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Friend or Foe? The Elusive Role of Hepatic Stellate cells in Liver Cancer

Bruno Cogliati[1,2], **Chittampalli N Yashaswini**[1], **Shuang Wang**[1], **Daniela Sia**[1], **Scott L. Friedman**[1]

[1]Division of Liver Diseases, Icahn School of Medicine at Mount Sinai, New York, NY, USA

[2]Department of Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, SP, Brazil.

Abstract

Liver fibrosis is a significant risk factor for the development and progression of liver cancer, which includes hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA). Recent studies utilizing cell fate-mapping and single-cell transcriptomics techniques have identified quiescent perisinusoidal hepatic stellate cells (HSCs) as the primary source of activated collagen-producing HSCs and liver cancer-associated fibroblasts (CAFs) in HCC, complemented in iCCA by contributions from portal fibroblasts. At the same time, integrative computational analysis of single-cell, single-nucleus, and spatial RNA sequencing data has revealed significant heterogeneity among HSCs and CAFs, with distinct subpopulations displaying unique gene-expression signatures and functions. Some of these subpopulations have divergent roles in promoting or inhibiting liver fibrogenesis and carcinogenesis. Here we discuss the dual roles of HSC subpopulations in liver fibrogenesis, as well as their contribution to liver cancer promotion, progression, and metastasis. We review the transcriptomic and functional similarities between HSC and CAF subpopulations, highlighting the pathways that either promote or prevent fibrosis and cancer, and the immunologic landscape from which these pathways emerge. Insights from ongoing studies will yield novel strategies for developing biomarkers, assessing prognosis and generating new therapies for both HCC and iCCA prevention and treatment.

Correspondence address: Scott L. Friedman MD, Division of Liver Diseases, Box 1123, Icahn School of Medicine at Mount Sinai, 1425 Madison Ave, Room 1170C, New York, NY 10029, Scott.Friedman@mssm.edu.

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Overview

Liver cancer is the third leading cause of cancer-related death and its incidence is increasing globally[1]. The most common types include hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA) which account respectively for 80-90% and 10-20% of all primary liver cancers[2]. In addition, the liver remains the dominant site of metastasis for extrahepatic tumors, representing 25% of all cases[3,4]. HCC and iCCA show fundamental differences in epidemiology and risk factors. While HCC more commonly develops in males in the setting of chronic liver diseases[5], iCCA typically arises in the absence of clear risk factors or underlying liver fibrosis[6], except in patients with underlying sclerosing cholangitis, which can predispose to iCCA. Common risk factors for HCC include chronic viral hepatitis, toxic exposure, and metabolic dysfunction from either alcoholic (ASH) or obesity-related nonalcoholic steatohepatitis (NASH)[7]. Although hepatitis B virus (HBV) vaccination and anti-viral therapies for HBV and hepatitis C virus (HCV) are reducing the incidence of viral-related HCC, the rising tide of nonalcoholic fatty liver disease (NAFLD) and alcohol-associated liver disease are fueling a new HCC epidemic that underlies the increasing incidence of this cancer[8].

Hepatic stellate cells (HSCs) are resident perisinusoidal vitamin A-storing cells that play vital roles in liver physiology and fibrogenesis[9]. During chronic liver injury, quiescent HSCs transdifferentiate into activated proliferative, fibrogenic myofibroblasts, mainly secreting extracellular matrix (ECM) components[9]. The associated chronic liver inflammation contributes to the tumor burden, since more than 90% of HCCs arise on a background of very advanced fibrosis or cirrhosis[5,7].

The tumor microenvironment (TME) is comprised of cancer cells, cancer-associated fibroblasts (CAFs), resident and recruited immune cells, and tumor-associated endothelial cells (TECs) embedded within a variable amount of remodeled ECM. In addition to tumor initiation, ECM remodeling can promote cancer progression by inducing invasion and metastasis[10-13]. iCCA frequently exhibits a dense desmoplastic fibrous stroma that may profoundly shape the progression of carcinogenesis through complex crosstalk with tumor cells[14]. CAFs play a crucial role in the deposition of collagen, one of the main components of the ECM, and thus, have been implicated both in the initiation and progression of liver cancer. Both HCC and iCCA express large amounts of ECM which primarily come from activated CAFs[5].

Emerging cell fate-tracing and single-cell sequencing studies have demonstrated that quiescent HSCs are the major source of activated HSCs and liver CAFs[15,16]. While HSCs only make up about 10% of liver cells[7], they express a remarkable degree of transcriptomic heterogeneity not previously appreciated[17]. In addition, distinct subpopulations have been described based on their spatial, zonal distribution. Briefly, quiescent HSCs are spatially zonated in a relatively static distribution[18-20], whereas activated HSCs can evolve in a time- and stage-dependent disease into subtypes that include collagen-producing myofibroblasts, or proliferative, intermediate activated/vascular, and inflammatory phenotypes[21,22]. Some subsets of HSCs contribute significantly to fibrosis and tumor development[23], and studies

exploring depletion of these pathogenic subsets reinforce the importance in understanding HSC heterogeneity, and the therapeutic implications of targeting specific HSC subsets. In this review, we discuss the dual roles of distinct HSCs subpopulations in liver fibrogenesis and their emerging contribution to liver cancer promotion, progression, and metastasis.

Hepatic Stellate Cells

Cell biology and functionality

HSCs are resident liver pericytes in the Space of Disse nestled between hepatocytes and the hepatic vessels created by liver sinusoidal endothelial cells (LSECs). HSCs, together with LSECs and Kupffer cells (KC), the liver resident macrophages, make up the bulk of the liver non-parenchymal cell (NPC) compartment, whereas HSCs account for roughly 15% of total resident cells in the normal liver[9]. Although most of the liver mass (~80%) is comprised of hepatocytes that carry out the organ's homeostatic functions[9], in chronic injury the NPC compartment expands and evolves in ways that can amplify injury, enhance fibrosis and promote cancer [24].

HSCs are embryologically derived from mesenchymal precursors within the septum transversum[25]. At ~ 4 weeks of gestation in humans and embryonic day 9.5 in mice, the mesenchymal precursors that are trapped between the invading hepatoblasts (hepatocyte precursors), and endothelial cells acquire morphological features that are characteristic of adult HSCs[25]. These features include dendrite-like processes that impart a "stellate" or star-like appearance, and cytoplasmic fat droplets that become the body's major storage site for Vitamin A[9,25]. HSC progenitors express mesenchymal markers including *Msx2* and *Alcam,* as well as the neuronal markers *Gfap* and *p75NTR*[26], whereas mature HSCs can be detected through their expression of *Des* and *Lrat*[16,26]. This developmental trajectory has been verified by HSC-selective Cre recombinase-mediated lineage tracing and cell depletion, and more recently using single-cell RNA-sequencing (scRNA-Seq) based cell trajectory predictions at different stages of mouse and human liver development[16,27-29].

In the healthy liver, quiescent HSCs play important homeostatic functions in addition to Vitamin A storage, including secretion of growth factors that promote the healthy turnover of hepatocytes and vasoregulation of hepatic blood flow owing to their contractile properties[9]. Following acute injury, HSCs undergo a dramatic activation, driven in part by *Tgfb* and *Pdgfrb* signaling, to transdifferentiate into activated myofibroblast-like HSCs that produce increased ECM and contribute to tissue repair[7]. When injury is chronic rather than self-limited, for example due to chronic viral infections, alcohol or NAFLD, activated HSCs continue to produce excessive ECM to generate scar, as demonstrated by recent single-cell transcriptomic studies[18,20,30,31], setting the stage for tumor development. If liver injury resolves, lineage tracing studies in mouse models indicate that half of the activated HSCs undergo apoptosis while the other half deactivate to return to an intermediate, quiescent-like state, whose phenotype is controlled by specific transcription factors such as *Tcf21, GATA4, and Lhx2,* among others[32-34]. The different states of HSCs – activated, apoptotic and de-activated, can be distinguished by unique profiles of protein and gene expression profiles that are regulated by distinct transcription factors[35].

In addition to HSCs, other less abundant cell types contribute to the pool of liver myofibroblasts, including portal fibroblasts, which express surface markers that are more closely aligned with fibroblasts of other tissues[36], and mesothelial cells[37-40]. These other cell types become more prevalent during cholestatic injury, which affects the portal triad, where the portal fibroblasts reside[41,42].

Emerging concepts of HSC heterogeneity

Single-cell (sc), single-nucleus (sn), and spatial transcriptomic (ST) RNA sequencing technologies have revealed an unprecedented resolution of cell subpopulations within the healthy and diseased liver[43-45]. From a technical perspective, scRNA-Seq analysis of NPCs may underestimate their numbers because liver tissue dissociation and cell isolation techniques favor recovery of other cell types[46]. In contrast, snRNA-Seq recovers a higher number of NPCs, including HSCs, and also enables gene expression analysis using cryopreserved tissues, which is not possible with scRNA-Seq[47]. Neither of these techniques can define the spatial localization of gene expression[48]. An additional challenge is that morphologic-based approaches such as immunohistochemistry and fluorescent *in situ* hybridization (FISH) are generally limited to a few candidate markers for validation, although the capacity for increased numbers of markers is rapidly expanding[49]. To overcome these limitations, spatial transcriptomics sequencing (ST-Seq) has been developed using barcoding technology to capture RNA information by performing *in situ* sequencing[50]. Although ST-Seq can efficiently localize gene expression within the liver's structure, complementary sc/snRNA-Seq methodologies are still required to reach single-cell based resolution[51]. In addition, the spatial tissue distribution of HSCs subpopulations can be archived by integrative *in silico* analysis and cell deconvolution with ST-Seq data[52]. Taken together, these findings highlight the technical limitations and potential applications of single-cell resolution methodologies in liver tissue, referring to the snRNA-Seq as an advantageous approach for HSCs analysis[31,47].

As noted, HSCs are far more heterogeneous then initially described when isolation techniques were first developed[53,54] (Table 1). In healthy liver, some scRNA-Seq studies have described only one HSC population[55,56], whereas in injury there are at least two subpopulations of HSCs based on the expression of genes related to retinoid storage- and myofibroblast function, which roughly correspond to quiescent and activated HSCs, respectively[31,47]. More recently, scRNA-Seq and spatial analyses have demonstrated the zonal distribution of two subpopulations of quiescent HSC based on *Ngfr* and *Adamtsl2* gene expression, termed 'portal vein-associated HSCs (PaHSCs)' and 'central vein-associated HSCs (CaHSCs)[18-20]. CaHSCs and PaHSCs have been proposed as major sources of activated collagen-producing HSCs in CCl_4-induced hepatotoxicity[15,18] and cholestatic[15] liver injury, respectively. On the other hand, activated HSCs derived from both zonated subpopulations are diffusely distributed in a mouse model of NASH[20]. These findings suggest that the pattern (i.e., pericentral, periportal or diffuse) and type (i.e., toxicity/cholestasis vs. metabolic) of liver injury can determine the recruitment and distribution of quiescent HSCs as they activate during liver injury, with changes in HSC phenotype tracking closely to the zones of injury.

Single-cell analyses have uncovered remarkable cellular heterogeneity of HSC subpopulations in humans and animal models of chronic liver disease[22,47] (Table 1). The heterogeneity of HSCs may reflect the complex multicellular and intercellular crosstalk within the liver microenvironment[19]. Lineage-tracing and/or scRNA-Seq analyses have revealed the transcriptional differentiation of HSCs in mouse models of liver fibrosis, demonstrating their transition from quiescent pericytes to activated myofibroblast phenotypes[21,22,57]. Furthermore, activated HSCs have been further characterized as distinct subpopulations, such as proliferative (pHSC), inflammatory (iHSC), intermediate activated/vascular (vHSC), contractile/migrative (cmHSC), and fibrogenic myofibroblasts (myHSC) (Fig. 1)[20-22,56]. As noted above, a de-activated HSC (dHSC) subpopulation has been described during the regression of liver fibrosis in a diet-induced NASH model in mice, highlighting an intermediary gene expression signature between quiescent and activated HSCs[20]. Other minor HSC subpopulations have been described that differentially express genes related to antigen presenting cells[19], and a chimeric HSC-endothelial cell type[58,59].

The single-cell transcriptomic and spatial methodologies have also revealed a cell-cell crosstalk among scar-associated macrophages, endothelial, and activated HSCs. In cirrhotic patients, pseudotemporal trajectory and RNA velocity analyses describe the differentiation of HSCs into scar-associated myofibroblasts[30]. In rodent NASH, a secretory HSC subpopulation has been reported that is regulated by autocrine IL-11 signaling and acts as a hub of intrahepatic signaling in endothelial and immune cells[45,59]. Interestingly, NASH-associated HSCs display an emerging autocrine signaling circuit in late-stage human and mouse NASH fibrosis, complemented by HSC crosstalk with cholangiocytes and endothelial cells[31]. In fact, two activated HSC subpopulations have been identified in human NASH, but only one expresses ECM-related genes[60]. Similarly, divergent HSC roles are seen in murine hepatocarcinogenesis, in which one population of HSCs limits HCC growth through cytokine- and growth factor-related pathways (cyHSCs), whereas another population of activated HSCs expressing *Col1a1* (myHSCs) stimulates liver stiffness via TAZ signaling that promotes proliferation of tumor cells and HSCs[23]. Liver stiffness has been correlated with activation of focal adhesion-upregulated pathways in an activated HSC subpopulation, increasing the deposition of ECM components and fibrosis progression[61].

While the reliance on single-cell methodologies has been transformative, there are also caveats to consider when leveraging data of this type. The perceived level of cellular heterogeneity is heavily dependent upon arbitrary cutoffs in the informatics pipeline and the actual number of cell type (HSC) subclusters. Ideally potential differences should be validated functionally. The measured extent of cellular heterogeneity is likely to increase further as technologies in single-cell transcriptomics advance, in part by increasing transcript recovery per cell.

Liver Cancer

Morphological and molecular features

HCC and iCCA are classically viewed as independent tumors that originate from hepatocytes and cholangiocytes, respectively, with resulting differences in histology and molecular features. Nonetheless, emerging genomic analyses[62,63] and *in vivo* genetic

lineage tracing studies[64-66] have identified multiple potential cells of origin for both tumor types (i.e., hepatocytes, cholangiocytes, progenitor cells), suggesting that HCC and iCCA encompass a continuum of primary liver cancers, which also includes subtypes with mixed histological and molecular features. Most HCCs display typical features, which include stromal invasion, increased cell density, intratumoral portal tracts, fatty changes and a pseudo-glandular pattern[67]. At the other end of the spectrum, iCCA is highly heterogeneous in both macro- and microscopic appearances, which are reflected in its histological classification[68]. Conventional iCCAs can be classified into small or large bile duct subtypes according to the size of the affected duct, with the former being characterized by a mass-forming growth pattern and the latter arising from large intrahepatic bile ducts with increased mucin production[69].

HCC and iCCA present distinct mutational profiles with frequent mutations in HCC of *CTNNB1*, *TP53* and the *TERT* promoter[70-72], whereas in iCCA *IDH1*/2 mutations and *FGFR2* fusions are common[73-80], which has established the basis for the first targeted therapies[81,82] for this malignancy. Both HCC and iCCA can be classified into two major transcriptomic-based phenotypic classes, termed ‘Proliferation’ and ‘Non-Proliferation’ in HCC[83-87], and ‘Proliferation’ and ‘Inflammation’ in iCCA[88,89], with some genomic resemblance between the two proliferative classes. Unique molecular and histological features differentiate the 2 HCC classes, with the Proliferation class (~50%) associated with HBV infection, poor differentiation, worse prognosis, pro-proliferative pathways and higher rate of *TP53* mutations. Conversely, the Non-Proliferation class (~50%) has a better outcome and dominant Wnt signaling associated with *CTNNB1* mutations[83-87]. Similarly, iCCAs assigned to the Proliferation and Inflammation classes display differences in outcome, histology and pathway activation, with well-differentiated tumors enriched in inflammatory pathways [88,89]. More recently, classifications based on immune features of either HCC or iCCA have emerged[90-93]. These efforts have identified mutations in the *KRAS* (~25-40%) and *CTNNB1* genes (~30%) as drivers of immunosuppression and escape in iCCA[12] and HCC[13], respectively, further elucidating our understanding of their molecular pathogenesis and underlying mechanisms of resistance to current immunotherapies.

Etiology-specific HCC microenvironments

Underlying chronic liver inflammation plays an important role in the pathogenesis of HCC, with new evidence implicating etiology-dependent, immune-related mechanisms of hepatocarcinogenesis. HBV and HCV trigger an immune response that can either promote or suppress carcinogenesis. Although T cells can efficiently recognize HBV-specific epitopes and potentially contribute to tumor control[94], HBV infection has mostly been linked to an immune tolerant microenvironment characterized by: 1) highly immunosuppressive regulatory T cells (Tregs) associated with poor survival[95]; 2) abundance of myeloid-derived suppressor cells (MDSCs) that potently inhibit T cells[96]; and 3) IL-10-producing immature B cells able to suppress suppressed HBV-specific CD8 T cell responses[97].

Similarly, HCV infection can induce selective loss of IL-2-secreting $CD4^+$ T helper cells[98], with dysfunctional T cells overexpressing exhaustion markers (i.e., TIME3, PD1)[99]. Further expression of exhaustion markers on T cells can be due to the secretion of galectin-9

by monocytes, which in turn promotes expansion of Tregs, reduction of effector T cells and suppression of HCV-specific T cell responses[100,101]. Unlike virus-induced HCC, in NASH a unique crosstalk between immune cells and hepatocytes has emerged as potential mechanism mediating carcinogenesis. A unique tumor-promoting role of $CD8^+$ T cells has been suggested, with recent studies in preclinical mouse models uncovering intrahepatic auto-aggressive $CD8^+$ $PD1^+$ T cells that induce liver damage in response to metabolic stimuli, rather than promoting immune surveillance[102,103]. Additional immune populations that contribute to NASH-HCC pathogenesis include $CD4^+$ $IL17A^+$ T helper 17 cells, which reportedly induce white adipose tissue neutrophil infiltration that mediates insulin resistance and fatty acid release[104]. Conversely, fatty acid-dependent oxidative damage induces selective loss of $CD4^+$ T cells, which accelerates hepatocarcinogenesis in mice[105].

Both immune and non-immune components contribute to the notoriously high heterogeneity and progression of HCC. Single-cell technologies have revealed different degrees of heterogeneity in malignant cells, within and between tumors, which contribute to the formation of a diverse TME. In this regard, single-cell analysis of 19 HCCs uncovered transcriptomic heterogeneity that is closely associated with genomic diversity, hypoxia and poor patient prognosis[60]. This study identified *VEGFA* as a candidate key player in hypoxia-induced tumor diversity that, through the potential polarization of CAFs, tumor-associated macrophages (TAMs), and TECs, ultimately drives tumor progression[60]. Of interest, *VEGFA* has been independently identified as a key mediator of TEC reprogramming towards a more oncofetal-like phenotype, characterized by the expression of $PLVAP^+$[106]. *PLVAP* regulates endothelial permeability and promotes the seeding of tissue resident macrophages during fetal liver development. $PLVAP^+$ endothelial cells are abundant in HCC compared to adjacent tissues[106], and have been also identified in cirrhotic livers, but not in healthy liver[30].

In addition to *VEGFA*, malignant hepatocytes contribute to intra-tumor heterogeneity of HCC through other ligand-receptor interactions that profoundly differ from non-neoplastic hepatocytes and pro-metastatic hepatocytes, as recently described in a large-scale single-cell analysis of 10 primary or metastatic HCCs from multiple etiologies[107]. According to this study, ligands highly expressed in pro-tumorigenic hepatocytes were mostly related to stress-response pathways (i.e., inflammation, interferon, p53, apoptosis), whereas those in pro-metastatic hepatocytes were related to EMT, *NOTCH* and myogenesis pathways. Conversely, in non-malignant hepatocytes, ligands were related to the physiological functions of hepatocytes (i.e., metabolism, adipogenesis, and complement system). T cell states were identified according to the underlying etiology, with $CD8^+$ T cell clusters and CTL exhaustion being enriched in HBV- or HCV-related HCCs compared to non-HBV/HCV-related HCCs, although caution is warranted in drawing conclusions because of the small sample size. It is unclear if these differences contribute to the purported differential sensitivity to immunotherapy in viral-associated HCC versus non-viral HCC (e.g., NASH). Future studies will continue to elucidate the intra-tumor heterogeneity at the single-cell level across distinct etiologies, and to further explore their interactions with CAFs.

The iCCA microenvironment

The iCCA microenvironment contains a variety of cells distributed within an intricate web of dense, fibro-collagenous-enriched ECM. This unique microenvironment promotes tumor growth, immune evasion and resistance to therapies[14]. The extent of desmoplasia in iCCA has clear prognostic and therapeutic value[14,108], as exemplified by the clinical associations observed between the amount of several ECM-related growth factors (*HGF*, *SDF-1*, *CXCL12*) ECM compounds (i.e., osteopontin, type 1 collagen, etc.) and outcomes[52]. More recently, two different types of stroma have been proposed based on their morphological appearance, with a more fibrous and less cellular stroma type associated with a better outcome[109]. In the last decade, the first scRNA-Seq studies in human and murine iCCAs have identified heterogeneous stromal subpopulations and critical interactions occurring between CAFs, the most abundant stromal cells, with tumor cells, TAMs and TECs, all profoundly affecting tumor growth[110]. Several epithelial mesenchymal transition (EMT)-promoting interactions between cancer cells and CAFs have been identified in human iCCA[111]. EMT is increasingly associated with tumor immune evasion[112], and these interactions could shape the immunosuppressive microenvironment, further contributing to the immune ‘cold’ phenotype (i.e., lacking immune cells) typical of iCCA.

Indeed, in large immunogenomic studies[93,111,113] the vast majority of iCCAs are non-inflamed tumors with a scarcity of T cells and abundance of immunosuppressive immune cells, including myeloid-derived suppressor cells (MDSCs) and Tregs. In particular, an extensive intra-tumor infiltration of $CD4^+$ Tregs with a hyperactivated and highly immunosuppressive phenotype has been described by high-dimensional single-cell technologies in 20 resected iCCAs and matched PBMCs. The infiltration of Tregs was accompanied by the loss of $CD8^+$ cytotoxic T cells and a much lower frequency of $CD8^+$ $CD39^+$ T cells, a marker recently linked to tumor-specific $CD8^+$ T cells in the TME, compared to highly immunogenic tumors, such as melanoma and lung cancers[114]. The presence of highly immunosuppressive Tregs in iCCA is consistent across single-cell-based studies aimed at better understanding the TME of this aggressive neoplasm[115,116]. In addition, unique cell–cell interactions between CAFs with an inflammatory phenotype and T/NK cells potentially lead to immune disruption through the regulation of chemokines involved in the recruitment of immunosuppressive or effector T cells[111]. Finally, accumulation of MDSCs in the iCCA TME has therapeutic implications based on orthotopic models of iCCA, where depletion of both granulocytic MDSCs and macrophages significantly potentiated responses to immunotherapy[117]. Both tumor-intrinsic (i.e., genetic alterations in driver genes[111]) and extrinsic factors (i.e., gut microbiome[118]) control the recruitment of MDSCs and promote iCCA growth.

The metastatic tumor microenvironment

The unique microenvironment of the liver is critical to supporting the seeding of tumor cells originating in other organs through the bloodstream or lymphatics. While a variety of primary tumors may be the origin of liver metastases, primary colorectal cancers account for half of the cases of metastatic liver cancer, followed by other primary gastrointestinal cancers (i.e., pancreatic, gastric carcinomas, etc.) and primary non-gastrointestinal cancers, including lung, melanoma and breast[4]. Strikingly, metastatic liver cancers occur 18-40 times

more frequently than primary liver cancers[4] and have drastically lower 5-year survival rates[119]. Mechanisms underlying liver metastasis are not well studied, and it is unknown whether their features are primary-tumor specific; however, crosstalk between tumor cells and resident liver cells or immune cells likely help generate a pro-metastatic niche[120]. Kupffer cells (KC) represent the first barrier against seeding tumor cells and contribute to their elimination by phagocytosis in the early stages[121,122]. However, in late stages, altered polarization of KCs induces secretion of growth factors that promotes cell growth and angiogenesis[42] or recruitment of immunosuppressive cells[123]. KCs can also activate HSCs, which can contribute to ECM remodeling and stiffness[124], as well as to generating an immunosuppressive microenvironment through the recruitment of Tregs and MDSCs that can suppress $CD8^+$ T cell functions[125]. Macrophages can further contribute to $CD8^+$ T cell depletion via Fas-FasL-mediated apoptosis leading to an immune desert phenotype that resists immunotherapy[126].

Activated HSCs (i.e., CAFs) can also contribute to a permissive microenvironment for metastatic tumor growth in liver by inhibiting tumor dormancy. Specifically, activated HSCs secrete the chemokine CXCL12, which, by engaging with its cognate receptor CXCR4 on NK cells reduces their activity, removing a brake in the dormancy of metastatic breast cancer to promote metastatic growth[127]. The authors have proposed that activated HSCs are a master regulator of tumor dormancy in liver, although it is unknown if they serve a similar role for all metastatic tumors, and whether this function is relevant to primary liver tumors as well. Nonetheless, HSCs are recognized as important immunoregulatory cells in several disease contexts (reviewed in[128]).

Liver Cancer-associated Fibroblasts

Origin of Liver CAFs

The interplay between tumor cells, CAFs, immune cells and other elements of the surrounding TME profoundly influences cancer growth. In particular, CAFs are the most abundant and critical component of the TME, which can influence cancer cells through complex and dynamic mechanisms. As in liver disease, scRNA-Seq technologies have revealed that CAFs in liver cancer are a heterogenous population[129] (Table 2), which provides a plausible explanation for the conflicting reports describing both tumor-promoting as well as tumor-restricting functions. As noted, CAFs derive from tissue resident mesenchymal cells (largely HSCs) (Fig. 1), followed by portal fibroblasts, bone-marrow-derived cells, and endothelial cells[15,110,115,130]. Similar to primary cancer of the liver, CAFs associated with liver metastases originate from HSCs[131], but the resultant CAFs differ between primary and metastatic cancers (see next section).

Heterogeneity of liver CAFs across tissues—The origin of CAFs was recently delineated in a pan-cancer analysis across ten common solid cancer types, in which three divergent CAF states were identified, each with specific functions[132]. In this study, CAFs were likely derived from the activation of local normal fibroblasts, whereas the presence of minor cell clusters with endothelial-like, macrophages-like or peripheral nerve-derived-like features could reflect alternative origins from other cell types, or transdifferentiation

from resident HSCs[132]. Despite the great phenotypic/functional heterogeneity and the distinct classifications or selection techniques applied, there are also common CAF subtypes across distinct cancers, including a predominant αSMA-expressing subset which has been consistently identified in pancreatic[133,134], colorectal[135], in breast[136] and in head and neck[137] cancer. Three main functional subtypes have been reported in both human[92,115] and mouse[110] iCCA including: i) myofibroblastic CAFs (myCAFs), enriched in genes involved in ECM and collagen production, ii) inflammatory CAFs (iCAFs) enriched in genes involved in the regulation of inflammatory response, and iii) antigen presenting CAFs (apCAFs), characterized by expression of major histocompatibility complex II (MHC-II) and CD74. Analysis of human iCCA samples has identified additional minor subsets including the so-called vascular CAFs, enriched in microvasculature signature genes and inflammatory chemokines (IL-6, CCL8), which seem to be the most abundant along with iCAFs[69]. Similar CAF subgroups have been reported in liver metastasis[131] and pancreatic cancers[133,134,138,139], while myCAF and iCAF subtypes have also been reported in breast cancer[140,141], melanoma[142] and bladder cancer[143]. Despite some commonalities, CAF subtypes differ between metastases and primary tumors[137] and between different cancer subtypes within the same organ[136], raising the question of how tissue-specificity is conferred, and underscoring the need for robust and standardized methods for isolation, enrichment and analysis of CAFs[129].

CAF subtypes are not static, but instead can undergo reversible changes according to tumor and microenvironmental cues, or in response to culture and treatment conditions; this plasticity adds to the difficulty in characterizing CAFs and clarifying their functions. For example, iCAFs can convert to myCAFs if maintained in vitro in 2D conditions in the presence of tumor organoid-conditioned medium, whereas trans-well conditions markedly increase iCAF formation, suggesting that myCAFs are contact-dependent[104]. Similarly, apCAFs acquire myCAF markers when cultured in 2D, confirming their plasticity, and further suggesting that apCAFs may require environmental cues to maintain their identity as a subpopulation[75].

In addition, tumor-secreted factors also dictate the CAF state, as recently demonstrated in pancreatic ductal adenocarcinoma (PDAC) models, where IL-1 stimulates activation of the JAK/STAT pathway to generate iCAFs, while TGFβ antagonizes this process by downregulating IL1R1 expression and promoting differentiation into myCAFs[144]. In another example, treatment *in vivo* with the JAK inhibitor AZD1480 induces a significant decrease in tumor volume accompanied by a marked increase in collagen deposition and αSMA expression, suggesting that JAK inhibition promotes a shift from an iCAF phenotype toward a more myofibroblastic state. However, in the same model, despite reducing myCAF activity, TGFβ did not reduce tumor growth, indicating that distinct CAF populations may have tumor-specific effects on progression.

These observations, albeit preliminary, have important therapeutic implications since they suggest that understanding the impact of cancer drugs on CAF subpopulations can improve treatment efficacy. More importantly, these data hint at the possibility that the reversion of CAFs to a more quiescent state could represent a novel and effective therapeutic strategy. For example, in PDAC models the administration of a vitamin D analogue induces

transcriptional reprogramming of activated pancreatic stellate cells to restore their quiescent state, resulting in reduced tumor burden and increased efficacy of gemcitabine-induced apoptosis[145]. Similarly, all-trans retinoic acid (ATRA), an active metabolite of vitamin A, restores quiescence in pancreatic stellate cells and inhibits local cancer cell invasion in 3D organotypic PDAC models[146], leading to its evaluation in clinical trials[147]. CAFs can also prevent $CD8^+$ cytotoxic T cell activity and promote their exclusion from the tumor nests by secreting CXCL12[148], TGFβ[149] or distinct matrix molecules[150], which can alter clinical responses to immunotherapy.

Contributions of CAFs to hepatic carcinogenesis and progression

CAFs can support tumorigenesis by directly stimulating cancer cell proliferation, promoting angiogenesis, and by remodeling the microenvironment. These functions can be exerted through ligand-receptor interactions, release of growth factors and inflammatory cytokines, and/or deposition of ECM components. In particular, CAFs mediate the secretion of collagen, fibronectins and proteoglycans among others, which, together with secretion of matrix-crosslinking enzymes, contribute to matrix remodeling, ultimately generating “rail tracks” that support cancer growth and invasion and increase tissue stiffness. Stiff substrates activate integrins[151], FAK[152], Src family kinases[153] and YAP/TAZ[154] signaling, which in turn promote expression of pro-proliferative and pro-migratory genes in both cancer cells and stromal cells[155]. In response to this signaling, cancer cells can further modify the ECM structural organization and composition. As an example, YAP target genes include ECM components and ECM-modifying enzymes[156,157].

A stiff ECM can also activate YAP within CAFs, establishing a feedback loop that helps maintain the CAF phenotype to support cancer growth and progression[158]. Increased stiffness combined with overexpression of integrins, cell surface receptors that mediate adhesion to the ECM, further contribute to tumor progression in various cancers[159], including HCC[160]. In breast cancer mouse models, the enzyme lysyl oxidase (LOX) mediates collagen cross-linking, which leads to ECM stiffening and an integrin-dependent invasive phenotype mediated by PI3K activation[12]. Activated CAFs produce hyaluronic acid (HA), a major ECM component, which in turn is required to promote CAF motility towards tumor cells[161-163]. Thus, HA plays a key role in the physical interactions between CAF and tumor cells, a feature associated with therapeutic resistance and poor prognosis[164]. The high deposition of collagen triggers the activation of the receptor tyrosine kinase DDR receptors, DDR1 and DDR2, which can both support or control tumor growth according to the context and cell source. For example, DDR2 deletion in HSCs leads to the trans-differentiation of activated HSCs and generates a pro-metastatic liver microenvironment through secretion of specific factors[165]. DDR1 expressed on breast cancer cells interacts with collagen I to promote stemness and multi-organ metastasis growth through the activation of STAT3 signaling[166]. On the other hand, upon deletion of DDR1 in luminal breast cancer cells, tumors grow faster, and have enhanced ECM deposition and lung metastasis[167].

The spatial arrangement within the tumor may also play a key role, with those CAFs in direct contact with tumor cells activating TGFβ signaling and collagen deposition, whereas those at greater distance from tumoral nests instead deposit HA and remodel the

immune TME by secreting IL-6 and other inflammatory mediators[168]. The deposition of HA further shapes the immune microenvironment through the recruitment of TAMs[169]. CAFs can thus generate a permissive TME through the recruitment of myeloid cells and immunosuppression of T cell activity, key hallmarks of cancer[170,171], and thereby decrease immune surveillance and promote tumor cell escape.

Under normal conditions, the rate of ECM production in the liver equals that of its degradation, resulting in no net accumulation of the matrix. On the other hand, chronic liver injury of any etiology leads to the accumulation of scar that generates hepatic fibrosis, which can either reverse if the cause of injury is eliminated, or progress to cirrhosis, and, in one third of cases, to HCC[5,7]. CAFs play a crucial role in the deposition of collagen, one of the main components of the ECM, and thus, have been implicated both in the initiation and progression of liver cancer. Both HCC and iCCA contain large amounts of ECM that is derived largely from activated CAFs. After injury, quiescent HSCs transdifferentiate into proliferative, fibrogenic fibroblasts[36] which are responsible for up to 80% of the collagen deposition in multiple models [13,16,172], ultimately leading to remodeling of the microenvironment that, depending on either the specific HSC subpopulations, disease stage or ECM state, can either promote or prevent tumor formation and progression[23,172].

The roles of HGF- and type-I collagen (Col1a1)-producing HSCs differ between iCCA and metastatic liver cancers, where HGF released by activated iHSCs promotes tumor growth. In two iCCA animal models, Col1a1 derived from the myCAF subpopulation of activated HSCs contributes to stiffness but not tumor growth. In contrast, myCAFs produce hyaluran synthase 2, the enzyme responsible for HA production, thereby promoting iCCA growth and progression without altering ECM stiffness[110]. The tumor-promoting role of Col1a1 in iCCA contrasts with liver metastasis and pancreatic cancer, where myCAF-derived Col1a1 suppresses tumor growth by mechanically restraining tumor spread[131] (Fig. 2). These different effects could be partially explained by the different collagen states, as recently described in PDAC where uncleaved collagen has a protective effect to halt cancer progression through the degradation of DDR, while cleaved Col1a1 triggers a downstream axis that engages DDR1–NF-κB–NRF2 to promote tumor growth[173].

CAFs may also support liver cancer by providing specific nutrients to support tumor growth. In pancreas, pancreatic stellate cells undergo autophagy to generate alanine that promotes tumor progression[174]. In addition to the similarity of pancreatic stellate cells to those of the liver, the finding is especially relevant to hepatic fibrosis and HCC, because autophagy of activated HSCs is essential to provide intracellular energy that fuels their activation[175]. More broadly, the regulation of cancer by cellular metabolic pathways in tumor as well as stromal cells is an extremely active area of investigation, and CAFs likely contribute to metabolic regulation of cancer through other mechanisms beyond autophagy that have not yet been explored.

Therapeutic implications

There are no FDA-approved treatments for hepatic fibrosis. The unmet need for antifibrotic therapies in chronic liver disease is most clear in NASH, where fibrosis is the only histologic

feature that correlates with clinical outcomes, especially liver failure and HCC[176]. Moreover, mitigation of underlying liver diseases, for example through treatment of HBV[177-179], cure of HCV[180,181], bariatric surgery in patients with NASH[182,183] or abstinence from alcohol[184] can yield substantial fibrosis regression, which is associated with improved outcomes, and, for HBV and HCV, a reduced incidence of HCC.

Therapies for NASH are advancing rapidly, with many drugs in clinical trials predicated on their ability to reduce fat and/or inflammation (reviewed in[185,186]). While these approaches might reduce cancer risk through an indirect impact on CAFs and fibrosis they are not discussed here, as we focus instead on therapeutic prospects for direct antifibrotic strategies in the following section.

New approaches to directly target fibrogenic cells are less advanced than etiology-directed therapies but could have a greater impact by attenuating the impact of CAFs on cancer development or progression. Animal models have demonstrated that stellate cells can be directly ablated by administering HSC-specific $CD8^+$ T cells or CAR T cells[187,188]. In particular, a CAR T strategy to clear only senescent stellate cells reduces fibrosis in experimental CCl_4 or NASH models, but its impact on HCC development has not yet been assessed. Senescent HSCs also may contribute to a pro-tumorigenic environment by secreting IL-33 through the gasdermin D, N-terminus initiated pore; blockage of this pore with disulfiram prevents release of senescence-associated secreted factors (SASP) from senescent HSCs, which reduces tumor burden in murine obesity-related HCC[189]. More recently, depletion of all stellate cells using engineered $CD8^+$ T cells has led to complete loss of hepatocyte regeneration[190], underscoring the importance of HSCs to liver homeostasis and the need to refine any depletion strategies so that only disease-promoting HSCs are removed, because HSCs also help maintain liver homeostasis[191-194], regulate liver regeneration[195], and accelerate wound healing[196,197].

Direct antifibrotic strategies also seek to reduce local activity of TGFβ, a master profibrogenic cytokine[198]. Because TGFβ is strongly antiproliferative towards hepatocytes, its systemic neutralization is not safe as this will promote cancer. An alternative is to instead target other molecules that regulate TGFβ activity locally, in particular integrins expressed by HSCs, for example alpha V integrin[199]; in fact, integrin antagonists are already in clinical trials to treat hepatic fibrosis but not liver cancer yet. Other emerging approaches to locally inhibit TGFβ including targeting caveolin (internalizes the TGFβ receptor)[200], hyaluronic acid synthase 2 (which activates HSCs)[201], CD147 (triggers HSC contraction, migration, and expression of fibrogenic genes)[202], hydrogen peroxide inducible clone 5 (important for myofibroblast differentiation)[203], and galectin-1 (promotes HSC migration)[204] (Fig. 3).

Specific clearance of CAFs within cancers is being investigated as well, because HSC-derived CAFs contribute to tumor growth and survival by creating an immunosuppressive environment[205,206], conferring anti-apoptotic properties to transformed hepatocytes[207], and promoting malignant cell migration[208]. Studies seek to inhibit crosstalk between CAFs and immune cells, or to prevent generation of CAFs from HSC[209]. CAF-specific therapeutics have focused recently on fibroblast activation protein (FAP), a type II transmembrane serine protease expressed on the cell-surface of fibroblasts, almost exclusively in disease states

including fibrosis, arthritis, and cancer[210-214]. Approaches to depleting FAP-expressing CAFs include DNA vaccines, CAR-T cell adoptive transfer, and oncolytic virus-based approaches [215-219]. Results in animal models suggest that these strategies can reduce tumor burden in mouse models of mesothelioma, as well as in cancers of the colon, pancreas, and lung. These efforts are reinforced by recent studies using lipid nanoparticles targeting fibrogenic cells that are laden with mRNA encoding an anti-FAP chimeric antigen receptor, which generates CAR T cells *in vivo* to reduce cardiac fibrosis[220] (Fig. 3).

Targeting of other hepatic cell populations that interact with CAFs is also being pursued. Once activated, HSCs engage in more crosstalk with macrophages, and can thus contribute to fibrosis indirectly by bolstering the activity of proinflammatory, pro-fibrotic macrophages[205,221-223]. While the source of matrix degrading proteases in liver fibrosis is still not established, macrophages are a likely source, and therefore regulation of their function by CAFs could alter their proteolytic activity.

There has been incremental progress in the medical therapy of HCC and iCCA, most recently with increasing success of immunotherapies that can restore immune cell killing of tumor cells [224]. These approaches have not yet been combined with therapies directed at CAFs, but combinations of this type may emerge first in NASH-HCC, whose incidence is rising more rapidly than HCC from other etiologies[102]. Cancer therapies that modify the tumor microenvironment via CAF-directed therapies could represent a powerful new approach to both prevent and treat HCC and iCCA. There are early indications that such an approach is rational[225].

Conclusions and Future Prospects

Despite their obvious presence within hepatic tumors, the identity and behavior of fibrogenic cells within the cancer stroma has been understudied and only recently has been scrutinized, powered by the development of single-cell technologies that have revealed a remarkable degree of cellular heterogeneity. At the same time, these technologies have helped resolve the divergent conclusions that support either protective or pathogenic roles of fibrogenic cells in carcinogenesis of the liver and biliary tree. While recent findings offer a deeper understanding of the CAF's contribution to cancer, they also make clear the significant obstacles to translating these findings into effective therapies. Because the CAF phenotype may be highly plastic, any treatments that modify their function must be restricted to subpopulations that promote rather than protect against cancer, although the cells' plasticity makes CAFs a moving target.

In liver, the clear origin of CAFs derived from activated HSCs in HCC, and also from portal myofibroblasts in iCCA, represent significant advances upon which future progress can build. The continued refinement of highly targeted immunotherapies, cell-specific delivery methods, cell-cell crosstalk, and identification of stable, phenotype-specific drug targets are anticipated, which will progressively transform the prospects for effective prevention and treatment of liver cancer.

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Key points

1. Hepatic stellate cells (HSCs) are the major source of activated myofibroblast-like cells in liver fibrosis and cancer-associated fibroblasts (CAFs) in both primary and metastatic liver cancer.
2. Single-cell resolution technologies are unveiling the transcriptomic and phenotypic heterogeneity of HSCs and CAFs in humans and mouse models of liver disease.
3. Specific subpopulations of HSCs and CAFs exhibit contrasting tumor-promoting and tumor-inhibitory functions based on their immunologic and tumor-specific context.
4. HSC subpopulations with unique secretomes, including activated HSCs, senescent HSCs and CAFs are promising targets for anti-fibrotic therapies that may reduce liver cancer, especially in non-alcoholic steatohepatitis (NASH).

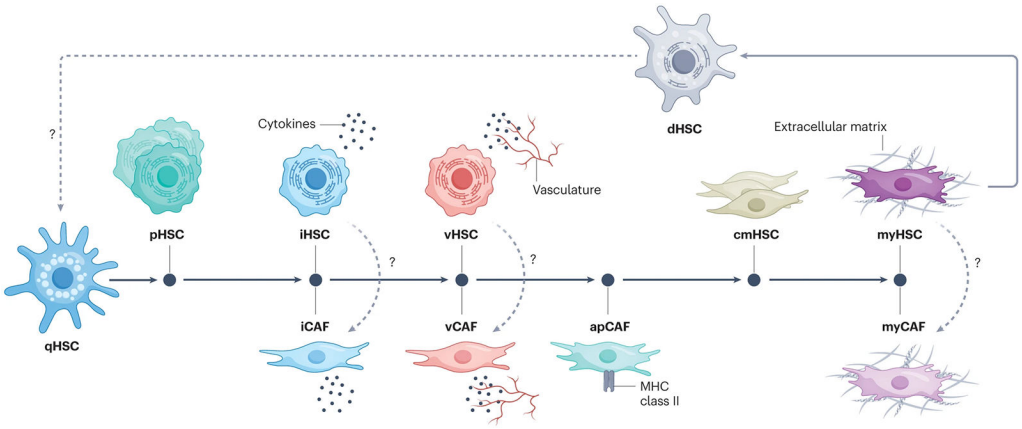

Figure 1. A model of HSC and CAF plasticity upon activation based on single-cell analyses.

Quiescent HSCs are the major source of activated HSCs/myofibroblasts in fibrosis and CAFs in both primary and metastatic liver cancer scRNA-Seq analyses have demonstrated the transition from quiescent HSCs to several activated HSC and CAF phenotypes. Furthermore, *in silico* analyses describe the transcriptional differentiation of activated HSCs into subtypes that include proliferative (pHSC), inflammatory (iHSC), contractile and migrative (cmHSC), and fibrogenic myofibroblasts (myHSC) phenotypes. Other subtypes expressing microvasculature genes are depicted within the subpopulation of vascular HSCs (vHSC), yet their origin(s) is (are) unclear. A de-activated HSC (dHSC) subpopulation appears during regression of liver fibrosis, characterized by an intermediary gene expression signature between quiescent and activated HSCs. However, full transdifferentiation of this subtype back to the quiescent phenotype has not been described. Similarly, CAFs display a transcriptional transition from an inflammatory (iCAF) to a fibrogenic phenotype (myCAF), with intermediary subpopulations such as vascular (vCAF) and antigen presenting CAFs (apCAFs). Due to the remarkable plasticity of HSCs and CAFs during liver fibrogenesis and carcinogenesis, their distinct subpopulations show complementary or ambiguous functions in response to specific chronic inflammatory and tumor microenvironments.

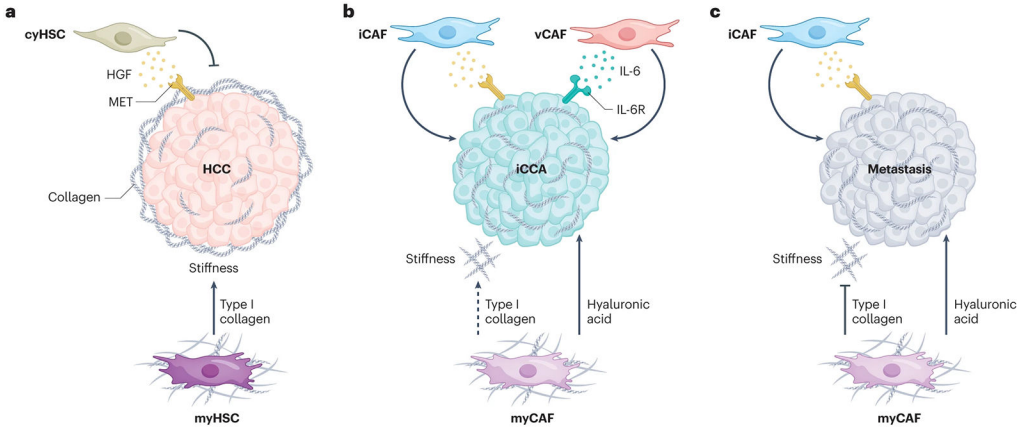

Figure 2. Roles of CAF subpopulations in the liver tumor microenvironment.

scRNA-Seq analyses have revealed significant heterogeneity of CAF subpopulation in HCC, iCCA and liver metastasis. The main CAF subtypes described in liver cancer are myofibroblastic (myCAF), inflammatory (iCAF) and vascular (vCAF). In HCC, HSCs expressing type 1 collagen (myHSCs) increase liver stiffness, which promotes proliferation of tumor cells. Conversely, HSCs expressing cytokines and growth factors (iHSC) suppress HCC growth via hepatocyte growth factor (HGF) and its receptor MET. In iCCA, myCAFs produce hyaluran synthase 2, the enzyme responsible for hyaluronic acid (HA) production, promoting tumor growth and progression. On the other hand, type 1 collagen produced by the myCAF subpopulation contributes to liver stiffness, but without effects on tumor growth. In human iCCA, the vCAF subpopulation promotes tumor growth via the interleukin (IL)-6/IL-6R axis. In liver metastasis, type 1 collagen derived from myCAFs suppresses tumor growth by mechanically restraining the tumor, whereas HA promotes tumor growth. In both iCCA and metastases, iCAFs promote tumor growth via the HGF-MET axis. Arrows indicate pro-tumorigenic effects and inhibitory arrows indicate suppression of tumor growth.

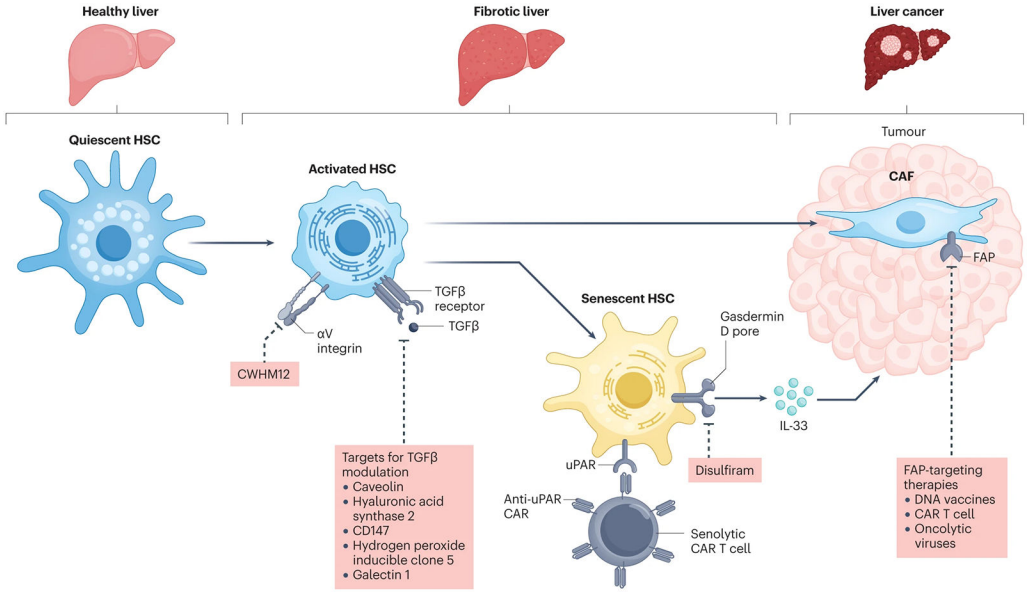

Figure 3. Hepatic stellate cell subpopulations are potential targets for anti-fibrotic and anti-tumor therapies in NASH.

Activated HSCs have elevated expression of alpha V integrin and TGFβ receptor, which can be targeted to reduce fibrosis. As proof of principle, a small molecule CWHM12 pharmacologically blocks alpha V integrin to attenuate fibrosis, and TGFβ receptor signaling can also be locally inhibited by targeting caveolin, hyaluronic acid synthase 2, CD147, hydrogen peroxide inducible clone 5, or galectin-1. After prolonged activation, HSCs can senesce and secrete the SASP component IL-33 via the gasdermin D pore, which promotes tumor development. Disulfiram reduces tumor burden in mice by inhibiting the gasdermin D pore. Senescent HSCs can be depleted by senolytic anti-uPAR CAR T cells to reduce liver fibrosis. HSC-derived CAFs might be depleted by targeting fibroblast activation protein (FAP) via DNA vaccines, CAR T cell therapy or oncolytic viruses, to potentially reduce hepatic fibrosis and tumor burden.

Table 1.

HSC subpopulations - Cluster subtypes and associated gene signatures and functions.

Tissue	Species	HSC Cluster	Gene Signature*	Phenotype and Functions
Healthy liver	Human	Periportal/ central HSC[19]	*GPC3, NTRK2, EFEMP1, GEM, CCL2, THBS1*	Quiescent phenotype ECM remodeling Metabolism of glycosaminoglycans
		Perisinusoidal HSC[19]	*DBH, HHIP, VIPR1, PTH1R, RAMP1, EDNRB, AGTR1A*	Quiescent phenotype ECM remodeling Antigen presentation
		HSC[55]	*ACTA2, COL1A1, TAGLN, COL1A2, COL3A1, RBP1*	ECM remodeling
	Mouse	HSC[21]	*Angptl6, Vipr1, Lrat, Ecm1, Trem56, Gucy1a1, Gucy1b1*	Quiescent phenotype Regulation of vascular tone
		HSC[15]	*Tcf21, Lrat, Des, Reln*	Quiescent phenotype Precursors of aHSCs and CAFs
		HSC[56]	*Fcna, Angptl6, Fgf21, Colec11, Tmem56, Plvap*	Quiescent phenotype
		PaHSC[18,20]	*Ngfr, Itgb3, Lrat, Reln*	Quiescent phenotype Precursors of aHSCs in periportal/ perisinusoidal fibrosis
		CaHSC[18,20]	*Adamtsl2, Rspo3, Lrat, Reln*	Quiescent phenotype Precursors of aHSCs in pericentral/ perisinusoidal fibrosis
		HSC[col-low 61]	*Fabp1, Bhmt, Adamtsl2, Col1a1*	Quiescent-like phenotype
Fibrosis	Human	Scar-associated mesenchymal cells[30]	*PDGFRA, COL1A1, COL1A2, COL3A1, TIMP1, CCL2*	Fibrogenic phenotype ECM producing
	Mouse	HSC[18]	*Lpar1, Col1a1, Col1a2, Col3a1, Acta2, Lox*	Fibrogenic phenotype ECM producing
		HSC[col-high 61]	*Col1a1, Col1a.2, Adamtsl2, Alcam, Acta2, Fhl2, Itga8, Vim, Hspb1, Palld*	Fibrogenic phenotype ECM deposition and stiffness
		Inflammatory HSC[21]	*Ccl2, Cxcl10, Cxcl1, Ccl7, Il11*	Inflammatory phenotype
		Contractile/ Migrative HSC[21]	*Acta2, Timp1, Vim, Tagln, Tnc, Tpm2*	Contractile and migrative phenotypes
		Fibrogenic HSC[21]	*Col1a1, Col1a2, Col3a1, Lox, Lum*	Fibrogenic phenotype
		Activated MFB[56]	*Acta2, Tglna, Col1a1, Col3a1, Col6a3*	Fibrogenic phenotype
		Trans-differentiated myeloid MFB[56]	*Slpi, C3, Saa3, Cd74*	Immunoregulatory phenotype
		Proliferating fibroblasts[56]	*Ap1, Jund, FosB*	Fibrogenic phenotype
		Portal fibroblasts[56]	*Mgp, Fbln1, Gas6*	Fibrogenic phenotype
		Proliferating HSC[22]	*Cdc20, Ccnb2, Cenpf, Birc5, Cenpa, Stmn1, Cks2*	Proliferative phenotype
		Myofibroblast HSC[22]	*Acta2, Tnnt2, Casq2, Fhl2, Serpin f1, Fgl2, Meg3, Mapf4*	Fibrogenic and contractile phenotypes
		Inflammatory HSC[22]	*Cxcl1, Cxcl2, CCl2, CCl7*	Inflammatory phenotype Inflammation regulation ECM remodeling
		Vasoactive HSC[22]	*Rgs5, Angptl6, Mest, Vipr1, Ifitm1, Bco1, Tmem56, Plvap, Igfbp3*	Vascular phenotype Vasoactive modulation Liver repair and immune regulation
NASH	Human	aHSC[60]	*ACTA2, IGFBP7*	Wound healing

Tissue	Species	HSC Cluster	Gene Signature*	Phenotype and Functions
		aHSC[60]	*RBP1, IGFBP7, COL1A1, COL5A2, COL4A1, COL3A1, COL1A2, COL4A2, TIMP1, TIMP3*	Fibrogenic phenotype ECM remodeling
		NASH-associated HSC[31]	*PDGFRB, TIMP1*	Fibrogenic phenotype ECM remodeling
	Mouse	NASH-associated HSC[31]	*Pdgfrb, Acta2, Col1a1, Col3a1, Mmp2, Timp1, Timp2*	Fibrogenic phenotype ECM remodeling
		Endothelial-chimeric HSC[58]	*Ptprb, Clec4g, Rgs5, Reln, Lum, Ecm1*	Vascular phenotype
		HSC[59]	*Col1a1, Dcn, Ecm1, Lum, Angptl6, Pth1r, Vipr1*	Fibrovascular phenotype ECM remodeling Cytokine signaling Vasoactive response
		Fibrogenic myofibroblast HSC[20]	*Col1a1, Col1a2, Col3a1, Acta2, Timp1, S100A6*	Fibrogenic phenotype ECM deposition
		Intermediate activated HSC[20]	*Irf7, Rgs5, Angptl6, Colec11, Serping1*	Intermediate phenotype
		Immune and inflammatory HSC[20]	*Cd36, Ly6c, Clec4g, Fcgr2b, Mrc1, Kdr, Cavin2, Aqp1, Irat*	Inflammatory phenotype
		Proliferative HSC[20]	*Cdk1, Mki67*	Proliferative phenotype
		De-activated HSC[20]	*Gabra3, Cxcl1, Fbln7, Bambi, Vipr1, ApoE*	De-activated phenotype

*Representative markers. HSCs: hepatic stellate cells; qHSCs: quiescent HSC; aHSCs: activated HSCs; PaHSCs: portal vein-associated HSCs; CaHSCs: central vein-associated HSCs; MFB: myofibroblast; ECM: extracellular matrix.

Table 2.

CAF subpopulations - Cluster subtypes and associated gene signatures and functions.

Tumor Type	Species	CAF subtypes	Gene Signature/ Pathways/ Markers Enrichment	Functions and Specific Markers
HCC	Human[226]	CAF_VSMC	Vascular smooth muscle cells	Unknown
		CAF_HSC	Hepatic stellate cells	Unknown
		CAF_port	Portal fibroblasts	Tumor-suppressing (PROLARGIN$^+$)
	Mouse[172,227] Human[227]	MyHSC	ECM molecules/pathways	Tumor-promoting (*Col1a1*$^+$)
		CyHSC	Cytokines & growth factors	Tumor-suppressing (*Hgf*$^+$)
		vCAF	Microvasculature genes	Unknown
		mCAF	ECM/collagen organization	Unknown
		lpmCAF	ECM/cholesterol metabolism Fatty acid metabolism	Tumor-promoting (*CD36*$^+$) through MIF induced immunosuppressive TME and cancer stemness
		lpCAF		
		apCAF	MHC II, antigen processing	Unknown
iCCA	Human[92,115]	iCAF	Inflammatory response	Potentially contribute to immune exclusion
		myCAF	ECM/collagen organization	Potentially tumor-promoting (*POSTN*$^+$)
		vCAF	Microvasculature genes	Tumor-promoting (*CD146*$^+$) through *IL6/IL6R*
		apCAF	MHC II, antigen processing	Unknown
		eCAF	Epithelium specific markers	Unknown
	Mouse[110]	iCAF	Inflammation & growth factors	Tumor-promoting (*Hgf*$^+$)
		myCAF	ECM and activation markers	Tumor-promoting (*Has2*$^+$)
		mesCAF	Mesothelial markers	Unknown
Liver Metastasis	Mouse[131]	iCAF	Inflammation & growth factors	Tumor-promoting (Hgf$^+$)
		myCAF	ECM pathways	Tumor-suppressing (col1a1$^+$) & promoting (Ha$^+$)
		mesCAF	Mesothelial markers	Unknown

CAFs: cancer-associated fibroblasts; VSMC: vascular smooth muscle cells; HSC: hepatic stellate cells; cyHSCs: cytokine- and growth-factor-expressing HSCs; myHSCs: myofibroblastic HSCs; mCAFs: matrix CAFs; myCAF: myofibroblastic CAF; iCAF: inflammatory CAF; vCAF: vascular CAF; lpmCAF: lipid processing matrix CAF; lpCAF: lipid-processing CAF; apCAF: antigen-presenting CAF; eCAF: epithelial-to-mesenchymal transition (EMT)-like CAF; mesCAF: mesothelial CAF; MIF: macrophage migration inhibitory factor.