Cancer stem cells in hepatocellular carcinoma: A review from origin to clinical implications

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Abstract

Hepatocellular carcinoma (HCC) is an aggressive disease with a poor clinical outcome. The cancer stem cell (CSC) model states that tumor growth is powered by a subset of tumor stem cells hidden in cancers. It explains clinical observations, including the almost inescapable recurrence of tumors after initial successful chemotherapy and/or radiotherapy, as well as the phenomenon of tumor dormancy and therapy resistance. In the past two decades, there has been a dramatic increase in research on the identification and characterization of liver CSCs, which has encouraged the design of novel diagnostic and treatment strategies for HCC. Novel aspects of liver CSC studies have newly emerged, opening opportunities for new research directions and potential therapies. In this review, we summarize the present knowledge of liver CSC markers and the regulators of stemness in HCC. We also comprehensively review the more recent developments in the liver CSC field with emphasis on experiments involving utilizing single-cell transcriptomics to understand liver CSC heterogeneity, lineage-tracing and cell-ablation studies of liver CSCs as well as the microenvironmental niche influences on liver cancer stemness, including CSC-immune system interactions. We also discuss the potential application of liver CSC-based therapies for treatment of HCC.

Key Points

- Liver cancer stem cells (CSCs), a unique subset of HCC cells with stem cell features, dictate a hierarchical organization and contribute to treatment resistance and tumor recurrence.
- Earlier studies using cell sorting and xenotransplantation techniques have identified various liver CSC markers that have laid important groundwork for our current research in the field.
- Recent lineage-tracing and cell-ablation studies in intact mouse tumors have provided insights into liver CSC plasticity, quiescence, renewal and therapeutic response.
- Liver CSCs are capable of sustaining tumors by altering intrinsic regulators that converge into common signaling pathways.
- Liver CSCs reside in dedicated niches where they interact reciprocally with cells/factors in the tumor microenvironment to regulate stemness.
- Understanding the key traits and mechanisms of liver CSC survival provide opportunities to improve patient outcomes through improving prognostic models and therapeutics.

Introduction

Hepatocellular carcinoma (HCC), the predominant form of primary liver cancer, remains a difficult-to-treat cancer. Globally, the disease is the fourth most common cause of cancer-related death and ranks sixth in terms of incident cases. HCC is associated with a number of etiological factors, including cirrhosis, non-alcoholic fatty liver disease (NAFLD), chronic hepatitis virus infection, diabetes, obesity and alcohol abuse. First-line treatment options for the disease include surgical resection and liver transplantation. However, because of the insidious growth nature of the disease, most HCCs are inoperable, as most patients are diagnosed at a late stage of disease progression. Even after surgery, the long-term prognosis of HCC remains unsatisfactory due to the high recurrence rate. Chemotherapy and radiotherapy have been the last resort for advanced HCC patients with unresectable tumors for decades, but their efficacy is limited due to the resistant nature of HCC. Sorafenib and lenvatinib are the only first-line molecular-targeted drugs approved by the US FDA for the treatment of advanced HCC until just very recently, when atezolizumab and bevacizumab combination was also approved for HCC patients with unresectable tumors. In the past two years, regorafenib, nivolumab, pembrolizumab, cabozantinib and ramucirumab have also been approved by the US FDA but only serve as second-line targeted drugs for HCC patients who progress on sorafenib. Although these drugs have proven useful as adjuvant therapy, the efficacy of these drugs has remained modest, extending patient's survival for only a few months at best. Because of the high incidence, frequent recurrence, severe casualty rates and limitations of current treatment regimens for HCC, this disease is recognized as a major public health concern.

HCC is a biologically complex and highly heterogenous disease. The bulk tumor consists of a diverse collection of cells harboring different molecular signatures with diverse levels of sensitivity to treatment. Heterogeneity provides the fuel for resistance and has extensive implications for cancer therapeutics and biomarker discovery. Evidence for a relationship between heterogeneity and clinical outcome is well-documented. Tumor heterogeneity has conventionally been explained by the clonal evolution of tumor cells resulting from the progressive accumulation of genetic and/or epigenetic events.[1] Alterations in the tumor microenvironment (TME) are also known to facilitate the development of tumor cell heterogeneity. In addition, there is now convincing evidence to show that the heterogeneity within a tumor is driven by a subpopulation of cells with stem/progenitor cell features called cancer stem cells (CSCs). CSCs can regenerate all properties of a tumor as a result of their unique stem cell-like capacity to self-renew and differentiate. There is now solid evidence to show that tumor growth of HCC is also fueled by CSCs, with these cells representing the root of tumor recurrence and therapy resistance.[2] This review summarizes the importance and knowledge of CSCs in the context of HCC and, more importantly, discusses recent developments in the liver CSC field, with emphasis on experiments involving single-cell transcriptomics to understand liver CSC heterogeneity, lineage-tracing and cell-ablation of CSCs in intact HCC tumors, as well as microenvironmental niche influences on liver cancer stemness, including the significance of CSC-immune system interaction. This review will also examine major advances in the development of CSC-based therapies, some of which are currently being implemented in the clinical setting, that can possibly be applied in the setting of HCC. **Figure 1** illustrates a timeline of events where milestones of cancer stemness related discoveries in HCC occurred.

Markers of cancer stem cells in HCC

Much of the work in earlier studies of liver CSCs has focused on identification of liver CSC markers by cell sorting and xenotransplantation assays in immunodeficient mice. Although one should not overly rely on markers for CSC identification, these earlier research works have no doubt laid the foundation for the current studies on liver cancer stemness. Often times, HCC development mimics fetal liver, normal liver and regenerating liver development in terms of the emergence of cells expressing certain stem cell markers and activation of signaling pathways. In fact, many of the liver CSC markers identified in HCC are oncofetal markers shared by hepatoblasts and hepatic progenitor cells. Our current knowledge of liver CSC cell surface markers including CD133, CD44, CD24, EpCAM, CD90, CD13, CD47, ICAM-1, LGR5 and calcium channel α2δ1; drug efflux markers including Side Population (SP), ABCB5 and aldehyde dehydrogenase (ALDH); as well as intracellular marker like keratin 19 (K19) have been comprehensively elsewhere[3,4] and summarized in **Table 1**.

Clinical prognostic relevance of CSC markers. While all of the known liver CSC markers are each separately reported to be correlated with more aggressive HCC and worse patient survival, a number of studies have also analyzed liver CSC biomarker signatures in HCC. Elevated expression levels of hepatic stem/progenitor cell biomarkers, including K19, ABCG2, CD133, Nestin and CD44, are tightly correlated with tumor angiogenesis and poor prognosis of HCC.[51] K19 and S2 gene signatures defined by MYC/AKT activation, AFP+ and EpCAM+ have also been found to predict the outcomes of HCC patients in relation to liver transplant indications.[52] EpCAM+ and AFP+ represents an HCC subtype that resembles hepatic stem cell-like HCC, that correlates with a poor prognosis, with tumors developing in younger patients with advanced tumor stages and invasion.[35]

New insights from single-cell transcriptomics. Advent of single-cell transcriptomics in recent years offers an excellent modality to define the intratumoral heterogeneity of HCC. Two recent studies conducted on HCC cell lines, freshly resected HCC tumors and HCC patient-derived xenografts have utilized single-cell transcriptome analysis to characterize CSC heterogeneity in HCC.[53,54] Both found liver CSCs at the single-cell level to be heterogeneous. Different CSC subpopulations contain discrete molecular signatures, and distinct genes within different CSC subpopulations are independently correlated with HCC prognosis, indicating that a diverse liver CSC transcriptome affects tumor progression and intratumor heterogeneity. The study by Zheng et al. analyzed three established CSC markers, i.e., CD24, CD133 and EpCAM, where they found gene signatures linked to CD133 and EpCAM, but not CD24, are independent predictors of HCC survival.[53] A separate study by Ho et al. identified two main HCC cell populations characterized by differential EpCAM expression and a CD24+CD44+ enriched stemness related subclone within the EpCAM+ cells that display a specific oncogenic gene expression signature.[54] Larger scale analysis on freshly resected HCC tumors is needed to draw a more definitive conclusion for how CSC heterogeneity contributes to molecular and biological diversity of HCC tumors and, thus, patient prognosis.

The origin of CSCs

The liver is a unique organ in that it exhibits remarkable regenerative potential and plasticity following injury. Depending on the type of injury (acute or chronic), liver regeneration may involve activation of either fully differentiated hepatocytes or hepatic progenitor cells (HPCs). Lineage-tracing studies suggest that hepatocytes are primarily responsible for liver maintenance under normal conditions and responses

to acute liver injury, like those inflicted by chemical damage or partial hepatectomy.[55] In the case where the replicative potential of hepatocytes are impaired as in the case of chronic liver damage (i.e. cirrhosis, fibrosis, steatosis, inflammation and viral infection), HPCs can be activated to supply new hepatocytes and maintain the organ's functional integrity. We now know that HPC activation can contribute to HCC progression. Yet with that said, a number of reports have found HCC to originate from hepatocytes but not HPCs or ductular cells (DCs), and a progenitor signature does not necessarily reflect progenitor origin but dedifferentiation of hepatic tumor cells.[56,57] A study by Shin et al. also shows hepatocytes, and not *Fox1-Cre*-marked cells that label HPCs, to be the cell of origin of HCC.[58] A more recent study finds HCC to predominantly originate from hepatocytes, while transformed hepatocytes can also activate HPC expansion in early stages of hepatocarcinogenesis and can also contribute to liver tumor heterogeneity.[59] Findings from a study by He and colleagues suggest presence of hepatic cancer progenitor cells (HcPCs) in precancerous regions of foci altered hepatocytes (FAH), which display CSC-like characteristics. They found multiple cell types, including CD44, EpCAM and Sox9 expressing cells to reside within the FAH, implying that FAH may serve as a site for CSC expansion. They also found IL-6 signaling to be necessary for the progression of HcPCs into HCC. They suggest HcPCs to be the precursors of CSCs and that FAH can shelter CSCs.[60] Although the authors did not perform lineage-tracing experiments to identify the cells of origin of HCC progenitor cells, correlative evidence suggests that differentiated hepatocytes are the cells of origin of HCC progenitor cells.

Lineage-tracing and lineage-ablation of CSC markers in intact HCC tumors. The recent few years have documented growing research that focuses on utilizing lineage-tracing and lineage-ablation of CSC markers, namely, Epcam and Lgr5, in intact HCC mouse tumors to better understand liver CSC origin. **Figure 2** provides a comprehensive summary of three lineage-tracing and cell-ablation studies of liver CSC markers suggesting liver CSCs to originate from both proliferating ductular cells and hepatocytes.

Intrinsic regulators of cancer stemness in HCC

Figure 3 summarizes the key intrinsic regulators and signaling pathways of cancer stemness in HCC.

Noncoding RNAs and epigenetic regulators. microRNA (miRNA) profiling of liver CSC marker-positive vs. liver CSC marker-negative cells or HCC tissue samples with CSC-like vs. CSC-unlike signatures has led to the identification of multiple miRNAs that are associated with the regulation of stemness pathways or the direct regulation of stemness markers. Dysregulation of miR-125b[61], miR-130b[22], miR-148a[62], miR-181[63], miR-192-5p[64], miR-150, miR-155, and miR-223[65], let-7c, miR-200b, miR-222 and miR-424[66], miR-429[67] and miR-1264[68] has been documented, with many of these miRNA dysregulations to converge in altering similar self-renewal pathways, including: (i) Wnt/β-catenin by the interplays of Oct4-driven miR-1246 on AXIN2 and GSK3β[68] as well as miR-181 and NLK[63] and miR-155 on EpCAM[65]; (ii) BMP/SMAD by miR-148a, which interacts with ACVR1, and miR-125b, which targets SMAD2 and SMAD4[61]; and (iii) ERK by miR-130b targeting TP53INP1 regulation of DUSP10[22,69]. Other deregulated miRNAs were shown to regulate stemness through altering transcription regulators of differentiation and cell cycle regulators such as miR-181 on CDX2 and GATA6[63], miR-192-5p on PABPC4[64] and miR-429 on RB1, RBBP4 and E2F1.[67] Downregulation of a set of miRNAs (let-7c, miR-200b, miR-222 and miR-424) in α2δ1+ HCC CSCs results

in their target PBX3 to be maintained above a certain thershold, which is required to maintain the liver CSC phenotype.[66]

The last five years have witnessed a surge of articles of long noncoding RNAs (lncRNA) to show differential expression in liver CSC marker-positive subsets and to be critical in the regulation of liver cancer stemness. Depletion of lncDILC was found in cisplatin-resistant hepatospheres, where it aided in the expansion of both EpCAM and CD24 liver CSC subsets through promoting autocrine IL-6/STAT3 signaling and mediating TNF-α/NF-κB and IL-6/STAT3 crosstalk.[10] LncHDAC2 and lncBRM have both been shown to be preferentially expressed in the CD13+CD133+ liver CSC subset, where they drive self-renewal through activation of Hedgehog-YAP signaling by recruiting the NuRD complex onto the promoter of PTCH1 to inhibit its expression to activate Hedgehog and initiating the BRG1/BRM switch to activate YAP1, respectively.[6,70] The same research group also found lncβ-Catm and lncTCF7 to be overexpressed in CD13+CD133+ HCC cells, where they both collectively contribute to activation of the Wnt/β-catenin signaling pathway.[7,71] A separate study found lncDANCR to contribute to stemness features of HCC by derepression of β-catenin contributing to Wnt activation.[72] LncSox4, preferentially expressed in EpCAM+ and CD133+ HCC cells, interacts with and recruits STAT3 to the SOX4 promoter to drive self-renewal.[26] Nanog-regulated lncICR is highly expressed in the ICAM1+ liver CSC subset and is found to maintain ICAM1 expression by enhancing the stability of its mRNA through RNA duplex formation.[73] Expression of lncPVT1 was also found to be elevated in HCC, where it promotes proliferation and stem cell-like properties by enhancing the stability of NOP2 proteins.[74]

Deregulation in epigenetic regulators has also been reported in the past five years. This includes altered expression levels of the posttranslational protein methylation modifier PRMT6[27], the chromatin remodeling enzyme CHD4[75], the histone variant macroH2A1[76], the histone deacetylase SIRT1[77] and the m6A reader YTHDF2.[78] Altered expression levels of these epigenetic regulators result in activation of common oncogenic, self-renewal and anti-apoptotic pathways, including MEK/ERK[27], PARP[75], NFκB-p65[76] and OCT4 signaling.[78] Two studies found SIRT1 to be critical in mediating self-renewal of liver CSCs by transcriptional regulation of SOX2 through alteration of histone modification and interaction with DNMT3A, resulting in hypermethylation of the SOX2 promoter[77] and NOTCH-mediated SIRT1 deacetylation of LSD1.[79] LSD1 was also found to promote β-catenin activation by inhibiting the expression of Prickle1 and APC in LGR5+ liver CSCs, by directly regulating the levels of mono- and di-methylation of histone H3 lysine-4 at the promoters of these genes.[39] BMI, a component of the polycomb repressive complex, has been found to play a crucial role in the maintenance of the tumor-initiating SP subpopulation.[80] The DNA methyltransferases DNMT1 and DNMT3β have also been implicated to alter CD133 and liver CSC properties. TGF-β regulates CD133 expression through inhibiting DNMT1 and DNMT3β expression, leading to subsequent demethylation of its promoter[81], while epigenetic reprogramming triggered by a transient DNMT1 inhibition generates an enduring effect, affecting both the tumor's malignant properties and the liver CSC pool.[82]

Transcription factors. In addition to the well-known OCT4, SOX2, NANOG and NOTCH that have now been extensively reported to regulate liver CSCs, a number of less-studied transcription factors have also been reported to play a role in the maintenance of stem cell-like features in HCC. ATOH8, a bHLH transcription

factor, was found to control the expression of the stemness-associated genes OCT4, NANOG, and SOX2, the differentiation-associated marker AFP and the liver CSC marker CD133.[83] OCT4 is also found to be controlled by the transcription factor ZIC2, where it recruits the NURF complex to the OCT4 promoter, thereby initiating OCT4 activation.[84] PBX3 has been shown to activate expression of genes critical for HCC stemness, including CACNA2D1, EpCAM, SOX2 and NOTCH3.[66] ZBP-89, is weakly expressed in EpCAM+CD44+ HCC cells, where it negatively regulates HCC stemness by inhibiting NOTCH1 signaling by competitive binding to the NOTCH1 intracellular domain with MAML1.[85] SOX9 is highly expressed in liver CSCs and is responsible for the cell division switch in liver CSCs that is important for sustaining self-renewal and tumorigenicity. SOX9 negatively regulates Numb expression, contributing to a feedback loop that sustains NOTCH activity and guides symmetrical cell division.[86] NOTCH2 signaling, which is required for stemness of liver CSCs, has also been reported to be negatively regulated by C8orf4, which is found downregulated in CD13+CD133+ HCC cells. Specifically, C8orf4 interacts with N2ICD, blocking its nuclear translation, to activate NOTCH2.[87] SALL4, a member of the zinc finger transcription factors, is found overexpressed in HCC tumors where it enhances tumor-initiating potential, correlating with increases in the expression levels of K19, EpCAM, ABCG2[88] and CD44.[36] SALL4 gene expression in hepatitis B virus (HBV)-related HCC is controlled by DNA demethylation, where demethylation of CpGs located within OCT4 and STAT3, downstream of the SALL4 transcription start site, enables OCT4 and STAT3 binding, recruitment of BRG1 and enhanced SALL4 transcription.[89] The embryonic stem cell-related ZFP42/REX1 is also found to play a role in CSC maintenance by binding to the promoter region of MMK6, thereby obstructing its transcription to result in altered p38 MAPK signaling.[28] YY1AP1, a coactivator of the ubiquitously expressed transcription factor YY1, was reported to be involved in epigenetic regulation of c-Myc, SOX2, NANOG, AFP and EpCAM gene expression, where specific histone demethylases contribute to YY1AP1 transcriptional networks in maintaining the viability of EpCAM+AFP+ HCC cells.[90] FoxM1 has also been shown to play an vital role in the progression of Ras-driven HCC, where it activates CD44 expression and regulates ROS-modulated survival.[91] MYCN is found to correlate positively with both liver CSC markers (AFP, EpCAM, CD133, DLK1 and GPC3) as well as Wnt/β-catenin signaling markers, but negatively with markers of biliary epithelial and mature hepatocyte, suggesting that MYCN expression is limited to hepatic stem cell-like HCC that is accompanied by activated Wnt/β-catenin signaling, yet not seen in mature hepatocyte-like HCC or bile duct epithelium-like HCC.[92]

Metabolic regulators. The lipogenesis pathway was found to be upregulated in drug-resistant hepatospheres, with SCD1, an enzyme that catalyzes the desaturation of lipids, ranking as a top-hit. SCD1 was shown to play a role in sorafenib resistance through regulating liver CSC function.[93] A separate but related study also found SCD1 to play a crucial role in fibrosis-induced HCC through a Wnt positive-feedback loop by stabilizing LRP5 and LRP6.[94] Separately, NANOG+ liver CSCs show elevated mitochondrial respiration and that SIRT1-mediated deacetylation of MRPS5 promoted the function of complex-I and the generation of NAD+ to boost mitochondrial respiration in liver CSCs.[95] TLR4/E2F1-mediated NANOG has also been shown to promote cancer stemness features of CD133+ HCC cells by reducing mitochondrial oxidative phosphorylation, promoting mitochondrial fatty acid oxidation.[96] A genome-wide RNAi screen found PMPCB, which plays a role in mitochondrial protein processing, to be EpCAM-dependent, regulating Wnt/β-catenin signaling through mitochondria-related ROS and FOXO activity modulation.[97] Emergence of the liver CSC phenotype is also associated with modulation of redox

homeostasis and energy metabolism through ANGPTL4 and mitochondrial PDK4, which are both critical players of metabolic reprogramming, suggesting that targeting liver CSCs by restoring mitochondrial pyruvate oxidation in combination with chemotherapy may be a new approach to treat HCC.[98] XOR, responsible for the catalysis of the last two steps of purine catabolism, has been associated with HCC. CD13+CD133+ liver CSCs are deficient in XOR, and its loss potentiates the expansion of liver CSCs through the direct binding of XOR to USP15 to promote deubiquitination and the stabilization of KEAP1, which leads to degradation of NRF2 and ROS accumulation in liver CSCs.[99] Elevated p62 expression is required for instigation of NRF2 and mTORC1, induction of c-Myc and protection of liver CSCs from oxidative stress-induced death.[100]

Kinases/phosphatases. Kinases and phosphatases have attracted growing interest in studies, as they are most targetable for therapy. Studies have found IRAK1 to regulate liver CSC traits through altering AP-1-mediated AKR1B10 signaling. Inhibition of IRAK1 by an IRAK1/4 inhibitor mitigated growth of established HCC tumors and sensitized the tumors to sorafenib.[101] SHP2 is also documented in CD133+ liver CSCs and CSC-enriched patient-derived hepatospheres, where it facilitated liver CSC growth by promoting the dedifferentiation of hepatocytes and self-renewal of liver CSCs through augmenting CDC73- and GSK-3β-mediated β-catenin signaling.[29] SHP2 blockade by SHP099 in combination with sorafenib was highly effective in the treatment of HCC.[102]

Secretory molecules. A flow cytometry cell sorting approach to compare CD133+ and CD133- cells found enhanced secretory proteins IL-8 and ANXA3 to be overexpressed in the liver CSC subset. IL-8 plays a role in promoting tumor angiogenesis, growth and self-renewal through a neurotensin-induced activation of the IL-8/ERK signaling cascade.[25] A series of a few studies by two independent research groups found ANXA3 to be important in promoting self-renewal and tumor growth of CD133+ HCC tumors through a deregulated HIF1α/Notch pathway[103,104] and augmented MKK4/JNK signaling.[105] A neutralizing antibody against ANXA3 was also successfully developed, where preclinical experiments found that ANXA3 neutralization suppressed the growth and self-renewal of HCC *in vivo*, sensitized HCC to cisplatin and sorafenib/regorafenib and eradicated the CD133+ liver CSC subset.[105,106] Studies in genetically modified HCC mice found CD133+ liver stem cells derived from preneoplastic livers of β2-spectrin $(\beta2SP)^{+/-}$ (SPTBN1) mice treated with IL-6 to be highly tumorigenic and metastatic compared to IL-6-treated wild-type mice demonstrating IL-6-mediated chronic inflammation as a major driver for reprogramming normal liver stem cells to metastatic HCC cells.[107] A separate study found oncostatin M (OSM), an IL-6 related cytokine, to be critical in the induction of hepatocytic differentiation of EpCAM+ HCC cells by inducing STAT3 activation, and with evidence of a decrease in stemness gene expression (EpCAM, AFP and K19) and a concomitant increase in albumin expression. OSM treatment also rendered EpCAM+ HCC cells to be more sensitive to 5-FU treatment.[108] Sorafenib-resistant HCC tumors that are enriched with liver CSCs showed elevated secretion of IGF and FGF.[109] Deficiency of ANGPTL1 will result in activation of the MET receptor, which in turn activates ERK/AKT-dependent EGR-1 signaling to contribute to cancer stemness and sorafenib resistance in HCC.[110] CD47+ HCC cells also preferentially secrete cathepsin S (CTSS) which regulates stemness through the CTSS/protease-activated receptor 2 (PAR2) loop.[16]

Other regulators. There are also a few regulators that cannot be broadly classified into the above groups yet play critical roles in the maintenance of liver CSCs. EIF5A2 contributes to the maintenance of a CD133+ liver CSC subset through regulating the binding of c-Myc on the promoter of miRNA-29b.[111] Expression of $p28^{GANK}$ is found expanded in the liver CSC subset, where it promotes self-renewal, tumorigenicity, metastasis and chemoresistance, and inhibits cell differentiation by preventing the ubiquitination and degradation of Oct4 by WWP2.[112] Similar observations could also be seen in a rat HCC model, where $p28^{GANK}$ enhanced liver CSC markers through binding and promoting the proteasome-dependent degradation of HNF4α, which determines hepatocyte differentiation status.[113]

Receptor-mediated signaling. Various receptor-mediated signaling pathways have also been identified to play a critical role in driving liver cancer stemness, including IGR/IGFR in NANOG-mediated self-renewal[114], antagonization of MET-receptor-mediated AKT/ERK signaling by ANGPTL1[110], a feed-forward loop of TLR4/NANOG-dependent suppression of TGF-β signaling in CD133+CD49+ HCC cells[30], TLR4 signaling transactivation of Twist1 by binding of NANOG and pSTAT3[31], TGF-β-regulated lncH19 signaling via SOX2[14] and TGF-β activation of AKT.[115]

Crosstalk and convergent signaling pathways. While an array of regulators subclassified above has now been identified to control liver cancer stemness, it is apparent that many of them do converge into commonly altered signaling pathways, including Wnt/β-catenin, which is frequently found altered in EpCAM+, CD133+ and SOX9+ HCC cells[116]; and promoted by deficiency of βII-Spectrin (SPTBN1)[117], overexpression of SCD[94] and overexpression of the GSK-3β inhibitor RBMY[118], etc. Wnt/β-catenin related AKT and AKT/GSK-3β signaling is also frequently altered in liver CSCs, including alteration of the AKT/BCL-2 pathway in mediating chemoresistance of CD133+ HCC cells[24], AKT modulation of ABCG2 transporters in SP+ HCC cells[48,119], sorafenib-enriched EpCAM+ HCC cells to promote tumorigenicity through the TSC-AKT cascade[38], and MAEL to promote stemness-associated gene expression by activating the AKT/GSK-3β/Snail pathway.[120] It has also been found that SP+CD44+ tumorigenic cells in AKT/β-catenin-driven HCC tumors can be targeted by JAK/STAT pathway inhibition[15], demonstrating that these seemingly independent pathways do interplay. The shift of predominant YAP expression upon TAZ depletion of the HIPPO pathway has been found to confer CSC-like behaviors in HCC and an enrichment of the CD90+ HCC cells.[121] BMP4 that is well-known to be associated with the SMAD pathway has also been reported to display a dual role in the liver CSC subset, where high-dose exogenous BMP4 promotes CD133+ HCC differentiation and inhibits aggressive cancer and stem cell-like behaviors by altering ERK1/2 signaling.[122]

Microenvironmental influences on liver cancer stem cells

Figure 4 summarizes the key players in liver CSC – stroma interaction.

Biophysical properties

Hypoxia. Oxygen deficiency is a common characteristic of the TME, and hypoxia is a condition that has long been known to promote stemness. Early work found that hypoxia can induce cisplatin resistance in tumorigenic hepatic progenitor cells through an AKT/HIF-1α and PDGF-BB autocrine signaling loop.[123] A separate study found that IL-6/STAT3 signaling increased CD133 expression through activation of HIF-1α, where STAT3 directly interacts with NFκB p65 and recruits it to the HIF-1α promoter in hypoxic HCC.[124]

Hypoxia can also enhance HCC stemness and CD24+ HCC cell maintenance in both HIF-1α- and HIF-2α dependent manners. The study also identified SENP1 to promote hypoxia-induced cancer stemness by HIF-1α deSUMOylation and a SENP1/HIF-1α positive feedback loop.[125] While it has generally been accepted that hypoxic conditions will enrich for CSC subsets in HCC, recent single-cell functional analysis identified distinct liver CSC subpopulations using single-cell surface markers to display appreciable biological differences in terms of response to hypoxia[53], suggesting biological plasticity of these cells. Plasticity of HCC cells is an important feature for tumor progression, especially when exposed to different tumor microenvironmental conditions. A better understanding of the underlying mechanism that drives CSC plasticity will help us comprehend how such events impact tumor microenvironmental heterogeneity and treatment response.

Glucose deprivation. In addition to hypoxia, rapidly growing tumors often experience glucose deprivation because of poor vascular supply. A study found that liver CSCs marked by CD133+ cells can preferentially survive restricted glucose treatment through enhanced expression of the glucose transporters GLUT1 and GLUT3 stimulated by IL-6/STAT3-dependent regulation to enhance glucose uptake.[126]

Extracellular matrix (ECM) stiffness. Physical properties such as the stiffness and tension of the microenvironment are also increasingly recognized as important players in the regulation of stem cell differentiation. Studies have found CD133+ HCC cells to express enhanced levels of MMP2 and ADAM9, both modifiers of ECM.[127] The ECM marker laminin-332 was also found elevated in SP+ HCC cells, where it sustains chemoresistance and quiescence as part of the hepatic CSC niche[128], suggesting an altered ECM in the liver CSC niche. The link between matrix stiffness and stemness has remained controversial. Low-level shear stress has been found to induce differentiation of liver CSCs via the Wnt/β-catenin signaling pathway.[129] Matrix stiffness has also been found to enhance higher expression of CD133+EpCAM+ and functional stemness by activating the integrin β1/AKT/mTOR/SOX2 signaling pathway.[130] Yet conversely, work by Schrader et al. found that chemotherapy enriched surviving cells from soft supports had enhanced clonogenic capacity than surviving cells from a stiff environment; which was associated with enhanced expression of CSC markers.[131] More recently, Tian et al. also found a soft matrix to enhance the expression of liver CSC markers CD133 and CD90 and the functional CSC phenotype of HCC cells.[132]

Stromal cells

Endothelial cells. K19, ABCG2, CD133, Nestin and CD44, along with markers of angiogenesis CD34, VEGF and PD-ECGF, were found to be capable of predicting high risk of tumor recurrence after surgery in HCC patients.[51] Anti-angiogenic metronomic therapy in HCC xenografts also resulted in the enrichment of residual dormant CSC foci marked by CD13 that are responsible for tumor relapse.[133] CD133+ HCC cells have also been reported to preferentially secrete IL-8 to promote tumor angiogenesis of neighboring endothelial cells in the CSC niche[25], and radiofrequency ablation-induced VEGF enriches for CD133+ cells and promotes tumor stemness and tumorigenesis in HCC in a manner dependent on NANOG and VEGFR2.[134]

Cancer-associated fibroblasts (CAFs). Since more than 80% of cases develop in the context of cirrhosis, in which activated fibroblasts are enriched due to chronic inflammation, CAFs are known to be critical for

HCC development and progression and to significantly impact patients' clinical outcome. Activated hepatic stellate cells (A-HSCs), which play a key role in liver fibrosis and cirrhosis, have been suggested to be associated with recurrence and poor prognosis of HCC patients and to promote HCC progression through interaction and alteration of monocyte activities within the liver microenvironment.[135] Our group previously reported on the role of CAFs in the regulation of liver CSC plasticity by driving c-Met/FRA1/HEY1 signaling through HGF secretion.[136] Consistently, a separate study by Rhee and colleagues found expression of K19 to be upregulated by fibroblast-derived HGF through a MET-ERK1/2-AP1 and SP1 axis.[137] Subsequently, CAFs have been found to promote stem cell-like properties of HCC through chemokine-activated Hedgehog and TGF-β[138], IL-6-induced STAT3/Notch signaling[139] and autophagic flux induction.[140] Jiang et al. found human peri-tumor tissue-derived fibroblasts will preferentially secrete cytokines to induce metastasis of HCC through recruiting CCR2+ and/or CXCR1+ EpCAM+ HCC cells.[141] Through direct cell-cell contact, CAFs has also been shown to activate Notch3-dependent self-renewal and tumor growth of liver CSCs through facilitating LSD1 deacetylation and stabilization.[79]

Adipocytes. Accumulation of adipocytes and inflammation are well-established hallmarks of NAFLD which is a major risk factor for HCC. Earlier work found a diet high in cholesterol and saturated fat (HCFD) in hepatitis C virus (HCV)-NS5A transgenic mice promoted the development of CSCs and tumorigenesis in mice. Mechanistically, HCFD and HCV-NS5A stimulated TRL4-NANOG and leptin receptor (OB-R)-pSTAT3 signaling, resulting in the promotion of Twist1-expressing CSCs.[31] A separate study found that adipocyte-secreted factors (IL-6, IL-8 and MCP1) stimulated the expansion of EpCAM+CD133+ HCC cells, which conferred a migratory phenotype and the ability to resist sorafenib through activation of c-Met, STAT3 and ERK1/2 signaling.[142]

Hepatitis virus

HBV. The hepatitis B virus X (HBx) oncoprotein is closely associated with HBV-associated HCC. There are now multiple studies that have shown HBx to trigger malignant transformation by promoting properties that are characteristic of CSCs, including functional stemness properties as well as altered expression of Oct4, Nanog, Klf4, β-catenin and EpCAM.[143,144] One study found that HBx promoted stemness by activating β-catenin and epigenetic upregulation of miR-181, by targeting EpCAM.[143] Another found that HBx induced EpCAM expression through active DNA demethylation directed by RelA, together with EZH2 and TET2.[144] Hepatic oval cell lines can also generate HCC following transfection with HBx gene and treatment with aflatoxin B1 *in vivo*[145], while a separate related study found that HBx protein promoted OV6+ HCC cells through a deregulated MDM2/CXCL12/CXCR4/β-catenin signaling axis.[146] Using a HBx transgenic mice model, higher titers of circulating IL-6, activities of IL-6/STAT3, and Wnt/β-catenin signaling pathways were also noted, suggesting that HBx may induce intrinsic changes in hepatic stem/progenitor cells by way of the above signaling, which enables them to obtain tumorigenicity potential.[147] Many of these studies are also supported by clinical evidence where HBx and stemness markers are closely correlated in HBV-HCC patients. In particular, high HBx expression in human HBV-related HCC is statistically correlated with enhanced EpCAM+ or OV6+ tumor cells and aggressive clinicopathological features.[147] While most studies in the field have centered around HBx, the Pres1

protein of HBV has also been recently reported to promote the appearance and self-renewal of liver CSCs.[148]

HCV. Both HCV core protein and HCV-NS5B have been shown to result in the acquisition of liver CSC traits, including upregulations of c-Kit, Lgr5, CD133, AFP, K19 and c-Myc.[149,150] As mentioned above, HCV-NS5A and HFCD work together to promote Twist1-expressing CSCs.[31]

Liver CSC-immune system interactions

Recent investigations have begun to elucidate the relationship of liver CSCs with immune cells. The enhanced ability of CSCs to initiate tumors suggests that these cells likely have an advantage in evading immune detection and evasion. Indeed, there is now accumulating evidence to show a role for major immune cell types in driving CSC expansion, CSC-specific avoidance of immune detection and destruction, as well as the ability of CSCs to elicit pro-tumorigenic immune cell activities.

Tumor-associated macrophages (TAMs). TAMs are one of the more well-studied immune cell types in relation to liver CSCs. Within the tumor bulk, liver CSCs, characterized by low proteasome activity and low intracellular ROS levels, facilitate the migration of macrophages and demonstrate metastatic potential by way of recruitment of macrophages to the tumor site via secretion of chemokines.[151] HCC derived IL-8, which we previously found to be preferentially secreted by CD133+ HCC cells[25], can stimulate M2 polarization of TAMs to promote HCC invasion.[152] In a reciprocal manner, TAMs can produce IL-6 to promote the expansion of CD44+ HCC cells and drive their tumorigenesis through an altered STAT3 signaling, which stimulates further cytokine production, leading to a feed forward loop contributing to CSC self-renewal.[153] TAMs can also produce TNF-α to promote EMT and cancer stemness through Wnt/β-catenin signaling.[154] Separately, TAMs can also promote CSC-like properties through TGF-β1 induced EMT in HCC.[155] A decrease in exosomal miR-125a/b secreted by TAMs has also been found to exert CSC-promoting effects in HCC cells through modulating CD90.[156] TAMs can also interact with A-HSCs to induce pro-tumorigenic features of HCC cells by enhancing migration and self-renewal.[135] However, in addition to the classical role of TAMs in supporting tumor growth, there has been increasing evidence that shows their anti-neoplastic activities in an immunosuppressive TME. Our group found the liver CSC marker CD47, a “don’t eat me signal”, to evade the phagocytosis of macrophages and that blockade of CD47/SIRPα signaling by a neutralizing antibody may be a promising approach to target liver CSCs.[16,157,158] CD24 has also been identified to be another “don’t eat me signal” in ovarian and breast cancers, where tumors expressing CD24 can promote immune evasion through its interaction with the inhibitory receptor Siglec-10, which is expressed by TAMs.[159] Since CD24 is also highly expressed in liver CSCs[8], it is possible that liver CSCs can also evade clearance of macrophages by interacting with the Siglec-10 receptor expressed on TAMs. Genetic ablation of CD24 or Siglec-10, as well as blockade of the CD24/Siglec-10 interaction using monoclonal antibodies, may be the way forward to immunologically augment the phagocytosis of CD24-expresing human tumors, but its efficacy and mechanism will need further exploration.

Tumor-associated neutrophils (TANs). TANs are classical inflammatory cells that are crucial for tumor initiation and progression. Through coculturing of TANs and HCC cells, Zhou et al. found TANs to be capable of expanding the liver CSC subpopulations through secreting BMP2 and TGF-β2, which triggered

miR-301b-3p expression and led to suppression of LSAMP and CYLD. Enriched liver CSC populations were hyperactive in NFκB signaling, secreted higher levels of CXCL5 and recruited more TAN infiltration, suggesting a reciprocal relationship between liver CSCs and TANs.[160]

Natural killer cells (NK cells). NK cells are a kind of cytotoxic lymphocyte critical to the innate immune system. They can distinguish and kill cancer cells directly, but their activity can be weakened by various inhibitory molecules expressed on the surface. Impairment of NK cytotoxicity is a mechanism to evade host immunosurveillance. One study on the hepatic oncofetal protein granulin-epithelin precursor (GEP), which has previously been linked to liver CSCs[161], found that its expression in HCC cells conferred the ability to evade NK cytotoxicity. GEP augmented production of soluble MHC class I chain-related molecule A (MICA), which suppressed NK activation.[162] A more recent study found that EpCAM+ HCC cells resisted NK cell-mediated cytotoxicity by upregulation of CEACAM1 expression.[163] GEP and CEACAM1 blockade by monoclonal antibodies sensitized HCC cells to NK cytotoxicity, facilitating tumor regression.

Myeloid-derived suppressor cells (MDSCs). MDSCs impair the functions of dendritic cells, and most importantly suppress T cell infiltration into tumors, hindering the efficacy of current immune checkpoint therapies. Drug-resistant HCC cells, which have been widely reported to be enriched with CSC features, can enhance the expansion and immunosuppressive function of MDSCs through preferential secretion of IL-6. Depletion of MDSC by administration of anti-Gr-1 antibody or blockade of IL-6 signaling sensitized HCC cells to 5-FU treatment[164], suggesting a role for IL-6 in drug-resistant HCC and that MDSC-targeting treatments may be a potential therapeutic strategy for overcoming HCC chemoresistance.

Dendritic cells (DCs). DCs are antigen-presenting cells that capture, process and present antigens on the cell surface along with proper costimulation molecules, leading to the development of an adaptive immune response against tumor. Accumulating evidence has shown the role of liver CSCs in the evasion of immune surveillance by altering the phenotype and impairing recruitment of DCs. The HCC subtype characterized by EpCAM+AFP+ expression displays features of hepatic stem/progenitor cells, which preferentially secrete AFP to the TME.[33] Pardee and colleagues reported on the role of HCC tumor-derived AFP in immune evasion by impairing DC differentiation and function. More specifically, HCC tumor-derived AFP-conditioned DCs expressed reduced levels of DC maturation markers, retained monocyte-like phenotype, displayed reduced levels of inflammatory mediators, and failed to promote T cell proliferative responses.[165] A separate study found that retinoic acid-inducible gene I (RIG-1)-deficient stemness high human HCC cells induced a functional change in DCs from immune-stimulatory to immune-suppressive through upregulating TGF-β1.[166] More recently, it has been found that β-catenin activation can promote immune escape and resistance to anti-PD-1 therapy in HCC, at least in part through impairing the recruitment of DCs.[167]

Tumor-infiltrating lymphocytes (TILs). TILs consist of all white blood cells that have invaded the tumor tissue, and their presence represents good prognosis in HCC patients. However, liver CSCs are capable of attenuating the action of these cells directly by altering their PD-L1 expression. Immune-high HCC tumors, characterized by increased B-/plasma-cell and T cell infiltration, have been found to be associated with poorly differentiated HCC progenitors marked by K19+ and/or SALL4+ expression. Immune-high HCC

tumors were also linked with elevated PD-L1 expression in tumor cells and immune cells, which was associated with a better prognosis.[168] A number of studies have found key liver CSC regulators that modulate the expression of PD-L1, including SOX2, which can bind on the promoter region of PD-L1 to promote its transcription.[169] A separate study found that the IL-6/JAK1 pathway drove PD-L1 phosphorylation at Tyr112, which recruits N-glycosyltransferase STT3A to catalyze PD-L1 glycosylation and maintain its stability, thus promoting cancer immune evasion.[170] Targeting IL-6 signaling may be a possible strategy to increase the efficacy of Nivolumab for HCC treatment. Apart from the direct intrinsic action on $CD8^+$ T cells, liver CSCs could also impair the function of these cells indirectly through modulating the activity of regulatory T cells (Tregs). In response to hypoxia, in which CSC subsets are enriched, CCL28 promotes recruitment of Tregs and tumor growth in HCC.[171] TGF-β signaling, which has been reported to be altered in CD44+ liver CSCs[172] have also been found to suppress miR-34a, leading to enhanced production of chemokine CCL2, which recruits Treg cells to enable immune escape and favors the colonization of disseminated HCC cells.[173]

Ectopic lymphoid structures (ELS). ELS are organized clumps of leukocytes resembling lymphoid organs, which infiltrate at the site of inflammation. Since the majority of HCC arises in the context of chronic inflammation, there is increasing awareness of a role of ELS in HCC. The presence of tumor-associated ELSs should in theory correlate with patient prognosis due to the presence of cells in adaptive immunity, yet this hypothesis has remained controversial. An early report showed that the presence of ELS was correlated with an increased risk of late HCC recurrence and poor prognosis. In a transgenic IKK activation mouse model, they further found that NF-κB activation induced ELS formation, which in turn functions as a microniche for the expansion of liver CSCs, leading to HCC initiation. This is the first study to show the potential role of ELS in the regulation of liver CSCs.[174] Subsequently, gene expression profiles comparing early hepatic lesions (EHL) to surrounding cirrhotic nodules found EHL to display ELS and elevated expression of genes relating to immunosuppression and immune exhaustion.[175] Another report found that ELS was associated with a lower risk of early relapse in HCC patients treated by surgical resection and that ELS may reflect the existence of on-going anti-tumor immunity.[176] These contrasting data may suggest the balance between expansion of liver CSCs and immune surveillance in driving different stages of HCC development and progression.

Therapeutic targeting of liver CSCs.

Figure 5 provides a thorough summary of the novel approaches to target liver CSCs, as well as various available CSC-based therapies that can be potentially translated for use in HCC. This includes targeting liver CSC surface markers, targeting intrinsic and extrinsic regulators of liver CSCs, as well as liver CSC-directed therapy.

Future challenges and perspectives

Despite our improved knowledge of liver CSCs in HCC, many questions remain that need to be addressed, including the origin of liver CSCs and challenges with plasticity and reversibility of liver CSCs that will complicate their identification and eradication. The target cell population of malignant transformation has been controversial in HCC. Several studies, including the more recent lineage-tracing work, have demonstrated that HCC may originate from hepatocytes as well as proliferating ductular cells. However,

an important consideration in carrying out CSC and tumor cell-of-origin studies is the emergence of CSCs through the process of dedifferentiation. Non-CSCs can acquire CSC-like traits; and thus a deeper understanding of CSC plasticity and recognizing the subpopulation of non-CSCs that can convert to CSCs will aid in dissecting tumor heterogeneity and finding CSC-specific therapeutics.

Our current knowledge of liver CSCs has largely been influenced by the biology of normal stem cells. Normal and CSCs alike share many of the identified activated markers and self-renewal signaling pathways, and thus eradication of liver CSCs through targeting these markers and signaling pathways may also result in the reduction of normal hepatic stem/progenitor cells, which may inhibit hepatic regeneration leading to hepatic failure. While clinicians have suggested that the ability of hepatocytes to regenerate is often greatly decreased in cirrhotic HCC patients, precautions should be taken to identify markers that are only expressed in the liver CSCs or to delineate nonredundant requirements of a specific pathway for normal liver regeneration and HCC.

Different approaches to targeting the interactions of CSCs with the immune system are actively being investigated, and various CSC-directed immunotherapies are presently in clinical development. These experimental therapeutic strategies comprise efforts to target CSC surface markers using antibody-based approaches, stimulate tumor-specific T cells and alter the immunosuppressive TME. A basis for further exploration has been placed above. Genetically engineered mouse models, including hydrodynamic tail vein delivery of oncogenic plasmids and sleeping beauty transposase, are valuable tools for the study of human CSC populations in immunocompetent mice. With the increasing success of patient-derived HCC and murine HCC 3D organoids, we can also exploit them further for coculturing with specific immune cells for subsequent functional studies. Ideal timing, sequence and combination of these CSC-specific, immune-based therapies will require more investigations prior to clinical applications. Perhaps the optimal time to utilize inhibitors against CSCs is before or concurrently with adjuvant therapy, or soon after diagnosis. It is now known that CSCs persist after adjuvant treatment, resulting in tumor relapse and poor prognosis. Thus, elimination of CSC clones at the time they are more vulnerable is of critical importance. There is also a need to develop more vigorous assays to measure self-renewal in therapy-treated tumors in the clinic. Currently, monitoring anti-CSC treatment effect is largely dependent on cell surface markers, which is not ideal given the aforementioned restrictions of marker-based selection and issues with CSC plasticity. Functional readouts, including those for self-renewal, is of paramount importance to confirm complete eradication.

Last, an area that may be well worth further exploration is the utilization of circulating CSC markers in liquid biopsies for diagnosis, prognosis and therapeutic response evaluation. Early work has found that CD45-CD90+ liver CSCs can be detected in 90% of blood samples from HCC patients, but none are detected in healthy individuals or patients with cirrhosis.[200] Increased numbers of CD45-ICAM1+ liver CSCs in blood samples of HCC patients have also been found to correlate with poor prognosis.[201] The presence of circulating EpCAM+ HCC stem cells in postoperative patients helps predict cancer relapse[202,203]. A multimarker circulating tumor cell detection panel, including EpCAM, CD90, CD133 and K19, showed high sensitivity and specificity in HCC diagnosis and great significance in predicting early recurrence of HCC after resection.[204]

Conclusions

Liver CSCs are now recognized to mark a unique subset of HCC cells with stem cell features that contribute to therapy resistance and tumor recurrence. As summarized in this review, earlier studies in the field have primarily used cell sorting and xenotransplantation techniques to identify and characterize liver CSC markers and altered signaling pathways that have laid important foundations for our current research. With the advent of single-cell sequencing, applications of lineage-tracing and cell-ablation techniques in CSC studies as well as the recognition of the importance of immune cells in the TME, recent research has revealed new information regarding liver CSC heterogeneity, origin, plasticity and novel treatment options. A deeper understanding of liver CSC traits and mechanisms will shed light on the development of rational combinations of therapeutic strategies that target liver CSC subsets as well as the tumor bulk. This will provide opportunities to improve patient outcomes through improving diagnostic and prognostic models and therapeutics.

Table 1. Markers of cancer stem cells in HCC.

	Involvement in Normal Cells/Stem Cells	Function in Liver CSC and Associated Signaling	Experimental Evidence	Refs
Cell Surface Marker				
CD13	Marks granulocytes, monocytes, mast cells and granulocyte/macrophage progenitor cells	- YAP1 signaling - Wnt/β-catenin signaling - ROS-induced DNA damage reduction	FACS and xenotransplantation	5-7
CD24	Marks lymphocytes, neutrophils and differentiating neuroblasts	- STAT3-mediated NANOG regulation - iNOS-mediated NOTCH signaling - IL-6/STAT3 signaling	FACS and xenotransplantation	8-10
CD44	Adhesion molecule of the extracellular matrix; a hyaluronan receptor	- TGF-β-mediated mesenchymal phenotype - CD44 interacts with TM4SF5 to promote STAT3/BMI1 signaling - TGF-β and H19 signaling axis - JAK/STAT signaling	FACS and xenotransplantation	11-15
CD47	Not reported	- CTSS-PAR signaling	FACS and xenotransplantation	16
CD90	Marks thymocytes, mesenchymal stem cells, hematopoietic stem cells, fibroblasts; functions in cell adhesion and cell communication	Not reported	FACS and xenotransplantation	17-18
CD133	Marks neural, hematopoietic and embryonic stem/progenitor cells; also expressed in fetal and regenerating liver	- AKT/PKB pathway - IL-8/ERK signaling - ANXA3/JNK signaling - Wnt/β-catenin signaling - STAT3-mediated SOX4 signaling - PRMT6-mediated MEK/ERK signaling - REX1-mediated p38 MAPK signaling - TLR4/NANOG signaling and defective TGF-β pathway - TLR4/NANOG and STAT3 signaling	FACS and xenotransplantation Linage-tracing and lineage-ablation	11,19-31
ICAM1	Marks endothelial cells and cells of the immune system	- NANOG de-regulation	FACS and xenotransplantation	32
EpCAM	Molecule that mediates epithelial cell adhesion	- Wnt/β-catenin signaling - SALL4 de-regulation - IL-6/STAT3 signaling - TSC2/AKT signaling	FACS and xenotransplantation Linage-tracing	10,29,33-38

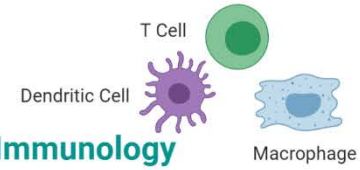
	Marks hepatic progenitors of biliary lineage			
LGR5	Marks adult stem cells like those of intestine, hair, etc.	- Wnt/β-catenin signaling	FACS and xenotransplantation Lineage-tracing and lineage-ablation	39-41
OV6	Marks resident stem cells in the adult liver; also expressed in regenerating liver	- Wnt pathway activation - SDF-1/CXCR4 signaling	FACS and xenotransplantation	42,43
α2δ1	Not reported	Not reported	FACS and xenotransplantation	44
Side Population (SP) / Drug Efflux Markers				
ALDH	Expressed in hematopoietic and neural stem cells; an intracellular enzyme involved in cellular detoxification by the oxidation of cellular aldehydes to protect stem cells	Not reported	FACS and xenotransplantation	45
SP	Marks hematopoietic stem cells	- Enhanced ABCG2, ABCB1, and CEACAM6 expressions - AKT signaling	FACS and xenotransplantation	46-48
Intracellular Markers				
K19	Marks bipotential cells in the human liver	- TGF-β/SMAD signaling	FACS (based on K19 promoter-driven enhanced green fluorescence protein gene) and Xenotransplantation	49,50

OV6, oval cell marker
ALDH, aldehyde dehydrogenase
SP, Side Population
K19, Keratin 19

Figure 1. Milestones of cancer stemness related discoveries in HCC.

Earlier studies using cell sorting and xenotransplantation techniques have identified various liver CSC markers that have laid important groundwork for our current research in the field. Following identification, an extensive amount of work has been dedicated to characterizing intrinsic regulators and microenvironmental influences that maintain cancer stemness in HCC. New developments involving single-cell transcriptomics, lineage-tracing and cell-ablation of CSCs in intact HCC tumors, as well as CSC-immune system interaction have only emerged in the last five years, providing exciting new avenues for exploration.

Milestones of Cancer Stemness Related Discoveries in HCC

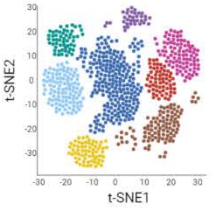


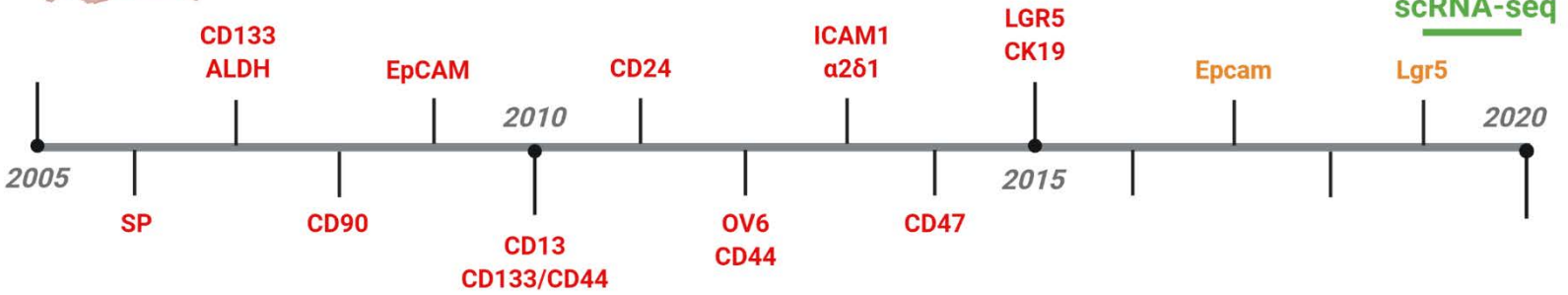
Liver CSC Immunology

Identification of Liver CSC Markers by FACS + Xenotransplantation

Cell of Origin and Lineage Tracing/Ablation Studies

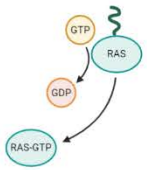
scRNA-seq



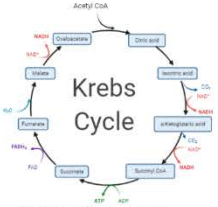


Altered signaling pathways

Epigentic regulators



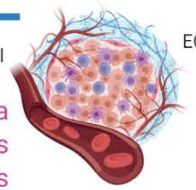
miRNA



Metabolic regulators

Characterization of Intrinsic Regulators

2008 onwards: Signaling cascades
2009 onwards: miRNAs
2010 onwards: Secretory molecules
2010 onwards: Epigenetic regulators (other than small RNAs)
2011 onwards: Kinases/phosphatases
2012 onwards: Receptor mediated signaling
2015 onwards: Transcription factors
2015 onwards: lncRNAs
2016 onwards: Metabolic regulators



2009 onwards: Hypoxia
2010 onwards: Endothelial cells
2011 onwards: ECM stiffness
2011 onwards: HBV and HCV
2016 onwards: Cancer-associated fibroblasts
2016 onwards: Adipocytes
2017 onwards: Glucose deprivation

Poor Vascularization - Hypoxia / Nutrient Deprivation

Adipocytes

Hepatitis Virus

Characterization of Microenvironmental Influences

CSC Targeted Therapy

Figure 2. Summary of the three lineage-tracing studies providing support for the liver CSC model. Details for each study are discussed in the text. The lineage-tracing tumor models are presented on the left and the main outcomes and conclusions summarized on the right. **(A)** This study sets out to determine the cell fate of Epcam+ ductal cells and their potential as tumor-initiating cells *in vivo*. By linage tracing of Epcam+ ductal cells in a chemically induced liver injury model followed by mutation induced by cytidine deaminase, they found that Epcam+ cells in the injured liver gave rise to HCC. They also showed that these Epcam+ ductal cell-derived HCC characteristically contained cholangiocarcinoma components, alluding to the divergent cell lineages of tumor cells. Findings support the role of Epcam+ ductal cells as cell origins of liver cancers that develop by inflammation-associated tumorigenesis.[37] **(B-C)** Genetic lineage-tracing and lineage-ablation studies by two independent research groups collectively uncover Lgr5+ hepatocytes as potential cells of origin in HCC development. Lgr5 is selectively expressed in a defined population of hepatocytes most adjacent to the central veins. They are long-lived and can contribute to their own lineage maintenance during postnatal liver development, homeostasis as well as acute liver regeneration. Moreover, Lgr5+ hepatocytes are found to be the main cellular origin of DEN-induced HCC and are highly susceptible to neoplastic transformation triggered by activation of the Erbb pathway.[41] Complementing the findings of this study, an independent study found Lgr5 lineage-ablation in intact DEN-induced HCC tumors to significantly inhibit organoid initiation and tumor growth.[40]

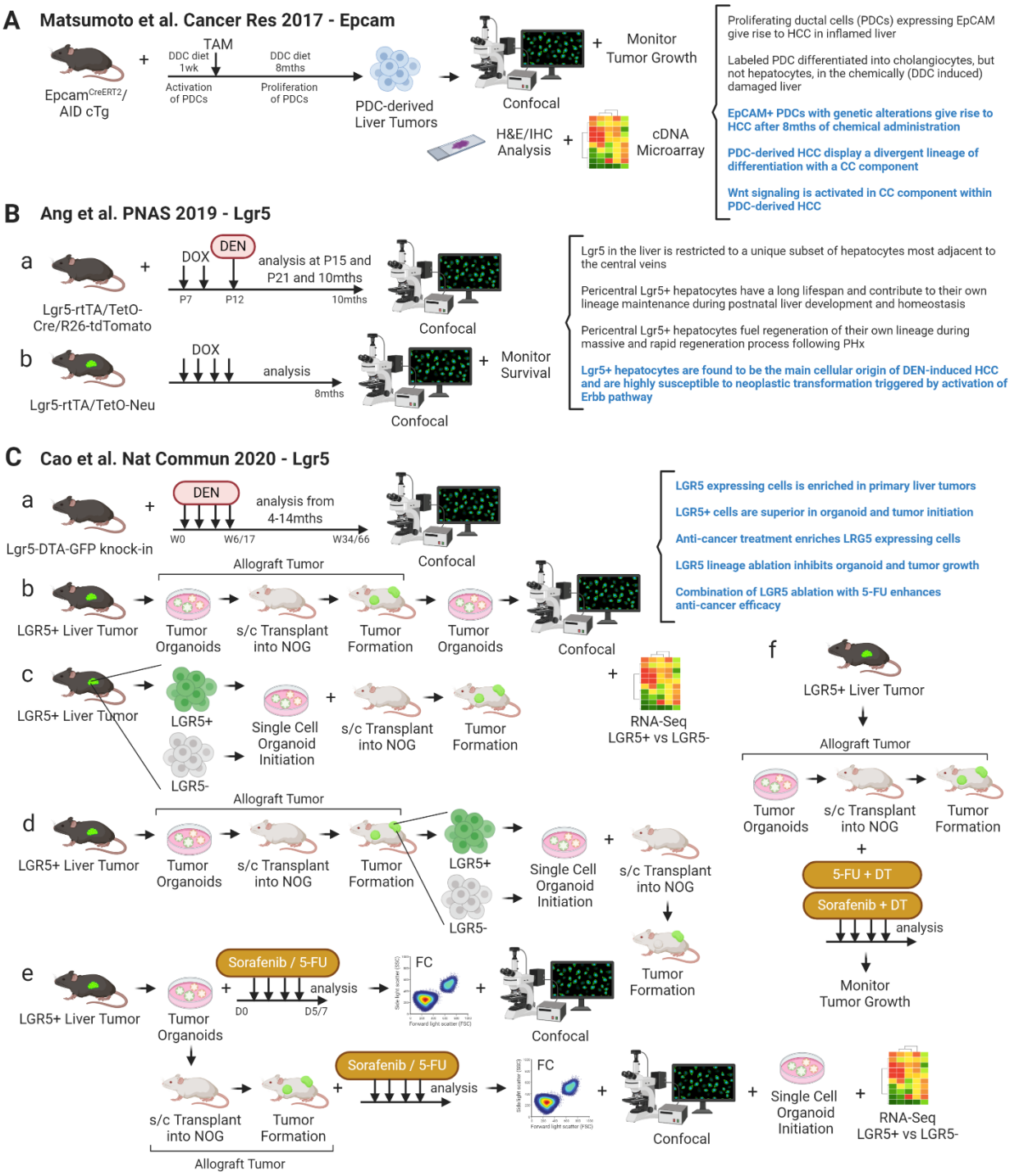
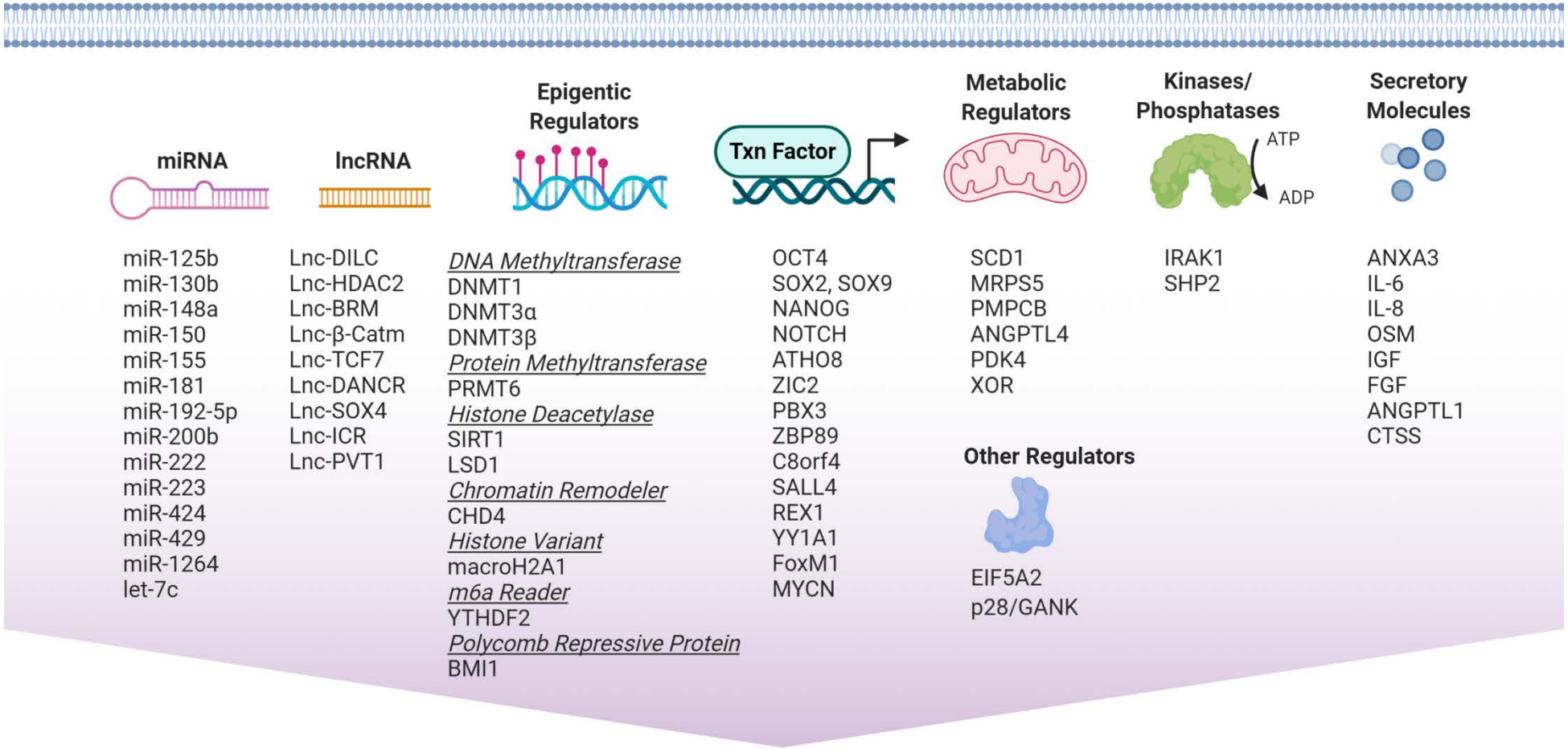

A Matsumoto et al. Cancer Res 2017 - Epcam
Epcam CreERT2/ AID cTg
TAM
DDC diet 1wk
Activation of PDCs
DDC diet 8mths
Proliferation of PDCs
PDC-derived Liver Tumors
Confocal
Monitor Tumor Growth
H&E/IHC Analysis
cDNA Microarray
Proliferating ductal cells (PDCs) expressing EpCAM give rise to HCC in inflamed liver
Labeled PDC differentiated into cholangiocytes, but not hepatocytes, in the chemically (DDC induced) damaged liver
EpCAM+ PDCs with genetic alterations give rise to HCC after 8mths of chemical administration
PDC-derived HCC display a divergent lineage of differentiation with a CC component
Wnt signaling is activated in CC component within PDC-derived HCC
B Ang et al. PNAS 2019 - Lgr5
a
Lgr5-rtTA/TetO-Cre/R26-tdTomato
DOX
DEN
analysis at P15 and P21 and 10mths
P7
P12
10mths
Confocal
b
Lgr5-rtTA/TetO-Neu
DOX
analysis
8mths
Confocal
Monitor Survival
Lgr5 in the liver is restricted to a unique subset of hepatocytes most adjacent to the central veins
Pericentral Lgr5+ hepatocytes have a long lifespan and contribute to their own lineage maintenance during postnatal liver development and homeostasis
Pericentral Lgr5+ hepatocytes fuel regeneration of their own lineage during massive and rapid regeneration process following PHx
Lgr5+ hepatocytes are found to be the main cellular origin of DEN-induced HCC and are highly susceptible to neoplastic transformation triggered by activation of Erbb pathway
C Cao et al. Nat Commun 2020 - Lgr5
a
Lgr5-DTA-GFP knock-in
DEN
analysis from 4-14mths
W0
W6/17
W34/66
Confocal
Allograft Tumor
b
LGR5+ Liver Tumor
Tumor Organoids
s/c Transplant into NOG
Tumor Formation
Tumor Organoids
Confocal
RNA-Seq LGR5+ vs LGR5-
c
LGR5+ Liver Tumor
LGR5+
LGR5-
Single Cell Organoid Initiation
s/c Transplant into NOG
Tumor Formation
d
LGR5+ Liver Tumor
Allograft Tumor
Tumor Organoids
s/c Transplant into NOG
Tumor Formation
LGR5+
LGR5-
Single Cell Organoid Initiation
s/c Transplant into NOG
Tumor Formation
e
LGR5+ Liver Tumor
Tumor Organoids
Sorafenib / 5-FU
analysis
D0
D5/7
FC
Confocal
s/c Transplant into NOG
Tumor Formation
Sorafenib / 5-FU
analysis
FC
Confocal
Single Cell Organoid Initiation
RNA-Seq LGR5+ vs LGR5-
Allograft Tumor
f
LGR5+ Liver Tumor
Allograft Tumor
Tumor Organoids
s/c Transplant into NOG
Tumor Formation
5-FU + DT
Sorafenib + DT
analysis
Monitor Tumor Growth
LGR5 expressing cells is enriched in primary liver tumors
LGR5+ cells are superior in organoid and tumor initiation
Anti-cancer treatment enriches LRG5 expressing cells
LGR5 lineage ablation inhibits organoid and tumor growth
Combination of LGR5 ablation with 5-FU enhances anti-cancer efficacy

Figure 3. Intrinsic regulators of cancer stemness in HCC.

A number of intrinsic regulators, many of which end up converging into common signaling pathways, have now been demonstrated as key players in promoting the acquisition of uncontrolled self-renewal and tumor-initiating potential in HCC. These regulators can be categorized into subclasses including miRNA, lncRNA, epigenetic regulators, transcription factors, metabolic regulators, kinases/phosphatases, secretory molecules and others.



Regulators Converge into Common Signaling Pathways

MAPK Signaling **WNT/β-catenin Signaling** **JAK/STAT Signaling** **HIPPO Signaling** **TGFβ Signaling** **BMP/SMAD Signaling**

IL6/STAT3 Signaling **NFκB Signaling** **Hedgehog Signaling** **JNK Signaling** **IGF/IGFR Signaling** **TLR4 Signaling** **Notch Signaling**

Figure 4. Microenvironmental regulators of cancer stemness in the HCC cancer stem cell niche. The tumor-associated stroma has been shown to play a significant role in regulating cancer stemness in HCC. CSC regulation by their niche operates through cell-cell interaction, secreted factors, cell-matrix interaction and biophysical properties of the niche. This figure illustrates key players in liver CSC-stroma interaction, including (1) biophysical properties - hypoxia and nutrient (glucose)-deprived niche, extracellular matrix remodeling, (2) stromal cells - endothelial cells, cancer-associated fibroblasts, adipocytes, (3) hepatitis viruses and (4) immune cells.

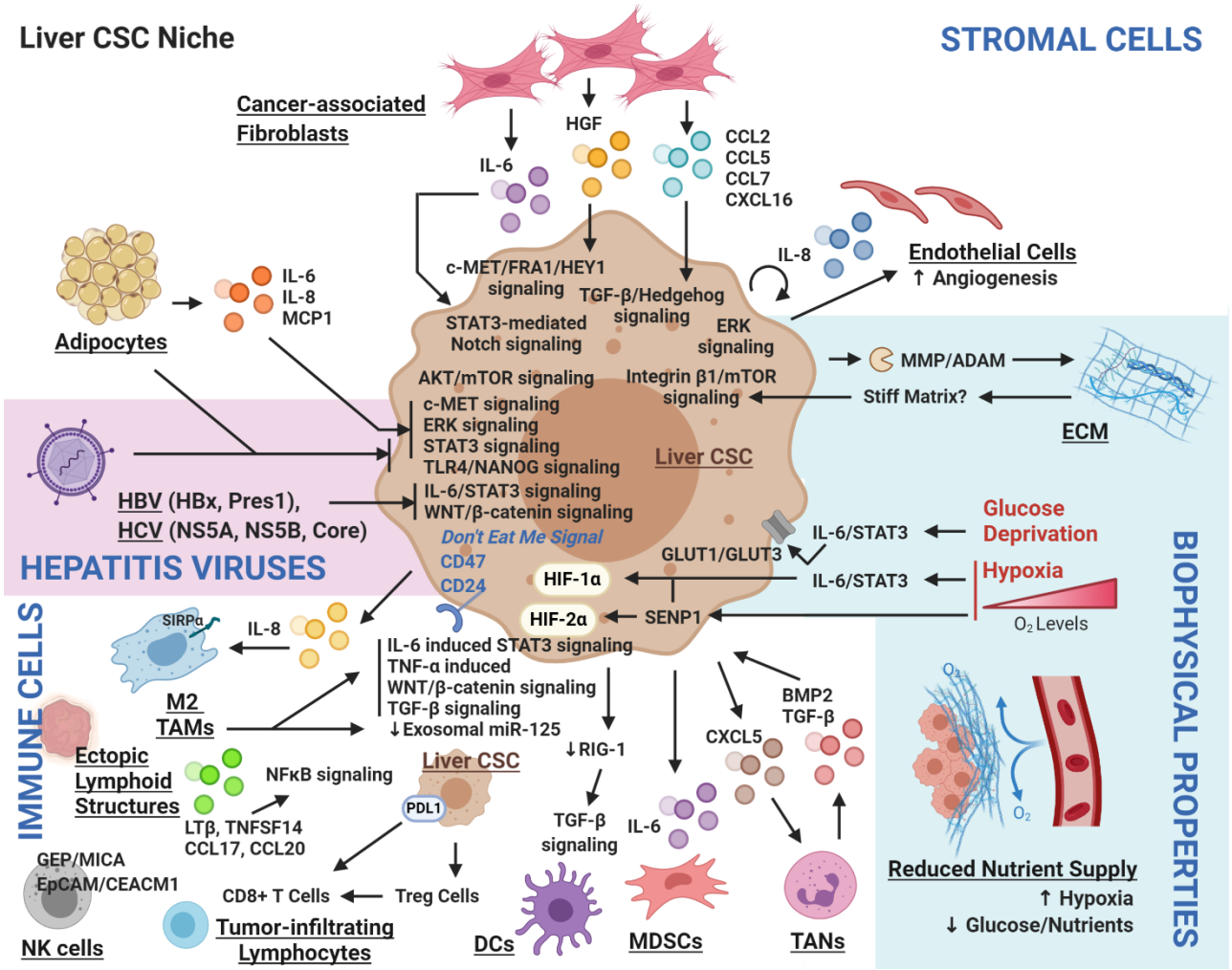


Figure 5. Proposed therapeutic approaches to target liver cancer stem cells.
This figures summaries novel approaches to target liver CSCs, as well as various available CSC-based therapies that can be potentially translated for use in HCC. This includes targeting liver CSC surface markers, targeting intrinsic and extrinsic regulators of liver CSCs, as well as liver CSC-directed therapy.

Targeting liver CSC surface markers.
CD13 - (i) Combination of a CD13 inhibitor and chemotherapy 5-FU reduced tumor volume compared with either agent alone in a mouse HCC model.[5] (ii) A CD13-targeting fusion protein, NGR-LDP-AE, composed of an NGR peptide and an anti-tumor antibiotic lidamycin, showed efficacy against liver CSCs and angiogenic endothelial cells, suggesting it to be a promising dual-targeting fusion protein for HCC therapy.[177] (iii) Using polyethylene glycol (PEG) as a drug delivery system, a recent study developed a novel polymer conjugate, namely, PEG-poly(lysine) block copolymer-ubenimex conjugate (PEG-*b*-PLys(Ube)), which can significantly inhibit CD13 activity and exert a potent anti-tumor effect in HCC.[178]
CD47 - Targeting CD47 by an antibody approach can mitigate HCC tumor growth, sensitize HCC cells to sorafenib and induce the phagocytosis of HCC cells.[16,157,179,180] Although the clinical testing of anti-CD47 antibody in HCC patients has not been carried out, a number of humanized anti-CD47 antibodies have entered clinical trials for the treatment of various other cancer types, including Hu5F9-G4 (Forty-Seven Inc.), IBI188 (Innovent Biologics), SRF231 (Surface Oncology), TTI-621 and TTI-622 (Trillium Therapeutics), and ALX148 (ALX Oncology).
CD44 - Anti-CD44 monoclonal antibody R05429083, which prevents the activation of various CD44-mediated signal transduction pathways by binding to the constant region of the extracellular domain of CD44.
EpCAM - Catumaxomab, a trifunctional antibody targeting EpCAM on tumor cells and CD3 on T cells that has the ability to redirect T cells and accessory cells such as macrophages, DCs and NK cells to the tumor site.[181,182]
CD24 - A bispecific protein rG7S-MICA targeted at CD24 is found to result in the recruitment of NK cells to accumulate in the tumor, inducing the release of cytokines and eventually leading to significantly increased anti-tumor activity in HCC tumors.[183]
CD133 - Bispecific antibodies that have dual-antigen-binding specificity to CD133 and CD3 has yielded promising results in preclinical studies in colorectal, pancreatic and hepatocellular carcinomas.[184,185]

Targeting intrinsic and extrinsic regulators of liver CSCs.
Liver CSC-associated self-renewal pathways - These drugs have been reviewed comprehensively elsewhere.[186]
IL-8 - A recent completed phase I clinical trial (NCT02536469) of a humanized anti-IL-8 monoclonal antibody (HuMax-IL-8, BMS-986253) in 15 patients with metastatic or unresectable locally advanced solid tumors found HuMax-IL-8 to be safe and well-tolerated.[187] Two recent back-to-back papers

published in Nature Medicine have found that elevated serum IL-8 was associated with enhanced intratumor neutrophils and intratumor myeloid cells and reduced clinical benefit of immune-checkpoint inhibitors, revealing the importance of assessing serum IL-8 levels in identifying unfavorable tumor immunobiology and as an independent biomarker in patients receiving immune-checkpoint inhibitors.[188,189] It will be meaningful to evaluate the efficacy of combining IL-8 blockade and immunotherapies approved for HCC against liver CSC and its niche.

ANXA3 - ANXA3 neutralizing monoclonal antibody cab suppress not only growth and self-renewal of HCC but also to sensitize HCC tumors to cisplatin and sorafenib/regorafenib, as well as to eradicate liver CSC subsets marked by CD133, CD24 and EpCAM.[105,106] ANXA3-transfected DCs to potently stimulate autologous T cells to preferentially kill CD133+ HCC cells.[104]

SHP2 - SHP099, an orally available SHP2 inhibitor, could suppress HCC tumor growth and override the adaptive resistance of sorafenib.[102] Numerous clinical trials on using SHP2 inhibitors to target various cancers are on-going, including TNO155 (Novartis), RMC-4630 (Revolution Medicines) and RLY-1971 (Relay Therapeutics, Inc).

STAT3 and NANOG - A recently completed phase 1/2 clinical trial (NCT02279719) tested the combination of BBI608 (STAT3 inhibitor, Napabucasin) and sorafenib or BBI503 (Nanog inhibitor) and sorafenib in advanced HCC patients with encouraging results and could potentially be explored in the targeting of liver CSCs in combination with sorafenib.

Differentiation inducers - Forced re-expression of HNF4α[190] and BMP4[122] as well as application of all-trans retinoic acid[191], have been shown to be effective in decreasing liver CSC subsets and chemoresistant subpopulations and improving the effect of chemotherapy.

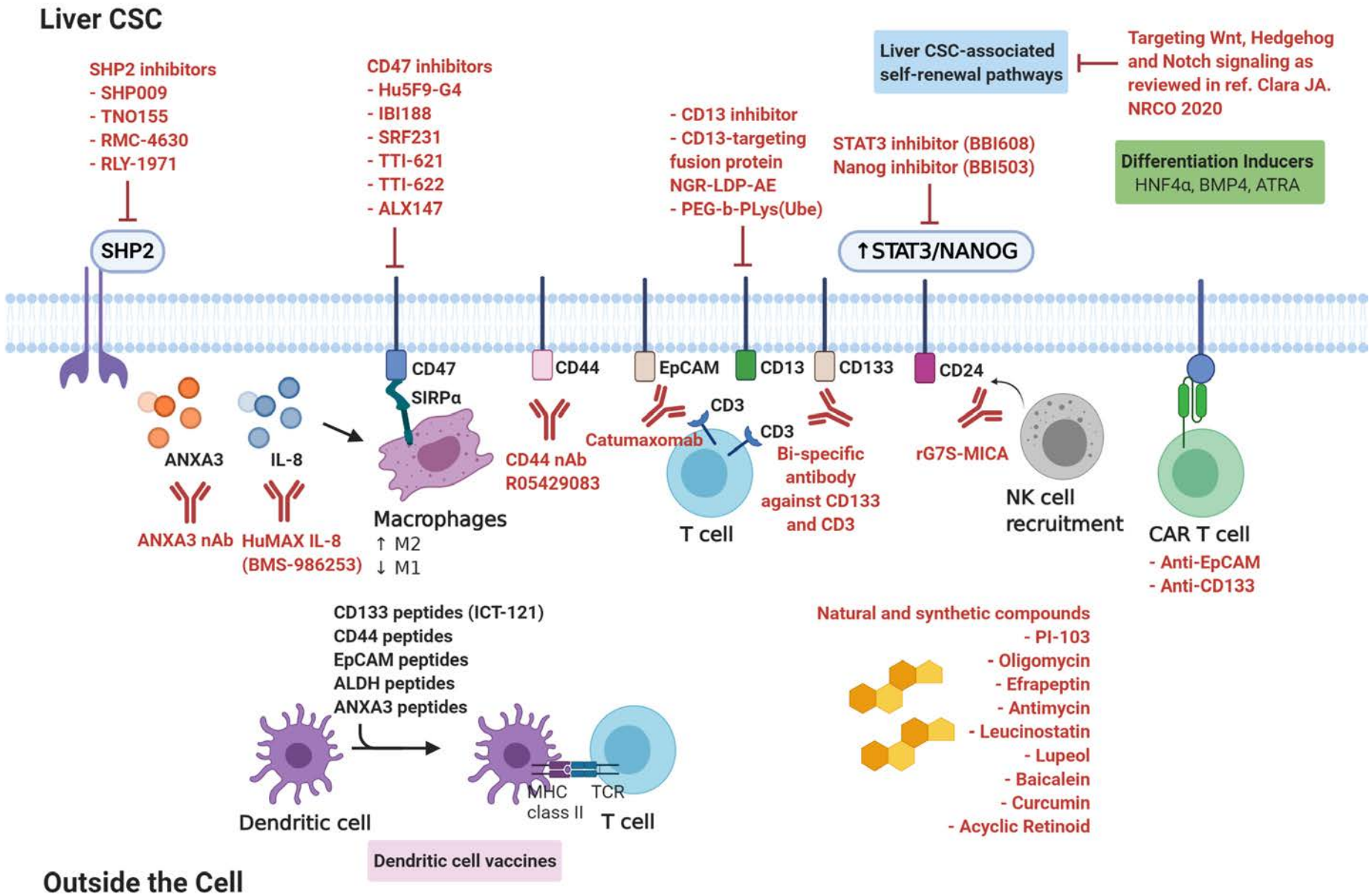
Natural and synthetic compounds - Small molecule (PI-103) and 4 natural compound analogues (oligomycin, efrapeptin, antimycin and leucinostatin) could selectively reduce viability of SALL4+ HCC cells through inhibiting oxidative phosphorylation.[192] Lupeol (a triterpene found in fruits and vegetables and a dietary phytochemical)[193], baicalein (a type of flavonoid found in plants)[194], curcumin (a chemical produced by plant roots)[195], and acyclic retinoid (ACR) (a synthetic vitamin A-like compound)[196] have all been separately demonstrated to target liver CSC through either inhibiting the PTEN/AKT/ABCG2 pathway[193], targeting SAR1B GTPase-mediated autophagy[194], hindering oncogenic NFκB-mediated HDAC signaling[195] or suppressing the transcription factor MYCN and the underlying Wnt/β-catenin signaling.[196]

Liver CSC-directed immunotherapy.

DC vaccines – (i) CD133 DC immunotherapy, ICT-121, shows promising phase 1 data (NCT02049489) by mounting a cytotoxic T cell response against CD133+ CSCs and suppressing tumor growth.[197] (ii) Pulsing DCs with CD44 and EpCAM peptides results in the efficient generation of mature DCS, thus enhancing T cell stimulation and generating potent cytotoxic T lymphocytes.[198] (iii) ALDH-presenting DCs have also demonstrated promising results in preclinical melanoma models.

CAR T cells - A recent phase I clinical trial tested the use of CD133-directed CAR T cells in the treatment of patients with CD133+ and late-stage metastatic malignancies, including HCC.[199]

Bi-specific antibodies – Against EpCAM, CD24 and CD133 as mentioned above.


Liver CSC
SHP2 inhibitors
- SHP009
- TNO155
- RMC-4630
- RLY-1971
SHP2
CD47 inhibitors
- Hu5F9-G4
- IBI188
- SRF231
- TTI-621
- TTI-622
- ALX147
Liver CSC-associated self-renewal pathways
Targeting Wnt, Hedgehog and Notch signaling as reviewed in ref. Clara JA. NRCO 2020
Differentiation Inducers
HNF4α, BMP4, ATRA
- CD13 inhibitor
- CD13-targeting fusion protein NGR-LDP-AE
- PEG-b-PLys(Ube)
STAT3 inhibitor (BBI608)
Nanog inhibitor (BBI503)
↑STAT3/NANOG
CD47
SIRPα
CD44
EpCAM
CD13
CD133
CD24
ANXA3
IL-8
ANXA3 nAb
HuMAX IL-8 (BMS-986253)
Macrophages
↑ M2
↓ M1
CD44 nAb R05429083
CD3
CD3
Catumaxomab
T cell
Bi-specific antibody against CD133 and CD3
rG7S-MICA
NK cell recruitment
CAR T cell
- Anti-EpCAM
- Anti-CD133
CD133 peptides (ICT-121)
CD44 peptides
EpCAM peptides
ALDH peptides
ANXA3 peptides
Dendritic cell
MHC class II
TCR
T cell
Dendritic cell vaccines
Natural and synthetic compounds
- PI-103
- Oligomycin
- Efrapeptin
- Antimycin
- Leucinostatin
- Lupeol
- Baicalein
- Curcumin
- Acyclic Retinoid
Outside the Cell

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