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REVIEW

Orchestration of hepatocellular carcinoma development by diverse liver cancer stem cells

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Abstract Hepatocellular carcinoma (HCC) is one of the world's most aggressive diseases and carries a poor prognosis for patients. Recent evidence suggests that HCC is organized by cancer stem cells (CSCs), which are a subset of cells with stem cell-like features. CSCs are considered a pivotal target for the eradication of cancer, and liver CSCs have been investigated using various stem cell markers. Several hepatic stem/progenitor markers have been shown to be useful for isolating putative CSCs from HCC, although the expression patterns and phenotypic diversity of CSCs purified by these markers remain obscure. Recently, we found that liver CSCs defined by different markers show unique features of tumorigenicity and metastasis, with phenotypes closely associated with committed liver lineages. Furthermore, our data suggest that these distinct CSCs collaborate to orchestrate the tumorigenicity and metastasis of HCC. In this review article, we summarize the recent advances in understanding the pathogenesis and heterogeneity of liver CSCs.

Keywords Hepatocellular carcinoma · Cancer stem cell · Tumorigenicity · Metastasis

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Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of death from cancer worldwide [1]. Its prevalence is mostly attributed to hepatitis B virus or hepatitis C virus infection, and high incidence is observed in Asia and Africa [2]. Increasing occurrences and mortality from HCC have also been observed in most industrialized countries [3]. Therefore, there is an urgent need to develop effective diagnostic and treatment strategies against this disease.

HCC is a heterogeneous disease in terms of morphology, biological behavior, response to treatment, and molecular profile [4]. This heterogeneity has traditionally been explained by the clonal evolution of tumor cells resulting from the progressive accumulation of multiple genetic and epigenetic changes [5, 6]. However, recent studies suggest that its heterogeneity may result from the hierarchical organization of tumor cells by a subset of cells with stem and progenitor cell features known as cancer stem cells (CSCs) [7]. CSCs are highly tumorigenic, metastatic, chemo- and radiotherapy resistant, responsible for tumor relapse after therapy, and able to divide symmetrically or asymmetrically to orchestrate the tumor mass [8]. Therefore, they are considered to be a pivotal target for eradicating HCC [9]. In this review, we summarize recent findings on liver CSCs in terms of heterogeneity and discuss an HCC treatment strategy that targets them.

CSC hypothesis

Cancer cells and stem cells have similar capabilities with respect to self-renewal, limitless division, and the generation of heterogeneous cell populations. The observation of these similarities many years ago led to the proposal that

cancer might be a type of abnormal stem cell disease [10], a concept which has recently been revisited [11]. The generally acknowledged definition of a CSC is a cell within a tumor that possesses the ability to self-renew and to give rise to heterogeneous lineages of cancer cells that comprise tumors in immunodeficient mice [11]. Experimentally, putative CSCs have been isolated using cell surface markers specific for normal stem cells. Stem cell-like features of CSCs have been confirmed by functional in vitro clonogenicity and in vivo tumorigenicity assays. Moreover, accumulating evidence suggests that CSCs play a role in perpetuating various cancers including leukemia and solid tumors [12–18].

In HCC, several markers are reported to enrich the CSC population, including the epithelial cell adhesion molecule (EpCAM), CD133, CD90, CD44, CD24, CD13, and oval cell marker OV6, as well as Hoechst dye efflux or aldehyde dehydrogenase activities [19–25]. Most of these markers are expressed in normal hepatic progenitors known as oncofetal markers [20–22, 26–35]. These marker-positive cells were experimentally confirmed to be more tumorigenic than marker-negative cells in immunodeficient mice using cell lines [9]. Among them, calcium channel α2δ1 isoform5, EpCAM, CD90, and CD133 are the markers confirmed thus far to enrich CSCs from primary HCCs [36, 37]. Recent studies have shown that some of these liver CSC markers are also functionally involved in the maintenance of CSC features (Table 1). EpCAM enhances Wnt signaling in ES cells and cancer [38, 39], and CD133 expression may maintain $CD133^+$ liver CSCs through the activation of neurotensin/IL-8/CXCL1 signaling [40]. CD44 regulates the redox status [41], while CD13 decreases cell damage induced by oxidative stress after exposure to genotoxic reagents [19]. Furthermore, a recent study demonstrated that the calcium channel α2δ1 isoform5, recognized by a monoclonal antibody 1B50-1, is expressed in liver CSCs and regulates calcium influx and ERK signaling [37]. Thus, the functional involvement of most liver CSC markers potentially makes them a good target for the eradication of liver CSCs. In particular, cell surface markers detected in liver CSCs may be good targets for immunotherapy.

Table 1 Cell surface markers in liver CSCs

Cell surface markers	Function in CSCs
Calcium channel α2δ1 isoform5	Calcium influx and activation of ERK signaling
CD13	ROS-induced DNA damage reduction
CD133	Neurotensin-interleukin-8-CXCL1 signaling
CD24	STAT3 mediated NANOG regulation
CD44	Regulation of redox status through xCT
CD90	Unknown
DLK1	Unknown
EpCAM	Activation of Wnt signaling
OV6	Unknown

Heterogeneity of liver CSCs

As described above, various hepatic progenitor markers have been detected in the population of liver CSCs. Purified cell populations using certain stem cell markers show CSC features such as high tumorigenicity, an invasive nature, and chemo- and radiotherapy resistance. However, it is unclear how these markers are expressed in primary HCC tissues or HCC cell lines. It is also unclear whether the CSCs expressing these markers exist in all HCCs or are restricted to a certain subtype. This is an especially important issue when treating HCC patients using molecularly targeted therapy against certain marker-positive CSCs.

In normal fetal livers, hepatoblasts express the biliary markers CK19 and EpCAM, as well as the hepatocyte markers albumin and alpha fetoprotein (AFP) [26, 27, 42, 43]. In addition, numerous studies have demonstrated that hepatic progenitor cells express a variety of markers putatively detected in various ectodermal or mesodermal lineages, including nestin, NCAM, CD34 and c-Kit, CD133, CD90, E-cadherin, and Dlk1 [44]. Hepatoblasts are also considered a heterogeneous population potentially organized in a hierarchical manner with various degrees of differentiation that may be related to their expression of stem cell markers [45]. Indeed, recent studies demonstrated that the characteristics of hepatic progenitors expressing different markers show distinct natures [32, 46]. Normal $EpCAM^+$ and $CD90^+$ oval cells represent two distinct populations: the former expresses classical oval cell markers such as AFP, OV-1, and CK19, and the latter expresses desmin and a-SMA but not AFP, OV-1, or CK19, which indicates that $CD90^+$ populations are more likely to be mesenchymal cells.

We explored the expression patterns of the representative liver CSC markers CD133, CD90, and EpCAM in primary HCC, and found that $EpCAM^+$ and $CD90^+$ CSCs show different gene expression patterns and cell morphology [36]. We further explored the tumorigenic capacity of sorted cells isolated from 15 primary HCCs and 7 liver cancer cell lines [36]. Although the number of samples analyzed was small, tumorigenic $EpCAM^+$, $CD133^+$, or $CD90^+$ CSCs were obtained in 26.6 % ($n = 4$), 20 % ($n = 3$), and 13.3 % ($n = 2$) of 15 HCCs, respectively, when xenotransplanted into NOD/SCID mice.

Interestingly, no EpCAM/CD90 double positive cells were detected in primary HCC, and $EpCAM^+$ and $CD90^+$ cells were distinctive with different tumorigenic/metastatic

capacities; that is, $EpCAM^+$ cells were associated with a high tumorigenic capacity and hepatic epithelial stem cell features, while $CD90^+$ cells had a metastatic propensity with mesenchymal vascular endothelial cell features. Importantly, the existence of $EpCAM^+$ cells correlated with high serum AFP values with a tendency for portal vein invasion, whereas the existence of $CD90^+$ cells was associated with a high incidence of distant organ metastasis. Furthermore, $CD90^+$ CSCs abundantly expressed c-Kit and showed chemosensitivity against the c-Kit inhibitor imatinib mesylate, whereas $EpCAM^+$ CSCs showed no such chemosensitivity. These data demonstrate that liver CSCs are not a single entity but exist heterogeneously with distinct CSC marker expression, suggesting that no common liver CSCs expressing particular stem cell markers exist in all HCCs. Our data also indicate that the presence of distinct CSCs is a key determinant of cancer phenotypes in terms of tumorigenicity and metastatic propensity, which may influence the clinical outcome of HCC.

The distinct nature of $EpCAM^+$ and $CD90^+$ liver CSCs raises the question whether these different types of CSCs originate from the same or different type of cells. This question remains elusive, but a recent study investigating three independent cell clones established from the same HCC specimen revealed that these clones maintain common karyotype abnormality but express EpCAM, CD90, and CD133 distinctively with different chemosensitivities against sunitinib [47], suggesting that distinct liver CSCs expressing different markers may originate from the same type of cells. In terms of liver CSC origin, a recent study demonstrated that acquisition of liver CSC properties is independent of the cell of origin, and liver CSCs can originate from hepatic progenitor cells, hepatoblasts, or adult hepatocytes in mice by forced H-Ras/SV40LT induction and subsequent oncogenic reprogramming [48]. In addition, another study has demonstrated the unexpected plasticity of normal mature hepatocytes to dedifferentiate into progenitor cells in rats [49], and this type of plasticity has also been reported in breast non-CSCs [50, 51]. Given the cellular plasticity reported in normal and cancer cells described above, it is reasonable to speculate that a similar plasticity may exist in $EpCAM^+$ and $CD90^+$ CSCs that can convert their tumorigenic/metastatic phenotypes and marker expression status. Further studies are required to clarify the role of cell plasticity on heterogeneity of HCC [36].

Interaction of distinct cell lineages in liver organogenesis and hepatocarcinogenesis

Embryogenesis is characterized by the ordered emergence of an organism made up of a multitude of stem and differentiated cells. Various signaling pathways play crucial roles in the dynamic cell proliferation and motility of organogenesis [52]. For example, in liver organogenesis, liver specification signaling is activated at the ventral endoderm (hepatic endoderm) by the paracrine secretion of fibroblast growth factor (FGF) and bone morphogenic protein (BMP) from the cardiac mesoderm and septum transversum, respectively [53–55]. Wnt/beta-catenin signaling may also induce hepatic specification [56]. Activation of these signaling pathways results in the formation of the liver bud from the hepatic endoderm. The liver bud is considered to be the earliest developmental stage of liver organogenesis, which coincides with the expression of albumin and AFP [57].

Once the hepatic endoderm is specified and the liver bud begins to grow, the cells become hepatoblasts and have the ability to differentiate into hepatic and biliary lineages as bipotent progenitors. Epithelial and mesenchymal cells located in the endoderm and/or mesoderm collaborate to orchestrate liver organogenesis [58] (Fig 1a). The importance of this was elegantly demonstrated in a recent in vitro study generating liver buds using induced pluripotent stem cells, human umbilical vascular endothelial cells, and mesenchymal stem cells [59].

Embryogenesis and tumorigenesis share similar features including autonomous cell proliferation, motility, homing, dynamic morphologic changes, cellular heterogeneity, and interactions with the microenvironment. Liver cancer development may partially recapitulate fetal liver development in terms of the emergence of cells expressing certain stem cell markers and the activation of signaling pathways during liver development (Fig 1b). Indeed, signaling pathways activated in normal liver development are known to be activated and may be involved in the development and maintenance of liver CSCs. FGF and Wnt signaling has also been implicated in the development of HCC [60–63], with the latter shown to regulate the self-renewal of hepatoblasts and liver CSCs [20, 31, 64–68].

Moreover, as observed in the process of normal liver development, the collaboration of CSCs with epithelial or mesenchymal cell features may play an important role in the tumorigenicity and metastasis of HCC (Fig 1b). Our data indicate that $EpCAM^+$ CSCs have no metastatic capacity for distant sites when subcutaneously injected into NOD/SCID mice. However, when $CD90^+$ CSCs were co-injected with $EpCAM^+$ CSCs, $EpCAM^+$ cells could metastasize to the lung, whereas subcutaneous primary tumors showed no difference in size [36]. Furthermore, although imatinib mesylate treatment had little effect on the size of primary subcutaneous tumors, it significantly suppressed lung metastasis potentially through the suppression of $CD90^+$ CSCs.

We found that the effect of $CD90^+$ CSCs on the enhanced cell motility of $EpCAM^+$ cells was mediated, at least in part,

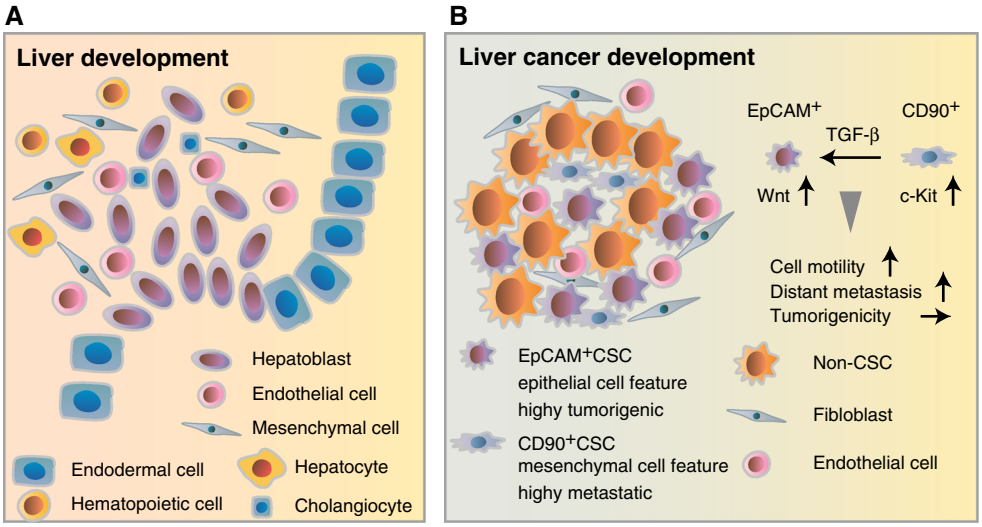

Fig. 1 Interaction of epithelial and mesenchymal cells in liver development and liver cancer development. **a** Liver bud formation is regulated by the activation of FGF, BMP, and Wnt signaling through the interaction of endodermal cells, endothelial cells, and mesenchymal cells. **b** Liver cancer development is regulated by the interaction of $EpCAM^+$ and $CD90^+$ CSCs. In primary HCC, $EpCAM^+$ and $CD90^+$ CSCs distinctively exist. $EpCAM^+$ CSCs show epithelial cell features with a high tumorigenic capacity and activated Wnt signaling, whereas $CD90^+$ CSCs show mesenchymal cell features with a highly metastatic capacity and activation of c-Kit signaling. In primary HCC where $EpCAM^+$ and $CD90^+$ CSCs co-exist, $CD90^+$ CSCs regulate distant organ metastasis through the activation of TGF-β signaling, but have no effect on tumorigenicity at primary sites which is mediated by $EpCAM^+$ CSCs

through the activation of TGF-β signaling by $CD90^+$ CSCs (Fig 1b) [36]. This suggests that $CD90^+$ cells are not only metastatic to the distant organ but also help the metastasis of $CD90^-$ cells, including $EpCAM^+$ cells, which have no distant metastatic capacity of their own. Our data further suggest that imatinib mesylate inhibits distant organ metastasis by suppressing $CD90^+$ metastatic CSCs, albeit with little effect on $EpCAM^+$ tumorigenic epithelial stem-like CSCs, which indicates the importance of $EpCAM^+$ and $CD90^+$ CSC interaction in the process of HCC development, especially in distant organ metastasis. These data suggest the limitations of a treatment strategy targeting only certain CSC marker-positive cells to eradicate HCC, as it is highly possible that marker-positive CSCs exist in each HCC patient with different chemosensitivities against molecularly targeted therapy. Interestingly, we have recently identified that EpCAM+ HCC cell lines show abundant expression of the transcription factor SALL4 and high histone deacetylase activity, and the histone deacetylase inhibitor successfully suppressed proliferation of EpCAM+ HCC cell lines but showed little effect on CD90+ HCC cell lines [69]. Further studies of liver CSC heterogeneity are required to provide better treatment strategies for HCC patients.

Conclusions

There is accumulating evidence that liver CSCs play a key role in the development and perpetuation of HCC, and the importance of targeting CSCs has become clearer. Understanding the diversity of liver CSCs will further the development of personalized medicine targeting patient-specific liver CSCs.

Conflict of interest The authors declare that they have no conflict of interest.

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