Review

Novel therapeutic Strategies for Targeting Liver Cancer Stem Cells

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Abstract

The cancer stem cell (CSC) hypothesis was first proposed over 40 years ago. Advances in CSC isolation were first achieved in hematological malignancies, with the first CSC demonstrated in acute myeloid leukemia. However, using similar strategies and technologies, and taking advantage of available surface markers, CSCs have been more recently demonstrated in a growing range of epithelial and other solid organ malignancies, suggesting that the majority of malignancies are dependent on such a compartment.

Primary liver cancer consists predominantly of hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC). It is believed that hepatic progenitor cells (HPCs) could be the origin of some HCCs and ICCs. Furthermore, stem cell activators such as Wnt/β-catenin, TGF-β, Notch and Hedgehog signaling pathways also expedite tumorigenesis, and these pathways could serve as molecular targets to assist in designing cancer prevention strategies. Recent studies indicate that additional factors such as EpCAM, Lin28 or miR-181 may also contribute to HCC progression by targeting HCC CSCs. Various therapeutic drugs that directly modulate CSCs have been examined *in vivo* and *in vitro*. However, CSCs clearly have a complex pathogenesis, with a considerable crosstalk and redundancy in signaling pathways, and hence targeting single molecules or pathways may have a limited benefit for treatment. Many of the key signaling molecules are shared by both CSCs and normal stem cells, which add further challenges for designing molecularly targeted strategies specific to CSCs but sparing normal stem cells to avoid side effects. In addition to the direct control of CSCs, many other factors that are needed for the maintenance of CSCs, such as angiogenesis, vasculogenesis, invasion and migration, hypoxia, immune evasion, multiple drug resistance, and radioresistance, should be taken into consideration when designing therapeutic strategies for HCC.

Here we provide a brief review of molecular signaling in liver CSCs and present insights into new therapeutic strategies for targeting liver CSCs.

Key words: Liver cancer stem cell, Hepatocellular carcinoma, Cholangiocellular carcinoma, Wnt/β-catenin signaling, TGF-β signaling, Notch signaling, Hedgehog signaling, BMI-1 signaling, EpCAM, Lin-28, miR-181, Self-renewal, Self-protection

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third leading cause of cancer death [1]. Despite some progress in the treatment of cancers, existing therapies are limited in their ability to cure malignancies and to prevent metastases and relapses. Surgery, radiofrequency ablation therapy and chemotherapy are all directed at reducing the bulk of the tumor mass. However, on

completion of therapy there is ultimately regrowth of tumor and relapse of diseases in the majority of cases [2-5]. Although the idea of tumor stem cells has been proposed for a number of decades, demonstration of their existence has only occurred within the last ten years. Recently, HCC progression has been thought to be driven by cancer stem cells (CSC) through their capacity for self-renewal, production of heterogeneous progeny, resistance to chemotherapy and to limitlessly divide. Advances were first achieved in hematological malignancies, with the first CSC demonstrated in acute myeloid leukemia. However, using similar strategies and technologies, and taking advantage of available surface markers, CSCs have been more recently demonstrated in a growing range of epithelial and other solid organ malignancies, suggesting that the majority of malignancies are dependent on such a compartment. Furthermore, many potentially and biologically significant surface markers and pathways that can modulate these stem/progenitor cells in cancer tissue have been successfully identified based on their dual role both in embryogenic stem cell development and tumor activation or suppression. In this review, we demonstrate a brief and up-to date review of molecular signaling in liver CSC and present insights into new therapeutic strategies for liver CSCs.

Liver stem cells in human liver regeneration

The liver is both an exocrine and an endocrine gland, which performs complex functions and has the phenomenal ability to regenerate. This process enables the recovery of the lost mass without endangering the viability of the entire organism [6, 7]. Many studies suggest that the existence of two basic types of liver regeneration [8, 9]. After acute liver injury, hepatic stem cells take part in normal tissue repair and homeostasis quickly [10]. In contrast, liver regeneration after loss of hepatic tissue does not depend on these kinds of cells, but on the proliferation of the existing mature hepatocytes, the parenchymal cells of the organ. In addition, other cells such as endothelial cells, Kuppfer cells, and Ito cells may also contribute to regeneration of the lost hepatic tissue [6].

The normal liver has been estimated to be replaced by normal tissue approximately once a year or more [11]. Therefore replacement rate of the normal adult liver was calculated to be 0.005-0.0025% at any time [12]. However, this slow normal renewal rate differs from the rapid proliferate response to loss of hepatic mass. In rodents, when two-thirds of the liver is resected (partial hepatectomy) the remaining remnant can regrow to the original liver size in approximately 10 days [7, 13]. In response to this stimulus, the normally quiescent hepatocytes leave G0 to enter the cell cycle under the influence of many growth factors. Hepatocyte proliferation begins in the periportal region of the liver and spreads to the centrilobular region. This regenerative response requires each hepatocyte to undergo only 2 rounds of replication to restore normal liver size. Hepatocytes are capable of large-scale clonal expansion within a diseased liver. Following very extensive liver damage or in situations in which hepatocyte regeneration after damage is compromised, a potential stem cell component located within the smallest branches of the intrahepatic biliary tree is activated. Hepatic progenitor cells (HPCs) amplify a biliary population of transit amplifying cells that are bipotential, capable of differentiating into either hepatocytes or cholangiocytes. These cells have been observed after severe hepatocellular necrosis, chronic viral hepatitis, alcoholic liver disease, and nonalcoholic fatty liver disease. It is thought that the activation of a potential stem cell compartment leads to the formation of reactive ductules, anastomosing cords of immature biliary cells with an oval nucleus and small rim of cytoplasm. Differentiation toward the hepatocyte lineage occurs via intermediate hepatocytes, polygonal cells with a size and phenotype intermediate between progenitor cells and hepatocytes. Intermediate hepatocytes become more numerous with time and extend further into the liver lobules. This sequence of changes suggests gradual differentiation of human progenitor cells into intermediate hepatocytes.

The Hepatocyte proliferation rate increases in chronic hepatitis with increased histological appearance of cellular damages until cirrhosis is reached, at which point the proliferation rate falls [14]. This fall probably reflects replicative senescence, although the diversion of blood flow through the liver probably plays a part [15]. The reduction in hepatocyte proliferation indices in chronic hepatitis occurs concurrently with the activation of HPCs [16, 17]. The development of an oval cell reaction in response to hepatocyte replicative senescence has been demonstrated in a transgenic mouse model of fatty liver and DNA damage [18]. In this model, mice developed fatty livers and large number of senescent hepatocytes. A striking oval cell response related to the number of senescent mature hepatocytes. The hepatocytes generated from oval cells in severely-damaged cirrhotic livers may have a high risk for neoplastic transformation.

Stem cells in the liver are proposed to be from two origins: endogenous or intrahepatic and exogenous or extrahepatic. Included in the intrahepatic stem cell compartment are the HPCs which are

greater in number but with short-term proliferative capacity. HPCs are thought to be localized within the canals of Hering, interlobular bile ducts [19, 20]. Included in the extrahepatic stem cell compartment are cells derived from bone marrow and peripheral blood cells; these cells are usually few but with long-term proliferation capacity [21-23].

Human Liver Progenitor Cells and Cancer Stem Cells

Human hepatic stem cells most likely give rise to HCC as well as ICC [7, 24-26]. The hypothesis that HCC arises from HPCs is supported by the finding that many tumors contain an admixture of mature cells and cells phenotypically similar to HPCs. Detailed immunophenotyping of HCCs revealed that 28-50% of HCCs express markers of progenitor cells such as CK7 and CK19 [27]. These tumors also consist of cells that have an intermediate phenotype between progenitors and mature hepatocytes. In fact, patients who have HCCs that express hepatocyte and biliary cell markers such as albumin, CK7 and CK10 carry a significantly poorer prognosis and have a higher recurrence rate after surgical resection and liver transplantation [28]. Cells resembling HPCs have also been noted in hepatoblastoma; the most common liver tumors in children which are widely believed to be stem cell derived given there can be both epithelial and mesenchymal tissue components. These tumors can even have structures mimicking intrahepatic bile ducts and form ductal plate-like structures [29] (Fig. 1).
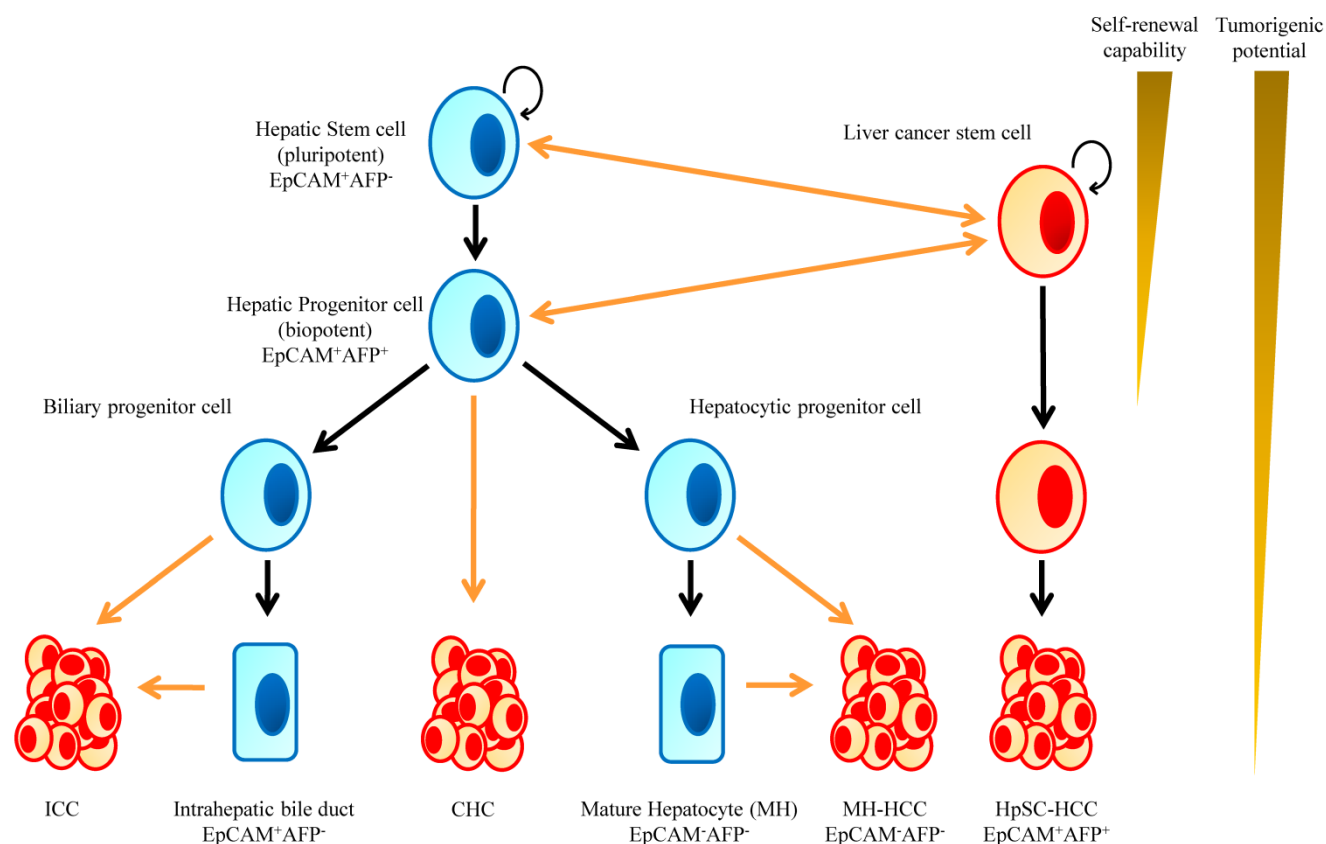

Figure 1. Implication of Stem Cells in Liver Development and Hepatic Tumorigenesis. Normal hepatic stem cells are characterized by their ability to self-renew and differentiate, which leads to formation of a normal liver tissues. Oncogenic mutations in normal stem/progenitor cells or even in differentiated cells enhance or endow the cells with self-renewal capability. Consequently, these cells function as cancer stem cells and contribute to the formation of bulk tumors.

Liver cancer stem cell and primary liver cancer

Hepatocellular carcinoma

The CSC hypothesis is based on the idea that stem cells are present also in cancer tissue and a hierarchy of cells is formed, as is the case with normal tissue. Tumor formation, growth, and propagation are maintained by a minute proportion of cells with stem cell-like properties. Now, CSCs in HCC can be identified by several cell surface antigens including c-kit, CD133, CD90, CD44, OV6, and CD326 (EpCAM), or by selecting the side population (SP) cells by Hoechest dye-staining.

SP cells in HCC cells possess high proliferation potential, tumorigenicity, and anti-apoptotic properties compared with those of non-SP cells [30, 31]. Furthermore, CD133+ cells isolated from HCC cells possess a greater ability to form tumors in vivo and have characteristics similar to those of progenitor cells including the expression of "stemness" genes, the ability to self-renew, and the ability to differentiate into nonhepatocyte-like lineages [32]. In addition, CD133+ HCC cells represented only a minority of the tumor cell population in human HCC specimens, and increased CD133 expression levels were correlated with increased tumor grade, advanced disease stage, shorter overall survival, and higher recurrence rates compared with patients with low CD133 expression [33] . Tumor associated calcium signal transducer 1 (TACSTD1), which encodes a pancarcinoma antigen epithelial cell adhesion molecule (EpCAM), was identified to be an early biomarker of HCC [34, 35]. EpCAM is a direct transcriptional target of the Wnt-β-catenin canonical signaling pathway. EpCAM+alpha-fetoprotein (AFP)+ HCC subtype had features of hepatic stem/progenitor cells, and EpCAM+ HCC cells were correlated with tumor progression and invasiveness. Additionally, this surface molecule is also highly expressed in premalignant hepatic tissues, HPCs and bile duct epithelium, but not in most adult hepatocytes [35-37].

Intrahepatic Cholangiocarcinoma

The origin of intrahepatic cholangiocarcinoma (ICC) is much less defined when compared with HCC. However, since that incidence and mortality of ICCs clarifying the origin of these tumors is important. Recent studies suggest that some ICCs could arise from liver stem cells rather than from mature cholangiocytes [38]. This concept is supported by the identification of a combined hepatocellular cholangiocarcinoma (CHC), which have morphological and phenotypical intermediate features between HCC and ICC [39]. The ability of HPCs to differentiate towards the biliary and the hepatocytic lineages gave rise to the hypothesis that transformed HPCs are the source of origin of intermediate primary liver carcinomas. Some animal models reveal that ICC can originate from HPCs [40, 41]. Furthermore, in a few cases of human ICCs, it has been reported that some tumor cells express specific markers of liver stem cells, indicating a possible stem cell origin [42, 43]. However, there is currently not enough data to make a statement regarding a stem cell origin of ICC and further immunohistochemical characteristics related to the expression of hepatic stem cell markers in ICCs should be elucidated.

Molecular signaling of Liver Cancer Stem Cells

Wnt/β-catenin signaling pathway

The Wnt/β-catenin pathway is an evolutionarily well-conserved pathway to be essential to normal cellular processes such as development, growth, survival, regeneration, and self-renewal [44]. Disruption of Wnt/β-catenin signaling results from both genetic and epigenetic changes is associated with a range of diseases and is frequently found in many cancers, especially colon cancer and HCC. Disrupted Wnt/β-catenin signaling by mutational and non-mutational events is observed in about one third of all HCCs which emphasizes the importance of this pathway in hepatocarcinogenesis [45, 46]. The Wnt pathway diversifies into two main branches, i.e., canonical (β-catenin-dependent) and non-canonical (β-catenin-independent), which play critical roles in specifying cellular fates and movements, respectively, during both embryogenic development and adult tissue regeneration [47-49].

Wnt ligands signal through binding to seven transmembrane Frizzled (Fzd) receptors and single transmembrane lipoprotein receptor-related protein (LRP) 5 or 6 co-receptors [50]. Canonical signaling mediated by ligands such as Wnt3a inhibits a multiprotein degradation complex consisting minimally of axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK3β) [51-54]. This inhibition culminates in nuclear translocation of β-catenin, enabling it to interact with T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors to regulate gene expression [55].

Non-canonical signaling, which is much less defined, is mediated by ligands such as Wnt11 that uses the same Fzd receptors [56]. The Wnt-Fzd complex interacts with heterotrimeric G and Dv1 proteins to activate phospholipase C, which then generates di-

acylglycerol and inositol-phosphatase from phosphatidyl inositol 4, 5-biphosphate and increase intracellular calcium concentration. The Wnt-Fzd-G protein complex can also stimulate p38 kinase and activate phosphodiesterase 6, which hydrolyzes cyclic GMP and results in the inactivation of protein kinase G and an increase in intracellular calcium. Wnt-mediated increase in intracellular Ca^{2+} activates protein kinase C and calmodulin-dependent kinase 2 (CamKⅡ). CamKⅡ can activate calcineurin (CAN), and subsequently the NF-AT family of calcineurin-dependent transcription factors, as well as TAK1-NLK kinases. Signaling through the TAK-NLK kinases are proposed to inhibit canonical Wnt signaling. This pathway stimulates the Jun NH2-terminal kinase (JNK), Ca^{2+}/CaMKⅡ and PKC pathways. Both pathways interact with each other, and in some cases, non-canonical signaling antagonizes the canonical pathway [57].

The Wnt receptor, FZD-7 is found to be overexpressed in up to 90% of HCCs [45, 58]. Twenty to 40% of HCCs bear abnormal cytoplasmic and nuclear accumulation of β-catenin [59, 60]. However, not all studies show a correlation between elevated nuclear β-catenin and expression of its transcriptional targets implying that the expression of these target genes is likely to be regulated by alternative signaling mechanisms [59, 60]. While most of the proceeding mutations have not been detected in allelotype analysis, it is salient to note that deletions in the AXIN1 locus (16p) have been described in HCC. Axin1 and β-catenin mutations have also been identified in approximately 25% of HCCs [45, 46, 61-64] , while overexpression of the FZD-7 receptor and glycogen synthase kinase-3 (GSK-3) inactivation can also lead to aberrant β-catenin pathway activation. Elevated expression of Wnt and its downstream mediators was also reported in EpCAM+ liver CSCs [35]. It has been demonstrated that murine hepatic stem/progenitor cells transduced with mutant β-catenin acquired excessive self-renewal capability and tumorigenicity in a similar fashion to BMI1. In addition, the Wnt/β-catenin pathway is activated in both rodent oval cells and OV6-positive tumor cells, and it leads to HCC chemoresistance [65]. These findings indicate that Wnt/β-catenin signaling plays an important role in the maintainance of CSCs.

Recently, the mechanisms leading to malignant transformation of stem/progenitor cells were effectively addressed in pediatric tumors [66]. Hepatoblastoma is a malignant embryonal tumor of the liver, which differs from HCC by distinct morphological patterns reminiscent of hepatoblasts, the bipotent

precursors of hepatocytes and cholangiocytes, and of their arrangement in the developing liver. Integrated molecular and genetic studies of hepatoblastoma disclosed two major molecular subclasses of tumors that relate early and late phases of prenatal liver development. It has been suggested that hepatoblastoma might arise from impairment of the normal liver differentiation program associated with excessive Wnt/β-catenin signaling [62]. In addition, the interplay of Wnt/β-catenin and Myc signaling in immature tumors activates a distinct transcriptional program that correlates with tumor aggressiveness. Correlation between stage of hepatic differentiation and clinical manifestation, notably vascular invasion, metastatic spread, and patient survival, was also established [66]. These finding highlight the important role of dysregulated Wnt/β-catenin signaling in the transformation of stem/progenitor cells.

Recently, EpCAM was identified as a direct transcriptional target of Wnt/β-catenin signaling in HCC [35]. Adult hepatocytes are EpCAM-, while the bile duct epithelium is EpCAM+. In addition, expression of EpCAM was observed during fetal liver development, liver regeneration, and liver repair associated with cirrhosis. Moreover, EpCAM is a marker of hepatic progenitors, suggesting that EpCAM+ HCCs are of hepatic progenitor cell origin [67].

The EpCAM signaling can be activated by regulated intramembrane proteolysis (RIP) and shedding of extracellular domain of EpCAM (EpEX) [68]. Sequencial cleavage of EpCAM by tumor-necrosis-factor alpha converting enzyme (TACE/ADAM17) and a gamma-secretase complex containing presenilin 2 (PS-2) result in release of EpEX into the culture medium, and release of an intracellular domain of EpCAM (EpICD) into the cytoplasm. EpICD becomes a part of a large nuclear complex containing transcriptional regulators β-catenin and Lef, both of which are components of Wnt/β-catenin signaling. Four and one-half LIM domain protein 2 (FHL2) is essential for signal transduction by EpCAM. FHL2 further regulates localization and activity of TACE and PS-2. Through its function as a co-activator of β-catenin, FHL2 links EpICD with specific DNA sequences and gene regulation. FHL2 has the potential to serve as a scaffolding protein for various signaling proteins used by EpCAM [69]. A number of EpCAM-regulated target genes have been identified including c-myc and cyclins, and additional genes involved in cell growth and proliferation, cell cycle, and cell death. Upon interference with E-cadherin, EpCAM may increase the availability of its interaction partner β-catenin in the soluble fraction. Cross-talk with the Wnt pathway is possible at the level of β-catenin and Lef-1 interactions

with EpICD, and known for induction of the EPCAM promoter by Tcf4. These findings indicated that expression of EpCAM strongly linked with proliferation of stem cells and cancer development by cancer initiating cells after aberrant EpCAM re-expression.

TGF-β family

The TGF-β signaling pathway appears to be most prominent at the interface between development and cancer in liver and gut epithelial cells [70]. Smad signaling has been shown to be pivotal for embryogenic hepatocyte proliferation, as well as in the formation of gastrointestinal cancers [71, 72]. Smad activation is modulated by various receptor- or Smad-interacting proteins that include ubiquitin and small ubiquitin-related modifier (SUMO) ligases, as well as multiple adaptor proteins that include Smad anchor for receptor activation (SARA), Filamin and β2-SPECTRIN. β2-SPECTRIN is crucial for the propagation of TGF-β signaling. Specifically, β2-SPECTRIN associates with Smad3, presenting it to the cytoplasmic domain of the TGF-β Type I receptor complex; followed by heteromeric complex association with Smad4, nuclear translocation and target gene activation [73]. Disruption of β2-SPECTRIN in mice leads to disruption of TGF-β signaling and results in a phenotype similar to Smad2^{+/-}/Smad3^{+/-} mutant mice, mid-gestational death due to gastrointestinal, liver, neural and heart defects, and loss of intrahepatic bile ducts. Interestingly, while the liver lineage is established, hepatocytes are poorly formed and liver architecture is lost with an absence of primitive bile ducts as in the Smad2^{+/-}/Smad3^{+/-} mutants. Moreover, bile duct formation can be induced in liver explants cultures treated with TGF-β [74].

Many studies have reported a reduction of TGF-β receptors in up to 70% of HCC [58, 75]. However, Smad proteins shown to be impaired in other cancers appear to play a minor role in HCC [76, 77]. Yet, TGF-β levels in serum and urine are increased in HCC patients [78, 79]. In addition, up to 40% of HCC have increased TGF-β expression based on immunohistochemical analysis [80, 81]. High TGF-β levels have been correlated with advanced clinical stage of HCC [82, 83]. This dual role of TGF-β signaling in HCC was explained by its effect on the tumor tissue microenvironment and on selective loss of the TGF-β-induced antiproliferative pathway [75]. Tumor cells that have selectively lost their growth-inhibitory responsiveness to TGF-β but retain an otherwise functional TGF-β signaling pathway may exhibit enhanced migration and invasive behavior in response to TGF-β stimulation. TGF-β signaling also has been shown to induce an epithelial to mesenchymal transition (EMT) process in tumor cells. EMT leads to enhanced migration and invasiveness [84]. Recently, loss of ELF, a TGF-β adaptor and signaling molecule, in the liver leads to cancer formation by deregulated hepatocyte proliferation and stimulation of angiogenesis [85]. More recently, it was reported that STAT3/Oct4-positive HCC cells, which have dysfunctional TGF-β signaling, are likely cancer progenitor cells that have the potential to give rise to HCC [86].

Notch pathway

The Notch signaling pathway plays an important role in stem cell self-renewal and differentiation [87-90]. However, other signaling pathways influence whether Notch functions as a tumor suppressor or oncogene in a particular tissue [91]. Notch signaling is activated through four receptors (Notch 1-4) that can interact in a redundant manner with five ligands of the Delta/Jagged family [49, 92]. Ligand binding triggers a γ-sevretase-dependent proteolytic cleavage of Notch receptor and the release of Notch intracellular domain (NCID) to the nucleus [93] , which in turn displaces the co-receptors associated with CSL transcription factors (CBF1 in humans; RBPJ in mice). Activating transcription factors are then recruited and expression of target genes such as Hairy and Enhancer of Split (HES1 and HES5) and Deltex1 is induced [92, 94].

Notch signaling plays a well-defined role in liver embryogenesis and bile duct formation. In addition, Notch family members are involved in angiogenesis and endothelial sprouting [95-97]. Increased expression of genes involved in this pathway has been shown in CD133^{+} liver cancer cells as compared to CD133^{-}. The activated intracellular form of Notch3, as well as the notch ligand Jagged, is highly expressed in HCC [98-100]. Notch-dependent transformation is associated with extracellular signal-regulated kinase activation downstream of the Ras pathway, which increases Notch mRNA stability and is required for transcription of the Notch target gene, Hes-1 [101, 102]. Conversely, Notch-1 has been reported to function as a tumor suppressor and participate in cross-talk with other signaling pathways such as Ras/Raf/MEK/ERK through the regulation of the PTEN tumor suppressor [103]. Recent evidence indicates that activation of Notch1 signaling increases the expression level of death receptor 5 (DR5) with enhancement of TRAIL-induced apoptosis *in vitro* and *in vivo* [104, 105]. Inhibitors of the NOTCH pathway are currently under investigation in clinical trials for treating solid tumors although the effectiveness of

NOTCH pathway inhibitors in treating liver cancer remains unclear.

Hedgehog signaling

Conserved from *Drosophilia* to humans, the Hedgehog (HH) pathway has a central role in embryonic development and adult tissue homeostasis by controlling cell fate specification and pattern formation [49, 106]. The functional importance of this pathway is illustrated by the multiple birth defects and malignancies associated with mutations and/or aberrant activation of the pathway [107, 108]. Three HH ligands Sonic (SHH), Indian (IHH) and Desert (DHH) have been identified in mammals that can bind interchangeably to two related twelve-pass membrane Patched (Ptc) receptors [109] . In the absence of ligand, Ptc antagonizes the pathway by preventing the activity of another transmembrane protein Smoothened (Smo) [110, 111]. Binding of HH ligands to Ptc relieves this inhibition and activates target gene transcription factors (Gli-1, Gli-2, Gli-3) [112, 113]. Like β-catenin, after ligand stimulation, Gli accumulates in the nucleus and induces transcription of genes related to cell cycle and growth including insulin-like growth factor-2 (IGF-2), cyclins, and β-catenin. The different Gli proteins exhibit activating or repressing transcriptional activators depending on proteolytic processing of the full-length proteins. Gli-1 and Gli-2 mainly act as transcriptional activators, while Gli-3 generates a repressor form (Gli3R) in the absence or inhibition of HH signaling [109, 114, 115]. Although functional significance of Gli-3 has been demonstrated by genetic inactivation [116] , the molecular mechanisms that control Gli-3 interactions and targets are largely undefined, whereas the dynamic interplay between Gli-1 and Gli-2 signaling is well documented.

Sonic is the predominant isoform in the liver. Up to 60% of human HCC express Sonic, and concomitant downregulation of Gli-related target genes are found after specific blockade of this pathway [117, 118]. Furthermore, tumorigenic activation of Smo can mediate overexpression of c-myc, a gene known to play an important pathogenic role in liver carcinogenesis [118]. Moreover, recent studies also showed that activation of Hedgehog signaling is critically related to CSCs and EMT features in many types of cancers including colon, gastric, esophagus, hepatic, and other cancers.

BMI1 signaling pathway

BMI1 is a part of the polycomb group genes (PcG) that are highly conserved throughout evolution. BMI acts as an epigenetic chromatin modifier and is known for its contribution to embryonic and stem cell self-renewal programs [119]. It is frequently overexpressed in different cancer types and disruption of BMI1 signaling has been linked to the activation of the hedgehog pathway in some cancers, such as medulloblastoma [120, 121]. Furthermore, BMI1 upregulation is associated with malignant transformation and acquisition of the malignant phenotype in HCC [122]. Aberrant BMI1 expression is reported in many CSC populations and it has been shown to have a critical role in maintaining and propagating the SP population in liver cancer. BMI1 is also highly expressed in CD133+ liver CSCs. The role of BMI1 in liver CSC maintenance is confirmed by ectopic expression of BMI1 in murine hepatic stem/progenitor cells. In these cells, BMI1 and the Wnt/β-catenin pathway regulate the self-renewal of normal or cancer stem cells in liver. Furthermore, BMI1 knockdown in SP cells completely abolished the tumorigenicity of SP cells [32, 123, 124]. Moreover, repression of targets of BMI1 plays a crucial role in the oncogenic transformation of hepatic stem/progenitor cells [125].

In addition to these signaling pathways, signal transducer and activator of transcription 3 (STAT3), mainly activated by IL-6 and its related cytokine, and IL-22 has been shown to play key roles in acute phase response, a protection against liver injury, the promotion of liver regeneration [126]. Furthermore, hyperactive STAT3 signaling results in expansion of oval cell numbers and trigger wound healing, cell migration, and proliferation [127, 128]. This signaling pathway may take part an important role of maintenance of CSCs.

Stem cell signaling network

Multiple studies have suggested that Wnt/β-catenin, Notch, Hedgehog, FGF, and TGF-β/BMP signaling network is implicated in the maintenance of tissue homeostasis by regulating self-renewal of normal stem cells as well as proliferation or differentiation of progenitor cells [129-133]. Especially, it is well established that Wnt/β-catenin and Hedgehog signaling pathways are critical for embryogenic development, as well as in the biology of CSCs and in the acquisition of EMT. Breakage of the signaling network for normal stem cells leads to the transformation to CSC. Alternatively, acquisition of self-renewal potential in progenitor cells due to epigenetic change or genetic alteration of stem cell signaling related genes gives rise to CSC. Detailed analyses on the dysregulation of Wnt/β-catenin, Notch, Hedgehog, FGF, and TGF-β/BMP signaling pathways in CSCs derived from a various type of human tissues or organ should be systematically investigated to

better understand CSCs themselves as well as the role they play in carcinogenesis.

miRNA

MiRNAs play critical roles in many biological processes including cancer by directly interacting with specific messenger RNAs (mRNAs) through base pairing and then inhibiting expression of the target genes through a variety of molecular mechanisms. MiRNAs can undergo aberrant regulation during carcinogenesis, and they can act as oncogenes or tumor suppressor genes. Disruption of miRNA expression levels in tumor cells may result from distorted epigenetic regulation of miRNA expression, abnormalities in miRNA processing genes and proteins, and the location of miRNAs at cancer-associated genomic regions. Consequently, abnormal miRNA expression is a ubiquitous feature of solid tumors including HCC [134]. In liver carcinogenesis, miRs have been found to have both tumor suppressive (miR-122, miR-26, miR-223) and oncogenic (miR-130b, miR-221, miR-222) activity [135-139]. Clearly, miRNAs play a critical role in carcinogenesis and oncogenesis. Emerging evidence suggests that certain abnormal miRNA expression induces CSC dysregulation, resulting in unlimited self-renewal and cancer progression. Therefore, miRNA expression is a vital key to CSC dysregulation.

Lin28 and let-7 signaling

Lin28 was first characterized in the nematode *Caenorhabditis elegans* as an important regulator of developmental timing [140, 141]. Recently, Lin28 was used together with OCT4, NANOG and SOX2 to reprogram human somatic fibroblasts to pluripotency [142]. Overexpression of these stem cell factors has been reported to promote oncogenesis by driving self-renewal and proliferation [142]. Moreover, poorly differentiated, aggressive human tumors have recently been shown to have an embryonic stem cell-like gene expression signature; these stem cell factors have also been reported to have roles in tumor progression.

LIN28 and LIN28B are overexpressed in primary human tumors and human cancer cell lines (overall frequency 15%) [143]. The mammalian homologs of lin-28, Lin28 and Lin28b, bind to the terminal loop of the precursors of let-7 family miRNAs and block their processing into mature miRNAs [144, 145]. In HCC, LIN28B-expressing tumors are associated with advanced stage [143]. Moreover, LIN28B-expression was associated with a significantly increased incidence of early recurrence. LIN28 is associated with an advanced disease and poor clinical outcome in HCC

[143, 146, 147]. The initiation of hepatocarcinogenesis is linked to chronic inflammation clinically and epidemiologically. The positive feedback loop involving NF-κB, Lin28B, let-7, and IL-6 is required for maintenance of the transformed phenotype and stem cell population [148] (Fig. 2).

miR-181

Mir-181 was first characterized in the patients with acute myeloid leukemia as a predictor of prognosis. Recently, we have identified a subset of highly EpCAM+ HCC cells from AFP+ tumors with cancer stem/progenitor cell features [149]. MiR-181 family members are up-regulated in EpCAM+AFP+ HCC cells. Moreover, miR-181 family members were highly expressed in embryogenic livers and isolated hepatic stem cells. Forced expression of miR-181 induces stemness of HCC cells while inhibiting miR-181 results in cell differentiation and inhibition of tumorigenicity. In addition, we identified three targets of miR-181, caudal-related homeobox 2 (CDX2), GATA6 and NLK. CDX2 and GATA6 are transcription factors and link to cancer stemness. NLK is a negative regulator of Wnt/β-catenin signaling. We propose that miR-181 maintains HCC stemness by inhibiting CDX2, GATA6 or NLK. MiR-181 could directly target hepatic transcriptional regulators of differentiation and an inhibitor of Wnt/β-catenin signaling [149]. Therefore, miR-181 and its signaling molecules could be molecular targets for inhibiting CSCs (Fig. 3).

Therapeutic target of Molecular signaling

The successful eradication of cancer requires anticancer therapy that affects the differentiated cancer cells and the potential CSC population [150, 151]. At present, conventional anticancer therapies include chemotherapy, radiation and immunotherapy kill rapidly growing differentiated tumor cells, thus reducing tumor mass but potentially leave behind cancer-initiating cells. Therapies that exclusively address the pool of differentiated cancer cells but fail to eradicate the CSC compartment might ultimately result in relapse and the proliferation of therapy-resistant and more aggressive tumor cells. An ideal drug regime would kill differentiated cancer cells and, at the same time, specifically, selectively and quickly target and kill CSCs to avoid toxic side effects for other cell types and to disrupt the self-protection potential of CSCs. Moreover, CSCs clearly have a complex pathogenesis, with the potential for considerable crosstalk and redundancy in signaling pathways, and hence targeting single molecules or pathways may have a limited benefit in treatment. The use of combinations of therapies may be needed to overcome the complex

network of signaling pathways, and ultimately inhibit the signaling that controls tumor growth and survival. In addition to the factors in which CSCs possess by themselves, microenvironment surrounding them is important for maintenance, such as angiogenesis, vasculogenesis, and hypoxia. Many new therapeutic strategies targeting CSCs at various stages of differentiation and microenvironment of CSCs have been tried. We will be discussed below (Fig. 4).
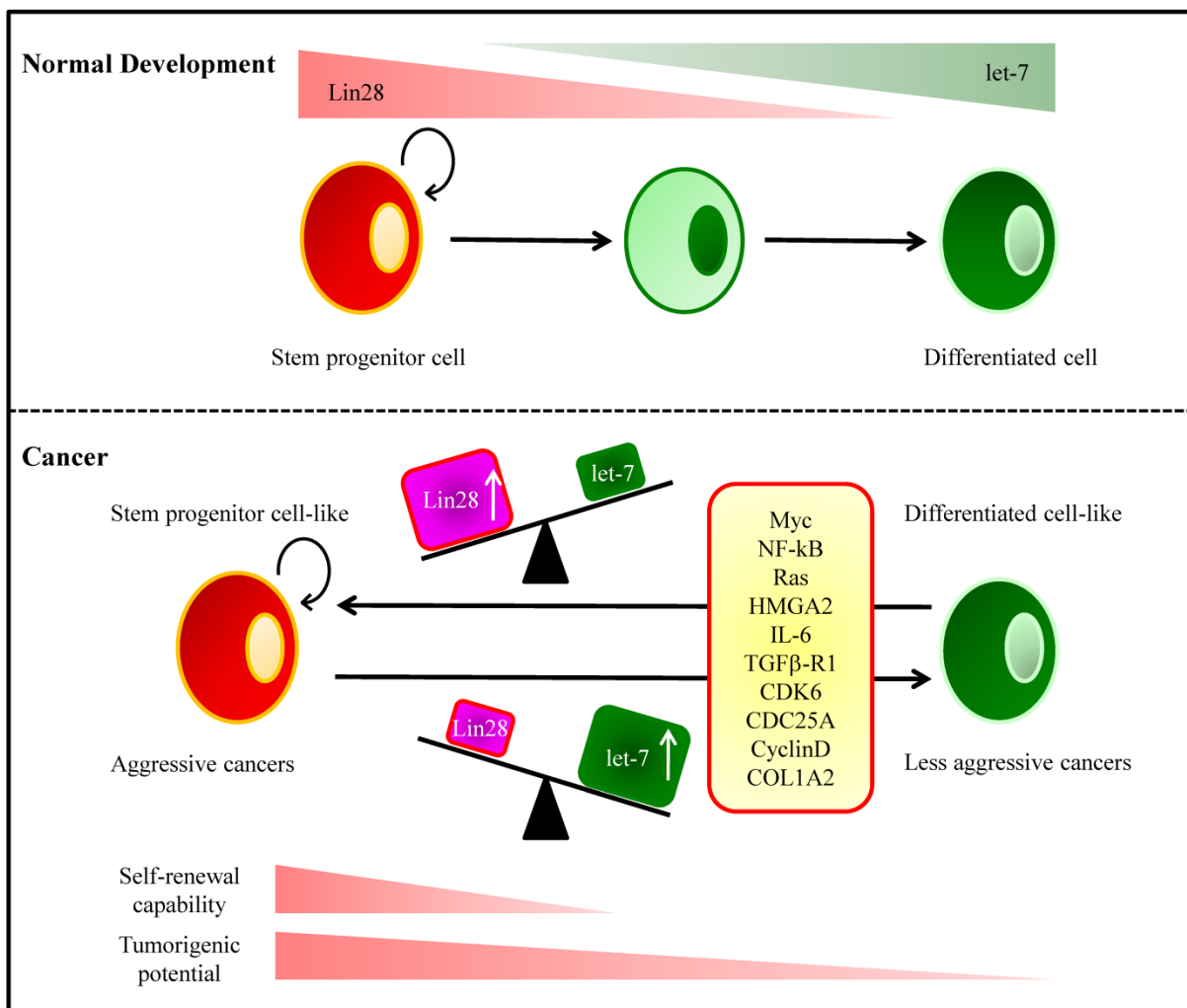

Figure 2. Let-7 and Lin28 in Development and Tumorigenesis. (Top) During normal development, the RNA-binding protein Lin28 is highly expressed in stem and progenitor cells. Lin28 blocks processing of let-7 miRNA precursor molecules into mature miRNAs, thereby maintains expression of genes that drive self-renewal and proliferation. As progenitor cells differentiate, Lin28 expression decreases, which allows let-7 processing and increased production of mature let-7 miRNAs. Let-7 miRNAs repress the expression of genes involved in self-renewal resulting in lineage commitment and terminal differentiation. (Bottom) Many molecules contribute to the balancing act of the Lin28/let-7 link in cellular differentiation and tumor progression. An imbalance between Lin28 and let-7 induced by these molecules can result in cellular transformation.
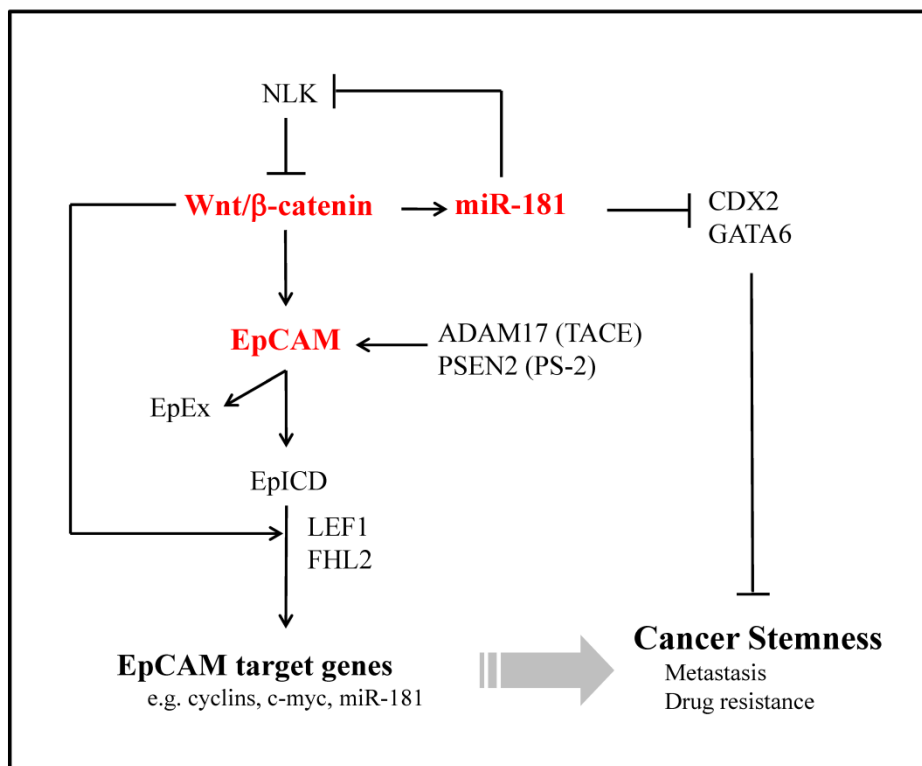
Figure 3. Potential Signalling Pathways of Wnt/β-Catenin, EpCAM and miR-181 Activated in Hepatic Cancer Stem Cells. Upon cleavage by TACE/PS-2, EpICD translocates into the nucleus in a multiprotein complex. Together with FHL2, β-catenin and Lef-1, EpICD contacts DNA at Lef-1 consensus sites. Owing to its ability to inhibit E-cadherin-mediated adhesion, EpCAM provides itself with β-catenin as a central interacting protein.
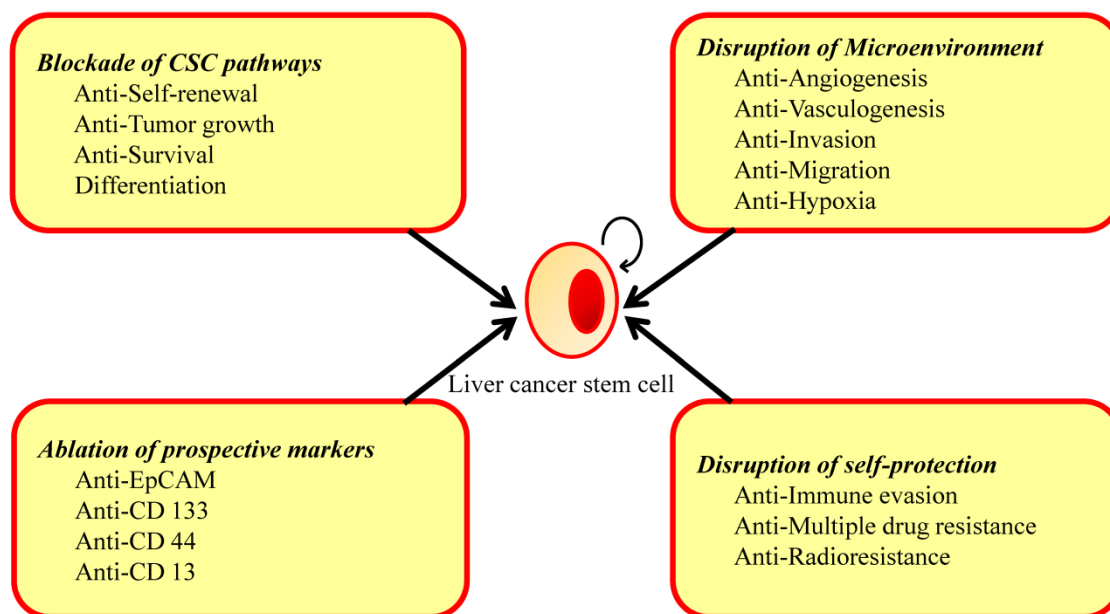

Figure 4. Strategies in Eradicate Liver Cancer Stem Cells. CSCs are protected from conventional therapies by changing their microenvironment and self-protection. Specifically targeting any of these areas may lead to the eradication of the CSCs.

Blockage of CSC pathways

Anti-Self-renewal

Targeting key signaling pathways for CSC self-renewal is one approach to therapy [123, 152, 153]. The Wnt/β-catenin signaling pathway is clearly important for the self-renewal and maintenance of stem cells [35]. Several experimental studies have demonstrated a decreased proliferation and an increased apoptosis resulting from inhibiting the Wnt/β-catenin signaling pathway [154]. The Wnt/β-catenin signaling pathway can be inhibited in a number of ways; for example, Dickkopf1 (Dkk1) binds to low-density LRP6 and prevents formation of the Fzd-Wnt-LRP6 complex [155]. A new approach to antagonize Wnt signaling has been the development of small molecules (XAV939) to inhibit the enzyme tankyrase that normally destroys the scaffold protein axin, a crucial component of the β-catenin destruction complex [156]. Furthermore, antibody-based therapeutic approaches targeting EpCAM are currently being developed [157, 158]. EpCAM-directed therapies will be efficacious in eradicating EpCAM-expressing CSCs.

The Hedgehog pathway is another potentially druggable target for CSC eradication. Several small-molecule modulators of Sonic hedgehog signaling have been used to regulate the activity of this pathway in medulloblastoma, basal cell carcinoma, pancreatic cancer, prostate cancer and developmental disorders [159]. In liver cells, suppression of the Sonic Hedgehog pathway by siRNA not only decreased HCC cell proliferation but also chemosensitized the cells to 5-fluorouracil (5-FU) and to the induction of cell apoptosis [160]. Furthermore, in hepatoblastoma, blocking Hh signaling with the antagonist cyclopamine had a strong inhibitory effect on cell proliferation of hepatoblastoma cell lines [161]. Thus, targeting intracellular pathways associated with self-renewal of CSCs remains a viable approach to be extended in the near future.

Anti-Tumor growth and inducing tumor cell differentiation

PTEN plays a role in the expansion of the CD133^{+} liver CSC population in liver-specific PTEN-deleted mice, which supports PTEN as a promising target in HCC therapy [162]. In addition, TGF-β family proteins have also emerged as key players in promoting the growth of stem cells in their undifferentiated state. A recent investigation revealed normal hepatic stem cells committing to malignant transformation due to aberrant TGF-β and activated IL-6 signaling [74, 163]. Therefore, inhibition of IL-6 signaling may be a potential therapeutic strategy in liver cancer treatment [163, 164].

CSC cells, which only make up a small proportion in cancer, have the capability to sustain tumor growth and are more resistant to conventional chemotherapy than other differentiated cancer cells. One approach to treat malignancies is to induce differentiation of the CSC cells. Differentiation therapy could force hepatoma cells to differentiate and lose their self-renewal property. Hepatocyte nuclear factor-4α (HNF4α), a central regulator of differentiated hepatocyte phenotype, suppresses a tumorigenesis and tumor development by inducing HCC differentiation, especially CSC cells [165]. Interferon therapy is effective for eradicating hepatitis viruses and also preventing the development of HCC. Interferon alpha treatment accelerated hepatocytic and biliary differentiation in oval cell lines [166]. Interferon could be applied to the treatment of HCC by targeting CSCs. In addition, oncostatin M (OSM), an interleukin 6-related cytokine known to induce differentiation of hepatoblasts into hepatocytes, could be used to effectively induce differentiation and cell division of dormant EpCAM[+] liver CSCs, and the combination of OSM and conventional chemotherapy with 5-FU efficiently eliminates HCC by targeting both CSCs and non-CSCs [167]. These findings indicate that combination of differentiation therapy and conventional chemotherapy may be an effective treatment of HCC.

Liver stem/progenitor cell markers

The identification of CSC markers and their exploitation in targeted chemotherapy is an important research goal. It has been shown that CSCs in HCC can be identified by several cell surface antigens, e.g., CD133, CD90, CD44, OV6, and EpCAM, or by selecting the SP cells by Hoechst dye-staining. Given the phenotypic similarities between CSCs and normal stem cells, it is reasonable to infer that the surface phenotype of CSCs resembles that of normal hepatic stem cells.

Anti-CD133

CD133/prominin-1, a pentaspan membrane glycoprotein, is an important cancer stem cell surface marker in various solid tumor types, including liver [168, 169]. CD133 expressing cells have been suggested to be critical tumorigenic progenitors in HCC, conferring chemoresistance by preferential activation of the AKT/PKB and Bcl-2 cell survival response [170]. The treatment of CD133^{+} HCC cells with an AKT1 inhibitor, specific to the Akt/PKB pathway, significantly reduced the expression of the survival proteins. In addition, suppression of CD133 by a mu-

rine antibody to human CD133 conjugated to a potent cytotoxic drug reduced the proliferation rate of Hep3B cells in vitro and delayed tumor growth in a SCID mouse model [171]. These findings suggest that targeting of CD133 might be a novel therapeutic strategy for liver tumors.

Anti-CD44

CD133+/CD44+ HCC cells were more tumoigenic than those of CD133+/CD44- cells *in vivo*. A recent study suggested that CSC phenotype could be precisely defined by co-expression of CD133 and CD44 cell surface markers [172]. CD133+/CD44+ HCC cells showed stem cell properties, including extensive proliferation, self-renewal and differentiation into the bulk of cancer cells. In addition, recent studies also revealed that blocking CD44 signaling using an anti-CD44 antibody might be a potential strategy to eradicate liver CSCs and consequently cure those patients [172].

Anti-EpCAM

Currently, several EpCAM-targeting antibodies are in clinical development, which include Catumaxomab (Fresenius Biotech) and Adecatumumab ((MT201) Micromet, Inc.). Clinical trials have been conducted in various cancers, including breast, prostate and colon cancers [157, 158]. In liver cells, RNAi targeting of EpCAM significantly decreased the CSC pool and reduced both tumorigenicity and invasive capacity of CSCs [37, 173]. Since EpCAM expression is a downstream target of Wnt/β-catenin, these results may have implications for development of novel target therapies.

Anti-CD13

Recently, CD13 was identified as a marker for semiquiescent CSCs in human liver cancer cells. In mouse xenograft models, combination of a CD13 inhibitor and 5-FU dramatically reduced tumor volume compared with either agent alone. 5-FU inhibited proliferating CD13+ semiquiescent CSCs, while CD13 inhibition suppressed the self-renewing and tumor-initiating ability of dormant CSCs. These results indicate that combining a CD13 inhibitor with a reactive oxygen species (ROS) -inducing chemo/radiation therapy may improve the treatment of liver cancer [174].

Disruption of Microenvironment

Hypoxia has been identified as a major cause of hypervascularization in HCC, and in patients with HCC, disease free survival is shorter when tumors express high levels of hypoxia-inducible factor-1α (HIF-1α). Hypoxia influences microenvironment in HCC and liver CSCs [175, 176]. Induction of tumor hypoxia combined with chemotherapy by transcatheter transarterial chemoembolization has been widely used in treating unresectable HCC, but tumor response rate is unsatisfactory and only a subgroup of patients benefit from this treatment [177, 178]. HIF-1α may be responsible for in this failure, as suggested by experimental findings obtained in a rat model of primary liver cancer [179]. Therefore, hypoxia-driven clonal selection of apoptosis-resistant tumor cells, together with hypoxia-induced MDR1 expression and angiogenesis, explain why hypoxic tumors are more resistance to conventional anticancer therapy. This justifies the current trials evaluating the use of anti-angiogenic therapy following HCC surgery. Several studies have established that tumor growth and invasion in HCC are dependent on dysregulated angiogenesis [180-182]. There is, therefore, a strong rationale for targeting growth factors that drive angiogenic process as a potential therapeutic strategy for the treatment of HCC. VEGF is a key angiogenic factor, and several agents that target VEGF or VEGFR are currently in development for the treatment of HCC [183, 184]. These agents include the tyrosine kinase inhibitors Vatalanib (PTK787) [185] and Cediranib (AZD2171) [186, 187], and the monoclonal antibody Bevacizumab (Avastin; Genentech, Inc., South San Francisco, CA, USA) [188], and multikinase inhibitors Sorafenib (Nexavar; Bayer HealthCare Pharmaceuticals Inc., Wayne, NJ, USA) [189-194], Sunitinib (Sutent; Pfizer Labs, New York, NY, USA) [195, 196], Brivanib (BMS-582664) [197-199], and Linifanib (ABT-869) [199]. In addition, ligands that bind to the EGFR, such as EGF, have a vital role in both tumor angiogenesis and proliferation, thought to be primarily through activation of the RAF/MEK/ERK and PI3K/AKT/mTOR pathways. Because of their efficacy in other solid tumors and the integral role of growth factors in HCC development and progression, it was hypothesized that agents specifically targeting EGF/EGFR signaling may also be beneficial in HCC. These agents include the tyrosine kinase inhibitors Erotinib [200, 201], Lapatinib (GW572016) [202, 203] and Gefitinib ((ZD1839) Iressa; Astrazeneca Pharmaceuticals LP, Wilmington DE, USA) [204-207], and the monoclonal antibody Cetuximab ((IMC-C255) Erbitux; ImClone LLC, New York, NY, and Bristol-Myers Squibb, Princeton, NJ, USA) [208, 209].

Disruption of self-protection

Anti-immune evasion

The observation that tumors progress in patients with HCC despite the presence of tumor-specific immune responses suggests that development of

HCC leads to a number of immunosuppressive mechanism, which are important to be considered when designing immunotherapy protocols. These mechanisms include production of immunosuppressive cytokines such as TGF-β and prostaglandins, impaired antigen-presenting cells, generation of inhibitory macrophages, increase in regulatory T cells and induction of myeloid-derived suppressor cells (MDSC). All these factors provide an environment that promotes angiogenesis, tumor survival and metastasis.

Targeting regulatory T cells has been of great interest to potentially remove the suppression of effecter T cells and enhance tumor-specific immune response. Depletion of regulatory T cells using anti-CD25 monoclonal antibodies or regulatory T cell-inhibiting agents, such as cyclophosphamide, has been shown to have anti-tumor effects in preclinical models [210-213]. In addition, MDSC suppress the cytokine production as well as the cytotoxic capacity of natural killer (NK) cells, playing a critical role in the host defense against cancer, in HCC patients [214]. Impaired NK cells can affect anti-tumor immune responses, which contributes further to tumor escape from both innate and adaptive immune responses in patients with HCC.

Anti-multiple drug resistance

Survival of stem-like cells in response to chemotherapeutic drugs is thought to be governed by the presence of active transmembrane adenosine triphosphate-binding cassette (ABC) transporter family members, such as MDR1, ABCG2 and ABCC2 [215]. It is believed that stem-like SP cells, which are known for their ability to efflux the DNA-binding dye Hoechst 33342, confer resistance to chemotherapeutic drugs, including cisplatin and doxorubicin, through expression high levels of such ABC transporters [216, 217]. In SP cells purified from HCC cell lines, inhibition of MDR1, ABCG2 and ABCC2 reverses their chemoresistance [218]. These cells have been shown to harbor other CSC-like properties, and may be related to the metastatic potential and chemoresistance of HCC [219]. Moreover, it was demonstrated that expression of granulin-epithelin precursor (GEP) and ABCB5 in liver CSCs is associated with chemoresistance and reduced survival times of patients with HCC [175].

Anti-Radioresistance

Several experimental and clinical findings provide evidence that the number of CSCs in a cancer affects its radiocurability. Recurrent tumors after radiotherapy could originate from one surviving CSC, and a permanent local tumor control requires inactivation of all CSCs [220]. Tumor cell hypoxia and tumor cell repopulation are the main factors causing radioresistance. Oxygen mediates the majority of the biological effects of sparsely ionizing radiation, and the response of cells to radiation depends strongly on the availability of oxygen. Various methods to deliver oxygen to cancer tissue have been studied. Enhanced tumor oxygenation has previously been achieved in an animal model using the synthetic heme-based oxygen carrier, albumin-heme which is a recombinant human serum albumin-Fe cyclohexanoil heme (rHSA-FeP). The rHSAFeP is a candidate radiation-enhancing drug, and arterial infusion of rHSA-FeP may serve as a local oxygeneation method that enhances the radiation effect [221].

Future Directions

The rapid development of the CSC field, combined with genome-wide screening techniques, has allowed for the identification of important new CSC markers and pathways, and these discoveries have contributed to one of the most important developments in cancer treatment. However, several important issues still remain to be resolved. Most of the key pathways important to CSCs are also shared by normal stem/progenitor cells and drugs targeting these pathways could have a detrimental effect on normal cells. For example, little is known about CSC directed therapies (e.g. targeting CD133 in CD133+ liver CSCs). Initial results are promising, but its potential short- and long-term side effects of these therapies are unclear. Such therapies will, if not specific for CSCs, lead to tissue and/or organ damage due to the depletion of the reserve/regenerative stem cells. Such treatment with off-target effects lead to acute and irreversible organ failure. Therefore, it is critical in delineating the molecular differences between CSCs and their tissue specific stem cell counterparts, to prevent damage to normal somatic stem cells and to ensure selectively targeting CSCs. This growing knowledge base has the potential to identify candidate genes and pathways that are important for CSC survival and propagation but are not important for normal stem cell function.

In addition, CSCs clearly have a complex pathogenesis, with the potential for considerable crosstalk and redundancy in signaling pathways, and hence targeting single molecules or pathways may have a limited benefit in treatment. Use of combinations of therapies may be needed to overcome the complex network of signaling pathways, and ultimately inhibit the signaling that controls tumor growth and survival. However, use of a combination regimen can lead to

tolerability and drug-drug interaction problems, and hence an alternative approach is to use molecularly targeted agents that have multiple modes of action. It is useful to understand which combination regimen is the most effective for inhibiting CSC survival and propagation with the least impact on normal stem cell function. When a sufficient number of CSC markers become available and an ideal combination therapy identify, CSC-specific therapies might be developed that spare healthy stem cells and thus reduce side effects and retain regenerative tissue capacities. Discoveries made in the CSC field will feed back into other areas of stem cell research because many marker gene products found in CSCs are shares with the normal stem cell population. It is also expected that a better understanding of the processes that control autonomous growth, differentiation and cell migration will contribute to novel regenerative-medicine-based treatments that will revolutionize therapeutic strategies and bring renewed hope to cancer patients.

Acknowledgements

Conflict of Interests

The authors have declared that no conflict of interest exists.

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