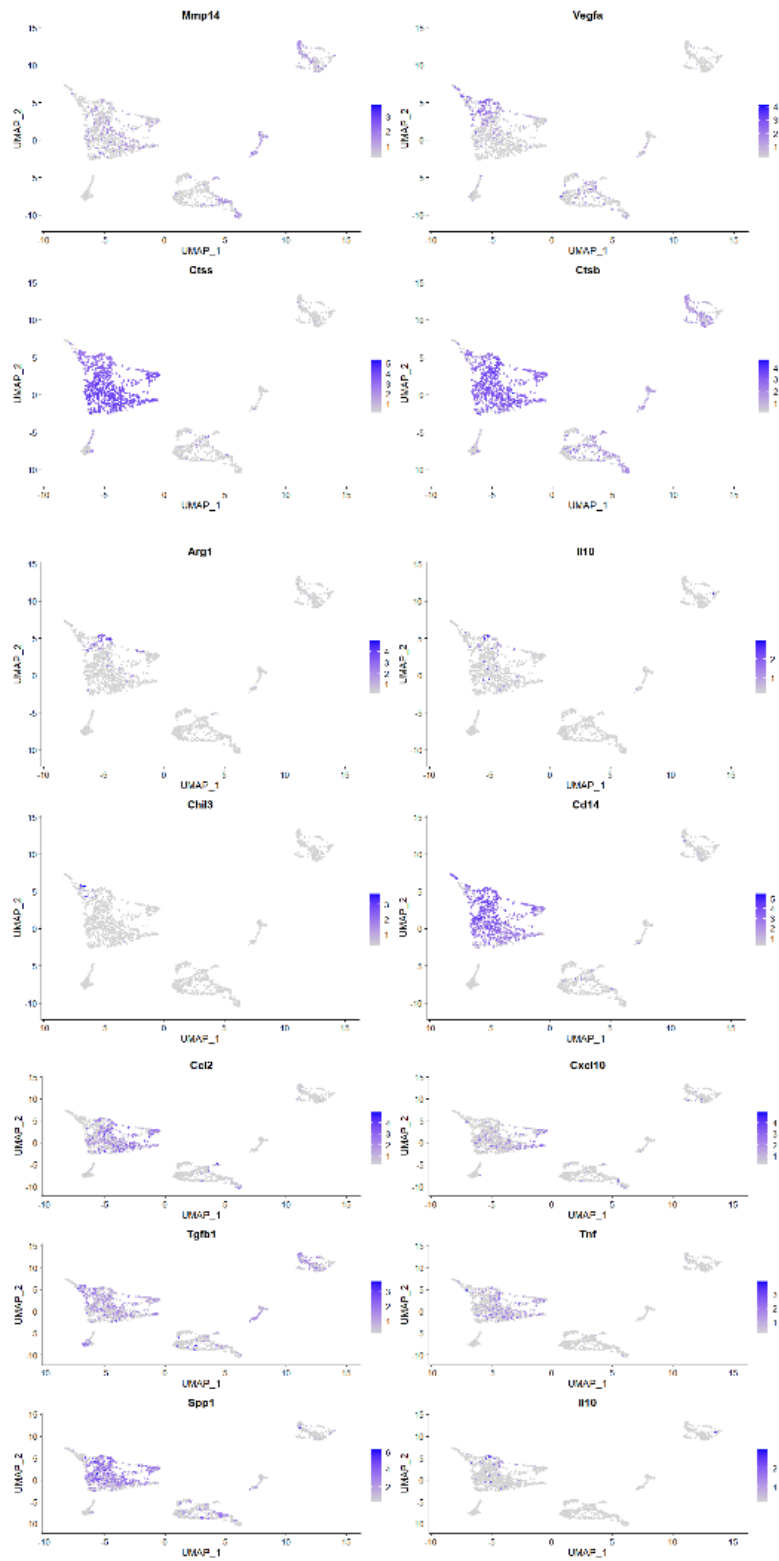


Figure 8: Identification of M2 Macrophages through M2 Biomarkers

Key M2 macrophage biomarkers are expressed in the overall macrophage cluster, with the wide spread of Cd14, Ctss, Ctsb, Ccl2, and Spp1 (coding for osteopontin) and the infiltrating macrophages expressing more M2 biomarkers than the proliferating macrophages and Kupffer cells. The overall macrophages show prominent M2 traits, while the infiltrating macrophages displaying greater M2 polarization than the Kupffer cells.

## 4.2 Endothelial Cells and Pericytes: Harbingers of Angiogenesis in the Tumor Microenvironment

In our study, prominent groups of endothelial cells (12.7%) and pericytes (5%) are identified. As endothelial cells and pericytes are heavily involved in vascular formation, the discovery of endothelial cell and pericyte populations leads us to hypothesize that angiogenesis is taking place in the HCC tumor microenvironment.

Angiogenesis is a crucial process in tumor progression as it provides nutrients for the expanding neoplastic cells, and facilitate tumor intravasation and metastasis. As the main architects of neovasculature, endothelial cells and pericytes play significant roles in neoangiogenesis. The formation of highly vascular structure in the HCC tumor microenvironment begins with HCC tumor cells activating inactive and dormant endothelial cells by secreting pro-angiogenic factors (e.g. Vegfa, Tgfb1, Egf), which attracts the endothelial cells to the site of tumor proliferation (S.-D. Wu et al., 2012). The recruited endothelial cells proliferate to form vascular structures around the tumor cells, after which the newly formed blood vessels are stabilized by pericytes (Current Strategies for the Treatment of Hepatocellular Carcinoma by Mo | IJN, n.d.). In our sample, the high expression of the biomarker genes, Eng (coding for endoglin, also known as Cd105), Dll4, Mcam (also known as Cd146), and Robo4 possibly illustrates that the endothelial cells in our sample are likely to be tumor-associated endothelial cells (TECs) (Fig. 9), which differ from normal endothelial cells by the phenotypes of leaky walls, genetic instability, increased vessel permeability, further promoting intravasation and metastasis (Current Strategies for the Treatment of Hepatocellular Carcinoma by Mo | IJN, n.d.; Dll4-Notch Signaling in Regulation of Tumor Angiogenesis | SpringerLink, n.d.; De Sanctis et al., 2018; Novikova et al., 2017; Tahmasebi Birgani & Carloni, 2017; Yang et al., 2011; Yoshikawa et al., 2008). Besides, several pro-angiogenic factors are detected, suggesting active endothelial cell recruitment and proliferation (Fig. 10). The pro-angiogenic factor Vegfa is highly expressed in macrophages that are highly likely to be TAMs and a small number of hepatocytes, which reveals the role of TAMs in contributing to angiogenesis. The high expression of the chemoattractant for pericytes, Pdgfb, mainly concentrates in the endothelial cells, Kupffer cells, and some macrophages and proliferating cells, which suggests the stabilization of vessel networks within the tumor microenvironment by TAMs and TECs.

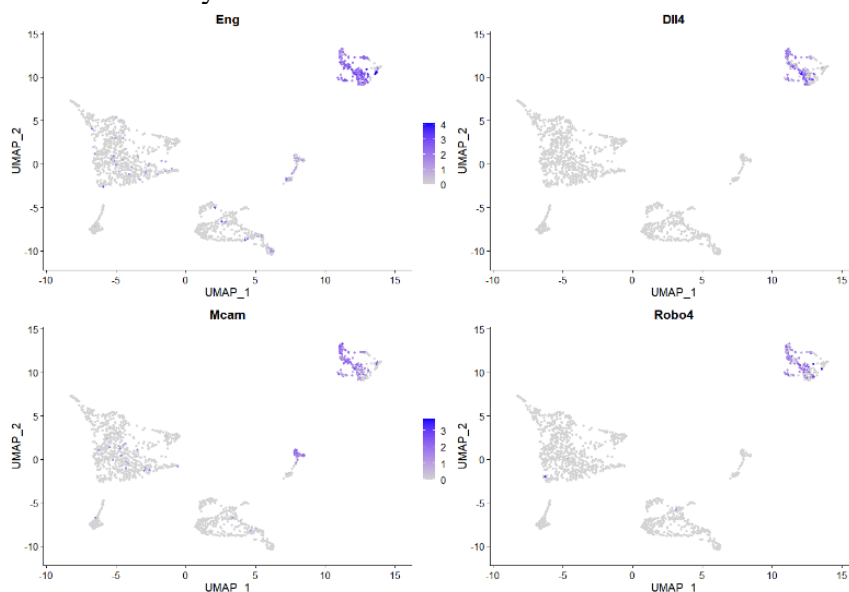


Figure 9: The Identification of TECs Based on The Biomarkers

The key biomarkers for TEC are all expressed in endothelial cells, with Eng having a particularly high expression.

Noticeably, Vegfa and Pdgfb expressed by TAMs attract endothelial cells, and the endothelial cells and pericytes are shown to express the chemokine Cxcl12 and the cytokine Tgfb1 which are involved

in immune cell recruitment and inflammatory response, potentially establishing a positive feedback loop that enhances neoangiogenesis and tumor growth (Pernot et al., 2022) (Fig. 10). Additionally, the heat shock protein Hspa12b, which is expressed in some of our endothelial cells, is demonstrated to potentially drive M2 polarization of macrophages, contributing to the TAM phenotypes (Fig. 11) (Zhou et al., 2020). This reveals TECs as a possible contributor to the transition from infiltrating immune cells into TAMs.

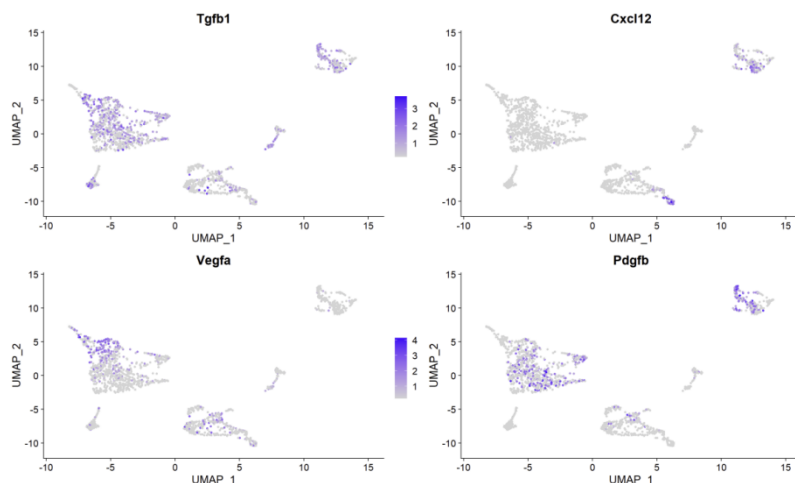


Figure 10: The Factors Contributing to The Interplay between TAMs and Endothelial Cells and Pericytes

TAM attractants Tgfb1 and Cxcl12 are expressed in endothelial cells, while Vegfa and Pdgfb that recruit endothelial cells are secreted by TAMs, possibly establishing a positive feedback loop.

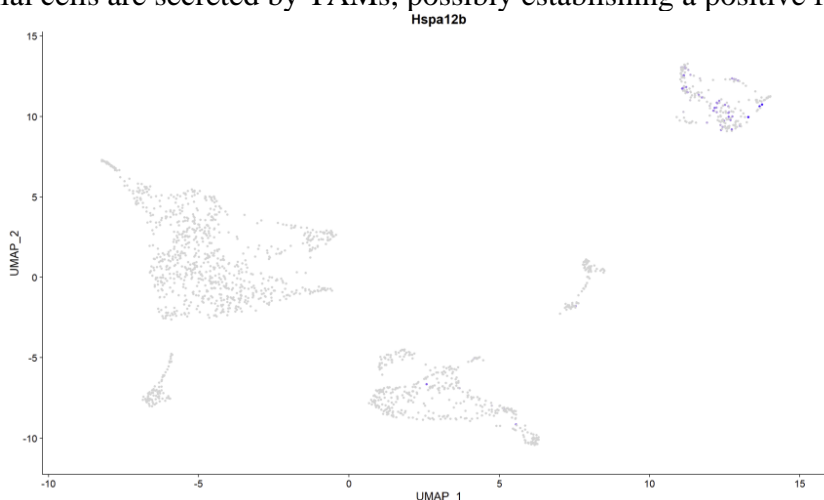


Figure 11: Hspa12b Expressed by The Endothelial Cells

The heat shock protein Hspa12b is expressed in endothelial cells, which possibly drive the M2 macrophage polarization, providing another explanation to the large number of potential TAMs in the tumor microenvironment.

Moreover, the identification of pro-angiogenic factors in the HCC tumor microenvironment and their corresponding receptors in endothelial cells and pericytes further illustrate the plausibility of slowing down HCC progression by manipulating endothelial cells and pericytes to inhibit neovascularization (Fig. 12). Namely, Vegfa and its corresponding receptor, Flt1, widely presented on the endothelial cells and some of the pericytes are detected, and Pdgfb and its corresponding receptor, Pdgfrb, presented mostly on pericytes and a few endothelial cells are also detected. Considering that a lot of currently developing treatments, including Linifanib, Vatalanib, and Sunitinib, are using enzyme inhibitors to target Vegfr and Pdgfr pathways to deter neovascularization, our study shed prospective light on those fruitful treatments (Mossenta et al., 2019).

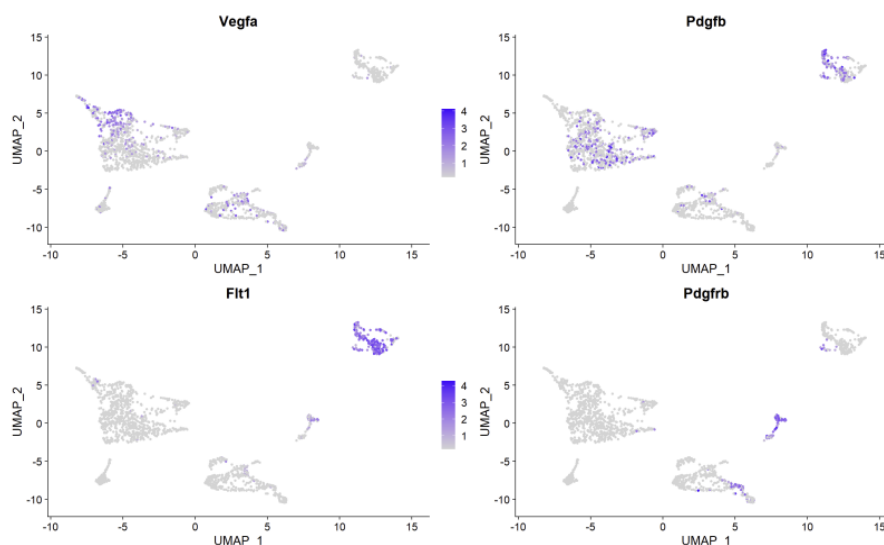


Figure 12: Pro-angiogenic Factors and Their Receptors

The pro-angiogenic factors Vegfa is highly expressed in macrophages, and its receptor Flt1 is highly expressed in endothelial cells. Meanwhile, the pro-angiogenic factor Pdgfb is expressed in macrophages, Kupffer cells, and endothelial cells, and its receptors are mainly expressed in pericytes.

To further examine the role of endothelial cells and pericytes in angiogenesis, there are several investigations future studies can perform. First, as TECs display many structural abnormalities, further studies can perform histological staining of the HCC tumor tissue and view the samples under microscopes to observe the morphological differences between TECs in HCC tissue, endothelial cells in normal tissues, and even compare it with TECs in other types of tumors. The pericytes can also be investigated under the microscope as it is hard to validate whether the pericytes have a closer relationship with the tumor than expected as the pericytes in our sample do not show high expression of VEGF and Il6 that is typical to pericytes of tumor vessels, but pericytes associated with neovascular structures show unique phenotypes of irregular shape and loose attachment (S.-D. Wu et al., 2012). Spatial transcriptomics can also be performed to visualize the topological distribution of pro-angiogenic factors secreting cells. Together with both observation under microscopes and spatial transcriptomics, the spatial examination of blood vessels in tumors can greatly enhance our understanding of how and where neoangiogenesis begins. Besides, proteomics and pathway analysis can further elucidate the pathways of angiogenesis, which may discover better targets and strategies for angiogenesis inhibition.

### 4.3 Pericytes: Possible Sources of Carcinoma-Associated Fibroblasts

In our exploration of the hepatocellular carcinoma (HCC) tumor microenvironment, we identified populations of pericytes (5%). Not only do pericytes have roles in promoting angiogenesis, but as tumor stroma, they have been postulated to serve as potential progenitors for carcinoma-associated fibroblasts (CAFs) (Cancer-Associated Fibroblasts: Overview, Progress, Challenges, and Directions | Cancer Gene Therapy, n.d.). CAFs, known to play a pivotal role in tumor progression, can arise from various cellular sources within the tumor milieu, especially the stromal cells. Given our findings, we hypothesize that pericytes may undergo specific transitions to contribute to the CAF pool in HCC.

Several positive CAF biomarkers, Acta2, Cspg4, Des, Pdgfra, Tgfb1, Tgfb2, and Il6, are identified in our pericyte clusters, and the negative markers, Krt8, Krt18, Krt1, and Pecam1 (cd31) are totally absent in most of the pericytes (Fig. 13) (Frisbie et al., 2022; Shiga et al., 2015). Surprisingly, the fibroblasts do not show high expression levels of these positive CAF markers except for Pdgfra and Tgbr2, considering that fibroblasts are usually the most prominent contributor to CAFs. This could be associated with the particular intratumoral heterogeneity in HCC tumors and the complex interactions between the various cells present in the tumor microenvironment, which are worthy of

further investigation. Future studies may enlarge the sample size of HCC and examine individually whether the pericytes always exhibit a more CAF-like phenotype than the fibroblasts.

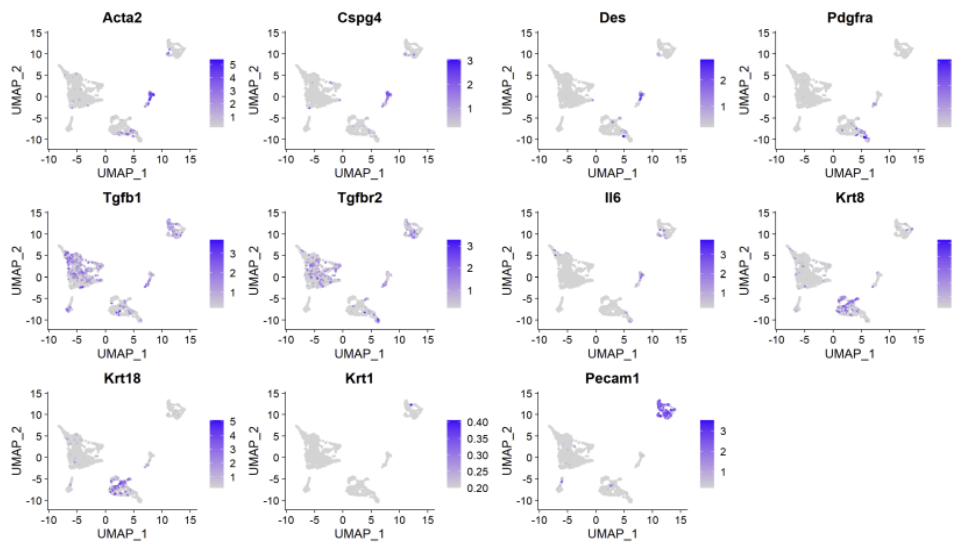


Figure 13: Identification of Pericyte-derived CAFs through Biomarkers

The positive CAF markers Acta2, Cspg4, Des, Pdgfra, Tgfb1, Tgfb2, and Il6, are upregulated in the pericyte cluster, while the negative markers, Krt8, Krt18, Krt1, and Pecam1 (cd31) are totally absent in most of the pericytes, demonstrating pericytes as possible CAFs.

A number of factors that drive the cellular transition to CAFs are identified in our sample (Fig. 14) (TGF- $\beta$  Promotes Pericyte-Myofibroblast Transition in Subretinal Fibrosis through the Smad2/3 and Akt/MTOR Pathways | Experimental & Molecular Medicine, n.d.; TGF- $\beta$  Promotes Pericyte-Myofibroblast Transition in Subretinal Fibrosis through the Smad2/3 and Akt/MTOR Pathways | Experimental & Molecular Medicine, n.d.; F. Wu et al., 2021). Noticeably, the macrophages, proliferating cells, and Kupffer cells contribute to most of the factors promoting transitions to CAFs, which highlights the role of these immune cells in controlling the tumor microenvironment and very likely in stimulating tumor growth and metastasis. The presence of these factors driving the transformation of normal stromal cells into CAFs suggests possible CAFs-targeted therapy by blocking the receptors for these factors (Yin et al., 2019).

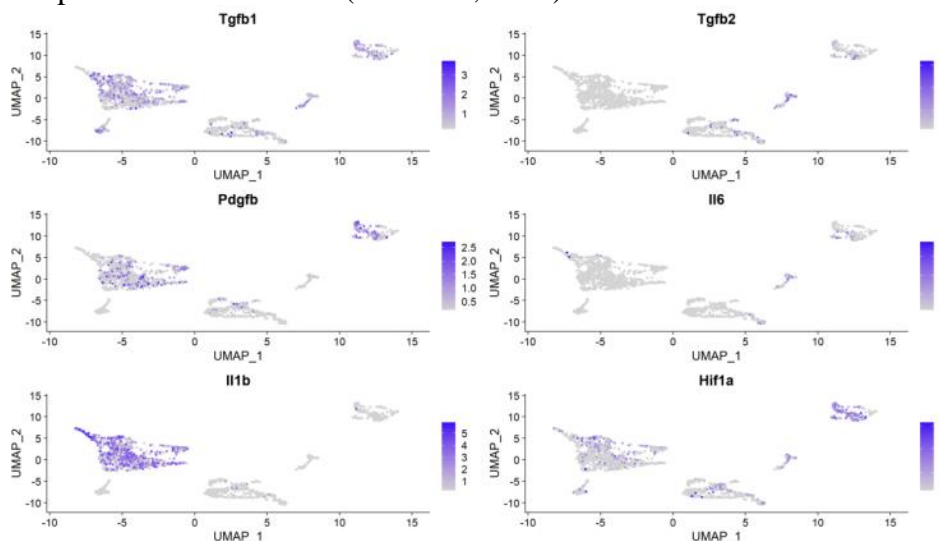


Figure 14: Factors Promoting Cellular Transition into CAFs

The feature plots show that the factors driving the transition from regular pericytes to CAFs are mainly secreted by macrophages which are likely to be TAMs and endothelial cells.

CAFs are major contributors to tumor progression as they promote tumor proliferation through the secretion of Tgfb, Fgfs, and Hgf, allow cooperative invasion by direct physical contact with the cancer cells, enhance drug resistance by acting as a physical barrier for drug delivery, and facilitate metastasis by driving cancer cells into displaying epithelial-mesenchymal transition (EMT) phenotype (Frisbie et al., 2022; Pernot et al., 2022). Particularly, CAFs derived from perivascular cells are presumed to have associations with metastasis (F. Wu et al., 2021). However, there is also evidence that CAFs can have a tumor-suppressing role (Pernot et al., 2022). The exact role the pericyte-derived CAFs in HCC play need further investigation to be determined. If the exact mechanisms determining the pro-tumoral and anti-tumoral nature of the CAFs are illustrated, novel therapies may target the anti-tumoral switch of the CAFs and make friends out of these foes.

In summary, the identification of pericytes as a likely source of CAFs and the factors driving CAFs transformation demonstrate complex interactions that modulate the HCC tumor microenvironment. For future studies to explore the CAFs in HCC and understand their roles in tumor progression at a deeper level, spatial transcriptomics and lineage tracing may be performed to identify the location and characterize the movements of the CAFs as the CAFs are often found to make close physical contact with the tumor cells (S.-D. Wu et al., 2012). Whether HCC has particular preferences for sources of CAFs, that is, whether fibroblasts, endothelial cells, or pericytes are more likely to transition into CAFs in HCC, can be closely examined, and it would surely deepen our understanding of HCC.

#### 4.4 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.

## 5. Conclusion

Our single-cell RNA sequencing analysis has provided intriguing insights into cellular heterogeneity and unveiled potential cellular interactions within the HCC tumor microenvironment, offering insights into the roles of various cell populations in tumor progression. The presence of diverse cell populations, the identification of cells that might function as TAMs, TECs, and CAFs, and the recognition of their potential interplay in driving tumor progression highlight the complexity of the HCC tumor landscape. These findings, though preliminary and necessitate experimental validation, provide a foundational understanding of HCC's cellular dynamics and set the stage for future investigations into their functional roles and therapeutic implications.

## Acknowledgement

We would like to express my deepest gratitude to Dr. Xudong Liu for his invaluable guidance and support throughout the course of this research. His expertise and insights have been instrumental in the completion of this work. We are particularly grateful for his provision of the mouse liver HCC tumor tissue scRNA-seq dataset, which formed the basis of my analysis.

## Contribution

The contributions to this research were as follows: The bioinformatic analysis and manuscript writing were carried out by Kejia Miao. The experimental data collection was conducted by Dr. Xudong Liu. Both authors reviewed and approved the final version of the manuscript.

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