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REVIEW

Road to stemness in hepatocellular carcinoma

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Abstract

Carcinogenic process has been proposed to relay on the capacity to induce local tissue damage and proliferative repair. Liver has a great regeneration capacity and currently, most studies point towards the dominant role of hepatocytes in regeneration at all levels of liver damage. The most frequent liver cancer is hepatocellular carcinoma (HCC). Historical findings originally led to the idea that the cell of origin of HCC might be a progenitor cell. However, current linage tracing studies put the progenitor hypothesis of HCC origin into question. In agreement with their dominant role in liver regeneration, mature hepatocytes are emerging as the cell of origin of HCC, although, the specific hepatocyte subpopulation of origin is yet to be determined. The relationship between the cancer cell of origin (CCO) and cancer-propagating cells, known as hepatic cancer stem cell (HCSC) is unknown. It has been challenging to identify the definitive phenotypic marker of HCSC, probably due to the existence of different cancer stem cells (CSC) subpopulations with different functions within HCC. There is a dynamic interconversion among different CSCs, and between CSC and non-CSCs. Because of that, CSC-state is currently defined as a description of a highly adaptable and dynamic intrinsic property of tumor cells, instead of a static subpopulation of a tumor. Altered conditions could trigger the gain of stemness, some of them include: EMT-MET, epigenetics, microenvironment and selective stimulus such as chemotherapy. This CSC heterogeneity and dynamism makes them out reach from therapeutic protocols directed to a single target. A further avenue of research in this line will be to uncover mechanisms that trigger this interconversion of cell populations within tumors and target it.

Key words: Cancer stem cell; Liver progenitor cell; Liver stem progenitor cell; Stemness; Plasticity; Cancer cell of origin; Cancer stem cells subpopulations

Core tip: In agreement with their dominant role in liver regeneration, mature hepatocytes are emerging

as the cell of origin of hepatocellular carcinoma. The relationship between the cancer cell of origin (CCO) and hepatic cancer stem cell is unknown. There is a dynamic interconversion among different cancer stem cells (CSCs), and between CSC and non-CSCs. Because of that, CSC-state is currently defined as a description of a highly adaptable and dynamic intrinsic property of tumor cells, instead of a static subpopulation of a tumor. This CSC heterogeneity and dynamism makes them out reach from therapeutic protocols directed to a single target.

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INTRODUCTION

The problem of multiple propositions for the cell of origin in liver cancer

The hepatocellular carcinoma (HCC) liver cancer subtype is more common (approximately 78%) than others, such as bile-duct cancer, hepatoblastoma, liver sarcoma and other carcinomas (approximately 22%)[1]. This carcinoma is one of the most important cancers; it ranks sixth in incidence and is the second leading cause of cancer-related death worldwide, with a high probability of increasing in upcoming years and an estimated 1 million cases anticipated by 2030[2]. In addition, HCC is a cancer that has a high number of risk factors, such as infection by hepatitis B or C virus, alcohol, and prolonged aflatoxin exposure[3].

Despite new data and increasing information about the origin of liver tumor cells, there is still controversy due to inconclusive evidence. Many studies support the hierarchical hypothesis, which suggests that liver tumors have specialized cells that begin and maintain the tumor, give anti-cancer drug resistance; these cells are denoted as cancer stem cells (CSCs). The limit of many studies is that cells are suggested to function similar to CSCs based on only some of their functional capacities or immunophenotypic characteristics. Some considerations must be taken because different models provide controversial results; CSCs are generally evaluated in late tumor stages, which means that the evaluation is limited to only one late period of tumor development and that some results are only clinical observations. Many questions are still unresolved: Are CSCs really the origin of liver tumors? Which is the cell of origin for these tumors? How are the selected markers considered to be adequate and representative of all subpopulations of CSCs in tumor masses? Are the subpopulations of cancer stem cells (SCs) similar in all kinds of tumors, independent of their etiology? In Supplemental Box 1, we summarize some current problems that are important in the identification of HCSCs in tumor. Hence, there is need for a better and more precise definition to help us clarify confusion about the concept of CSCs.

Since the evidence of CSC was consolidated during pioneer work on leukemia mouse models, subsequent studies have reported the participation of CSCs in various solid tumors, including liver tumors[4-6]. A divergent position is the case of hepatocellular carcinoma (HCC); a few years ago, it was believed that tumor formation was exclusively due to the main parenchymal cell, the hepatocyte[7]. Later findings change the idea of the cell of origin, proposing as a candidate the progenitor cell[8]. Additionally, the existence of SCs in the adult liver is still heavily debated. Nevertheless, some evidence suggests the participation of CSCs in HCC, for example, when tumors have high recurrence after anticancer treatments, such as surgery, radiation or chemotherapy. Additionally, CSCs are considered responsible for tumor metastasis, and it has been reported that a distinct subpopulation of cells exhibit properties that are consistent with stemness, such as self-renewal, cell proliferation and cell survival. Examples of distinct subpopulations include a side population (SP) of the human HCC cell lines MHCC97-H, MHCC97-L, Huh7 and HCCLM3 that give rise to tumors when transplanted into immunodeficient mice[9]. In addition some pluripotency transcription factors are expressed in these SP cells[10]. Moreover, in 2006, Durnez and coworkers used HCC immunophenotyping in HCC and found that 28% of HCCs express liver progenitor cells (LPC) markers. These authors proposed that HCCs may have a progenitor origin[11]. Finally, one study proposed that 40% of HCCs are probably clonal and could potentially arise from stem SCs[12].

Based on the recent literature, the present review has the following aims: to summarize the current knowledge of HCSCs; discuss many pertinent, current questions about CSCs; analyze the significance and limitations of CSC studies; determine the cellular origin of HCCs and discuss other controversial concepts about HCSCs. Finally, we suggest future research perspectives that pertain to not only tumor biology but also clinical development or specific therapies that effectively target HCSCs.

Similarities and differences between two hypotheses of cancer composition

Thus far, there have been two hypotheses, or major schools of thought, related to the cellular composition of cancers. The first hypothesis, denoted as the "stochastic theory" or the clonal-evolution model of carcinogenesis, suggests that tumor development is

initiated by any cell that has suffered mutations by a random process. This hypothesis supports the idea that the accumulation of mutations and epigenetic changes that trigger cancer development may occur in any somatic cell. At some point during tumor progression, a clonal selection of cells may exist that will eventually form cancer. This hypothesis supports the fact that any cancer cell can participate in tumor development, resistance, and recurrence because the tumor could be considered relatively homogeneous; therefore, all cells can function as tumor-supporting cells and are then targets for treatment. Nevertheless, some studies contradict this hypothesis. For example, an autotransplant assay performed in the 1960s demonstrated a low frequency of tumor formation from ovarian and cervical cancer cells. When the cells were injected subcutaneously, the researchers obtained only positive data when they used a high number of tumor cells ($> 1 \times 10^6$)[13]. Another injecting cell assay supported the hypothesis of a small tumor cell subpopulation is responsible for the initiation and maintenance of cancer[14]. Considering the variability of different markers in the tumor, specifically SC markers, some authors support the idea that tumoral heteroigeneity is a consequence of continuous degeneration following sequential genomic insults and epigenetic changes that trigger different signature patterns.

The second hypothesis postulates that there is a tumor mass hierarchy, and indeed, this hypothesis proposes that different populations form the tumor. Similar to the stochastic model, this model proposes that any single mutated cell (the cell of origin) can gain the ability to proliferate but that this situation creates a heterogeneous tumor because most progeny cells will form subpopulations that do not contribute to tumor growth to the same degree and that cannot form secondary tumors. The CSC hypothesis fits with the inefficient and ineffective treatment of cancer because anti-cancer drugs do not discriminate between all tumor cells. If indeed these drugs are not specific, then they could not eliminate the cells that are responsible for sustaining tumors. Evidence from tumorgenicity assays confirms this phenomenon. For example, in pancreatic adenocarcinoma, a cell population enriched with $ALDH^{(high)}$ and CD133+ has an enhanced tumorigenic potential relative to $ALDH^{(low)}$ and CD133+ or $ALDH^{(high)}$/CD133-[15], showing the existence of a distinct cell subpopulation as part of the tumor. In another study, a research group demonstrated that metastasis was established by tumor cells that had characteristics related to quiescence, mesenchymal and stemness in breast cancer [16]. Additionally, it was found that approximately 68% of patients who were subjected to hepatic resection after being diagnosed with HCC showed a pattern of having blood cells expressing the SC markers CD90+, CD45-, and CD44+, indicating that these cells were liberated from the tumor and probably responsible for metastasis[17]. It was reported that a small population in HCC cell lines (HCCLM3, MHCC97H and MHCC97L) have high expression levels of CSC markers, including CD90, EpCAM and CD24; these subpopulations were associated with stronger invasive ability[18]. Moreover, one meta-analysis suggested that CSCs markers were associated with a less differentiation grade in histopathology analysis and decreased survival in HCC patients[19]. This evidence supports the existence and participation of CSCs in solid tumors, including HCC.

Another discrepancy regarding CSCs is the terminology. Some permutations used in this field are varied, such as "stem-like cell", "tumor-initiating cell", "tumor-progenitor cell", "propagating cell" and "CSC". Nevertheless, some authors use the terms "tumor-initiating cell" and "CSC" to describe the same population or considered equal[20]. According to the hierarchical model of cancer biology and the definition proposed by the European Consortium for Stem Cell Research, SC is defined as a cell that can potentially produce unaltered descendants and has the capacity to produce daughter cells with different and restricted progeny. A progenitor cell is defined as any cell that produces progenies composed of transit-amplifying cells that are fated for differentiation or initially uncommitted and that retain self-renewal capabilities, although the existence of progenitor cells is still being debated[21]. CSC is considered a self-renewing cell that is responsible for sustaining cancer and producing differentiated progeny that form the bulk of cancer cells[22].

Cancer cell of origin (CCO) is an interesting term used when any normal somatic cell (progenitor cell, differentiated cell or normal SC; n-SC) that acquired the first cancer-promoting mutation(s) gains self-renewal capacity and can generate the full repertoire of tumor cells (both tumorigenic and non-tumorigenic cells, nTC)[23]. Then, one difference between CCO and CSC concerns their functional status as cancer-initiating cells or cancer-propagating cells, respectively. Until now, the relationship between CCO and CSC is not well understood, but some authors propose that the phenotype between them may be different and change in a dynamical way. Despite all the uncertainly about which cell is the CCO, the use of terminology based on the operational functions helps to clarify some questions. It is better to classify a CSC according to its functional definition instead of as a group of markers, and continuous changes in the definitions of CSC-hypotheses that are based on experimental evidence and conclusions will very likely materialize in the upcoming years.

Although current discoveries have provided com-

Table 1 Liver cancer stem cell markers in hepatocellular carcinoma

Marker	Another name	Function	Ref.
CD133	Prom-1	Neurotensin/IL-8/CXCL1 signaling	[33,186]
OV6	Oval cell marker	Unknown until know, but it is considered to be an inducible progenitor cell marker in rodent models.	[8,38]
EpCAM	Epithelial cell adhesion molecule	Activation of Wnt signaling	[187,188]
CD13	Cluster of differentiation 13	ROS-induced DNA damage reduction	[71,72,189]
CD24	Heat-stable antigen	STAT3-mediated NANOG regulation	[18,34,150]
CD90	Thy-1	Glycophosphatidylinositol (GPI) anchor	[18,32]
CD44	Hyaluronic acid receptor	Participation in epithelial mesenchymal transition	[33,69,190]
ALDH	Aldehyde dehydrogenase	Group of intracellular enzymes that oxidize aldehydes (thereby serving a detoxifying role)	[15,39]

pelling evidence that CSC populations in solid cancers are key contributors of tumor maintenance, recurrence and therapy failure, further research is required to understand how these cells participate in HCC. It is important to note that both hypotheses have significant differences, but they cannot be considered mutually exclusive or lacking cooperation in cancer.

One marker is not enough to characterize HCSCs

Evidently, surface markers are not enough to define a CSC. Indeed, is important to note that none of the markers used to isolate normal and cancerous SCs are expressed exclusively by the SC itself. Moreover, markers used to identify SCs from one organ are not useful for identifying SCs in other tissues, and identifying a marker in SCs from one kind of tumor does not mean that it will be present in other tumors; it could be present in the same kind of tumor but in a different context[24]. For example, CD133 is used to successfully enrich SC fractions from various solid tumors, but it is also present in normal brain SCs and is not restricted to only SCs[25]. CD44 is now considered a marker of CSCs, although determining which isoform is representative and dominant is still under debate. However, CD44 has been implicated in many physiologic processes, especially leukocyte homing, activation and migration, because it is considered the major adhesion molecule in the extracellular matrix. Furthermore, aldehyde dehydrogenases (ALDHs) are an enzyme family in charge of catalyzing the oxidation (dehydrogenation of aldehydes) and detoxification of aldehydes. The identification of the isoform responsible for this process and that has high activity in SCs and CSCs was unknown until now. ALDH1A1 is expressed by hepatocytes in the centrilobular region of healthy mice livers[26]. Thus, the conceptualization that CSCs only have one specific pattern of markers needs to be re-evaluated because, as it was mentioned before, the previously considered phenotype is no specific to the population of CSCs and is not exclusive to SCs.

The first thing to consider for a cell to be a CSC is its phenotype. Until now, there have been various accepted markers used to identify HCSCs, such as Hoechst 33342 dye staining, ALDH, CD133, CD90, CD44, EpCAM, and CD13 (Table 1, Figure 1). Briefly, cell sorting after Hoechst 33342 dyeing is used to identify CSCs, including HCSCs. Because CSCs overexpress adenosine triphosphate (ATP)-binding cassette (ABC) transporters, they can efflux Hoechst 33342 dye, which results in low levels of staining with Hoechst. Additionally, Hoechst- cells have been related to stemness gene expression and depict higher tumorigenicity ability. However, one problem with this staining is that Hoechst 33342 dye is toxic[27]. The ALDH family is composed of cytosolic isoenzymes that are responsible for oxidizing intracellular aldehydes, thus contributing to the oxidation of retinol to retinoic acid in early SC differentiation. The human ALDH superfamily is composed of cytosolic isoenzymes, which includes 19 putatively functional genes at distinct locations on chromosomes. Their function consists in the oxidation of retinol to retinoic acid in the earliest stages of SC differentiation. Cells that are ALDH+ express CSC markers, are invasive and clonogenic[28]. Recently, it was proposed a system denominated label-retaining cells (LRCs) to identified slow-cycling cells in HCC lines and HeLa tumors. These cells were evaluated *in vitro* and *in vivo* and showed a functional behavior of CSC, unlike no selected cells[29]. It would be very interesting to further study HCC tumoral LRCs in order to determine if they express CSC markers, and their role in HCC. Ma and colleagues identified for first time CD133 in HCSCs, this transmembrane glycoprotein is also named Prominin 1[30]. CD133 was reported to be expressed in approximately 1%-3% of human HCC specimens, however in peripheral tumor tissue the expression was between 0.025%-0.1%. In addition, it was showed that HCC cells expressing CD133 have some survival pathways activated and consequently high resistance to anticancer therapy[31]. CD90, or Thy-1, is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein with a role in cell-cell and cell-matrix interactions and is expressed predominantly in leukocytes. Yang and coworkers found that $CD90^+$ HCC cells have greater tumorigenic and metastatic potential than $CD90^-$. CD90+CD45- cells found in tumor and blood samples from HCC patients were evaluated in a model of immunodeficient mice and were able to form tumors. The fact that CSCs circulate in the blood

indicates that HCSCs have a probable role in preparing a new place for colonization, but until now, no reliable data have been reported[32].

EpCAM is a glycoprotein with the three domains, a N-terminal extracellular, a single transmembrane domain and a short C- terminal fragment. This protein is one of the most representative and successful markers of HCSCs, and it is also considered an early biomarker for HCC. Its coexpression with alpha feto-protein (AFP) is indicative of a poor prognosis in patients. EpCAM+ is considered a molecular signature of hepatic progenitor cells and/or stem/progenitor markers, whereas EpCAM- is a characteristic of mature hepatocytes in HCC probes. In HCC EpCAM is a Wnt/β-catenin target gene, and this pathway activation increases the EpCAM+ cell population. Another important marker in HCC is CD44, which adds properties to the CD133+ or CD90+ CSC population. Fewer CD133+ CD44+ cells are required to initiate tumor growth in immunodefficient mice when compared with CD133+ CD44- cells[33]. Similarly CD90+ CD44+ cells are more metastatic than CD90+ CD44- cells in immunodeficient mice[32].

Recently, CD24 was identified as a possible HCSC marker. CD24+ cells possess more CSC properties of tumor initiation, self-renewal, chemoresistance, high metastatic capacity and differentiation than CD24- cells in an HCC mouse model. Indeed, CD24 overexpression showed a significantly correlation with poor survival in one study with HCC clinical samples. Finally, some stemness genes have a good correlation with CD24 in HCC cell lines and clinical samples. These genes were identified as downstream effectors of CD24 signaling, which acts through STAT3 activation to mediate tumor initiation and self-renewal[34].

Another HCSC marker is OV6, or the oval cell marker. The oval cells were first identified by Farber in carcinogen-treated rats[8]. These cells co-express several hepatic lineage markers [*e.g.*, AFP, Albumin, cytokeratin (CK)-7,-8, and -19][35]. Since then, several studies have found OV6+ cells in HCCs and in hepatoblastomas[36]. Additionally, Yang and coworkers demonstrated that $OV6^+$ HCC cells possess greater tumorigenic ability and chemotherapeutic resistance[37]. In addition, the $CD133^+$ population was significantly enriched for OV6+ cells[38].

All of these markers have been tested based on the abilities and qualities that are recognized for CSCs. A considerable problem is that some authors place higher importance on some markers over others based on functional assay results in their respective models of study. For example, in one study where CD133+ was isolated in HCC cell lines, CD44 and CD34 showed higher expression when compared with CD133- cells, but both subpopulations displayed similar expression of CD49f and CD117. This casted doubt as to whether these subpopulations should be considered definitive CSCs[30]. Others authors have demonstrated the participation of only one marker, such as CD133 in human HCC and HCC cell lines, and these cells were evaluated based on other abilities, such as to self-renew, to create differentiated progenies and to form tumors. CD90+CD45- cells present in blood from liver cancer patients were tumorigenic, however CD90-CD45- cells were not evaluated[32]. One study provided evidence of the existence of a hierarchical organization that bears tumorigenic potential in the order of CD133+ALDH+ > CD133+ALDH- > CD133-ALDH- in HCC cells. The authors considered the presence of both markers as a more specific method of characterizing the tumorigenic HCSC population[39]. Another example is the association of coexpression of CD90+ and EpCAM+ with poorly differentiated morphology. This classification might help to determine a clinical outcome and a therapeutic approach in HCC patients, but the authors did not consider other markers in relation to the selected markers[40].

Main signaling pathways that govern the stemness

In addition to surface markers and enzymatic activities, other hallmarks are important in characterizing CSCs. Signaling pathway behavior is considered an important hallmark of every cell. The signature of CSC pathways corresponds to the pattern of pluripotency-associated transcriptional networks. For example, in normal hepatic SCs, the high expression of Sox9, Oct4, Sox2, STAT3 was considered, in addition to some hepatocyte markers, such as CK18, ABCG2, E-cadherin, CD117 and/or CD49f[30]. It is well known that n-SCs and CSCs share pathways for sustaining the property of stemness. Some important signaling pathways related to self-renewal characteristics include Wnt/β-catenin, Notch, sonic hedgehog (Shh), TGF-β, and AKT (Figure 1).

The Wnt family consists of inter-cellular signaling molecules that are involved in the regulation of embryonic development and zonation, which is required for spatial separation of the diverse metabolic functions. These molecules are frequently altered during the processes of epithelial to mesenchymal transition (EMT), postnatal hepatic growth adult liver homeostasis, liver metabolism, and carcinogenesis. Wnt/β-catenin is an important signaling pathway involved in the tumorigenesis, progression, invasion and maintenance of CSCs[41]. This pathway is disrupted by mutational and non-mutational events in approximately one-third of HCCs[42]. Finally, the Wnt pathway involves the translocation of the β-catenin component to the nucleus, which induces the transcription of prominent targets, such as CD44[43], cyclin D1[44], and c-Myc[45], which is also a major target of EpCAM signaling. Current data indicate that the protein c-Myc is important in the

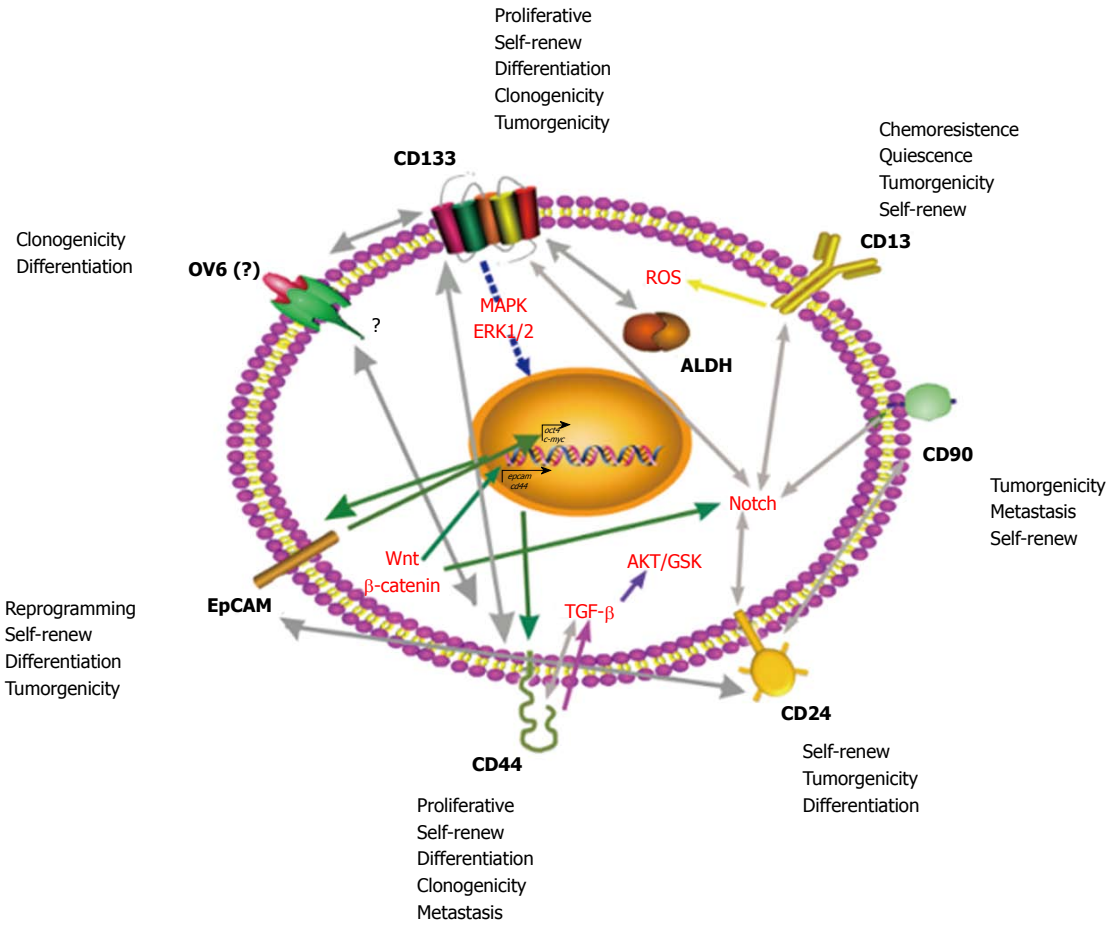

Figure 1 One marker is not enough for designate one cell as cancer stem cell. Some markers are reported as representative of HCSCs (CD133, CD13, CD90, CD24, CD44, EpCAM, OV6 and activity of the ALDH family) nevertheless there are main signaling pathways dictating the stemness state. OV6 structure and function is still unknown. Properties or characteristics related of every HCSC marker are listed besides each marker (self-renew, chemoresistence, quiescence, tumorgenicity, *etc*). Gray lines with double arrows indicate correlation in expression (*i.e.* Notch and CD90, CD133 and CD13 are overexpressed in the same CSCs population). Straight lines indicate that the molecule or pathway affects directly another molecule or pathway (*i.e.* CD44 affects directly TGF-β pathway, and TGF-β pathway affects directly AKT/GSK pathway). Doted line indicates that it affects indirectly the molecule or pathway (*i.e.*, CD133 expression affects IL-8/CXCL1 *via* MAPK/ERK indirect activation).

switch from adult to embryonic SCs[46,47].

The Notch signaling pathway plays an important role in SC self-renewal and differentiation. Notch receptors are single-pass transmembrane proteins. These receptors are processed in the endoplasmic reticulum and in the Golgi, resulting in cleavage and a glycosylated heterodimer. The processed receptors are translocated to the membrane where they could bind to their respective ligands, members of the Delta-like and Jagged family[48]. In breast cancer, for example, the activated intracellular form of Notch3 and the Notch ligand Jagged are highly expressed[49]. Notch signaling plays an essential role in liver embryogenesis and bile duct formation. CSCs CD133+ overexpress Notch1 compared to that of CD133- in HCC. Moreover in HCC, Notch3 and the Notch ligand Jagged are overexpressed[50]. An expansion of Hedgehog-responsive cells in hepatitis B/C virus (HBV/HCV) infection, promotes liver fibrosis and cancer. Increased expression of the Hedgehog pathway was identified in CD133+ HCSCs[51]. Another example of the participation of the Hedgehog pathway in CSCs was described in pancreatic cancer where CSC-like cells were treated with the inhibitor GDC-0499, which resulted in a decreased of CSC-like cells[52].

Another important pathway is the AKT signaling pathway, which is involved in regulating homeostasis and chemoresistance of HCSCs. During hypoxia, the AKT pathway significantly increases the expression of hypoxia-inducible factor-1 alpha (HIF-1α), and in a positive loop, HIF-1α will affect the phosphorylation of AKT. The positive interaction between AKT and HIF-1α leads to overexpression of platelet-derived growth factor (PDGF)-B, vascular endothelial growth factor (VEGF) and to the expression of ABCG2, which are important for homeostasis and chemoresistance of CSCs. In addition, the activated AKT pathway is associated with high levels of cell survival proteins, such as Bcl-2 in $CD133^+$ HCSCs[53].

The TGF-β signaling pathway is prominent at the

interface between liver development and cancer in the liver. Smad signaling is pivotal for embryonic hepatocyte proliferation and cancer proliferation. TGF-β is a cytokine that controls proliferation and differentiation in both normal and cancer SCs. It had been reported that TGF-β regulates the expansion of CD133+ though the induction of EMT. Additionally, this cytokine can activate differentiation programs and inhibit cell-cycle progression during early carcinogenesis through intermediary SMAD proteins. The association and cooperation between TGF-β and oncogenic RAS activates the nuclear β-catenin signaling pathway, which causes neoplastic hepatocyte loss differentiation and directs them to become immature progenitor cells, which facilitates tumor recurrence; this transition supports the idea that CSCs participate in HCC. Moreover, the loss of the TGF-β adaptor b2-Spectrin triggers malignant transformation of liver SCs and LPC by growth-arrest signals and dedifferentiation. Finally, Mishra and colleagues found that there is a functional link between IL-6, a major SC signaling pathway, and the TGF-β signaling pathway in HCC and that suppression of IL-6 signaling, through the generation of mouse knockouts, resulted in a reduction of HCC in mice[54].

Functional assays for the evaluation of different stem cells

CSCs have properties that differ from n-SCs: (1) the ability to develop a malignant tumor; (2) resistance to chemotherapeutic agents used to treat malignant tumors, thus making them responsible for recurrence; and (3) the ability to give rise to distant metastases. These properties can be evaluated by some assays *in vitro* and *in vivo*. While some authors consider one of the *in vitro* assays sufficient to determine that a cell is CSC-like, until now, the best assay has been *in vivo* evaluation.

Some authors consider the *in vivo* tumorgenicity assay the gold standard in identifying CSCs; the tumor cell population of interest is transplanted into animal models, followed by an evaluation of the tumor-propagating capacity of the cells[55]. This assay has been useful because the selection of specific subpopulations of CSCs yields different results, indicating the ability for cells to have different tumor behaviors. A question then arises of whether tumorigenicity assays can be used to search for the potential CCO. The problem is that this assay only suggests that a specific cell population can be transformed into a tumorigenic cell nevertheless may not necessarily be the genuine CCO *in vivo*[56]. Nevertheless, some conflicts exist about this kind of assay. One issue is that this assay uses immunodeficient animals (NOD/SCID); therefore, the original context of tumor development is not similar to the normal immune system of a healthy animal. Additionally, this assay has only been analyzed by the selection of some markers, which hides some probable differential expression of the total population. Finally, it is important to consider that these models do not have the same tissue microenvironment. Xenotransplants differ in architecture and stroma compared with their native niche. Indeed, one study performed by Quintana and colleagues confirmed this affirmation when they showed that the tumor-initiating capacity may be an artificial consequence of the conditions employed in xenograft mouse models. For example, when these authors changed some parameters, such as the extracellular matrix in immune-compromised mice, they obtained better results[57].

The limiting dilution assay, or clonogenicity assay, is an in vitro cell survival assay based on the capacity of a single cell to grow into a colony. This assay tests every cell in the population for its ability to divide an unlimited number of times. Currently, this assay is used to determine responsiveness to cell death; cancer therapies, such as cytotoxic agents or ionizing radiation; and tumor recurrence[58]. In addition, this assay has given information about tumor survival, resistance and some extracellular matrix components, such as fibronectin and laminin[59]. It is possible to distinguish between effects on cell viability and cell proliferation by including colony size as an endpoint in the assay. However, this assay does have limitations. For example, this assay cannot measure the effect of cell-cell communication on cell proliferation because cells are plated at low densities to form colonies; therefore, there is a loss of the three-dimensional environment. Moreover, this assay is not applicable when a substance concentration decreases cell growth without affecting cell cycle progression and/or DNA synthesis. Additionally, this assay is considered inappropriate for some agents. Last, quality control is another problem, especially because it is difficult to distinguish cell clones from cell clumps in this assay[60].

The sphere assay is an *in vitro* method for assessing the self-renewal and multipotency capacity of a cell population in a low adherence system in the absence of serum. The result is the selection of undifferentiated cells that have a high self-renewal capacity[61]. Under these low-adherence and non-differentiating conditions, it is hypothesized that CSCs are able to form three-dimensional spheroids containing a heterogeneous population of progenitor cells with the competence to differentiate into multiple cell types. The self-renewal capacity of a cell population is assessed by the ability of the cells to form tumorspheres upon multiple passages, and this capacity is proportional to the number of spheres formed. Another advantage of the spheroid assay is that it minimizes the solid tumor characteristics in

its inside area during hypoxia and the low pH and interior cell characteristics that may be inaccessible to metabolites and drugs in comparison to exterior cells. Moreover, this assay has been used to evaluate the migration and invasive ability of CSCs, which correlates with their ability to metastasize. Even though the tumorsphere assay has been useful for self-renewal phenotype studies associated with CSCs, several limitations exist for this assay[62]. The first drawback is the inconsistency to form and maintain spheroids of uniform size; it was reported that the size and morphology of spheroids are different in different HCC cell lines. Some cells are more viable during the formation of spheroids, and the formation of spheroids varies according to the methodology used (*e.g.*, spinner flasks, non-adherent surfaces, hanging drop technique and microfluids)[63]. The second disadvantage is the variability in the number of cells that are necessary to form spheroids. It is difficult to extrapolate data from all kind of cells. Finally, analyzing the spheroids as if they were tissue is sometimes not representative for drug testing because spheroids cannot give the same information and do not have the same metabolism as other organs or tissues. Thus, we cannot obtain all the data that would be collected in a complete organism[64].

Determining whether CSCs are heterogeneous

In the 1980s, morphological intratumor heterogeneity became evident through histological analyses. Data from many investigations about CSCs showed evidence for the existence of different subpopulations of CSCs that had a great degree of heterogeneity. CSCs isolated from different stages of the same type of tumors are distinct. For example, some CSCs isolated from the primary tumor differ from those found in metastatic growths[65]. Even though different subpopulations of CSCs share the same niche, they usually coexist and may interconvert. Recently, a new concept was proposed that CSCs are a cell 'state', not a fixed 'category' of cells. CSCs are not an immutable, frozen cell population. Because of cellular plasticity, CSCs and non-CSCs coexist and can interconvert in a dynamic equilibrium[66]. Non-CSCs can acquire CSC properties by reprogramming or dedifferentiating, and they can interconvert in a similar manner as EMT to MET and vice versa.

Intra-tumor heterogeneity has been described in pancreatic cancer with regard to metastatic progression. By next-generation sequencing of the primary tumor and corresponding distant metastatic lesions, it was found that a primary pancreatic tumor is composed of spatially and genetically distinct subclones that give rise to lung or liver metastasis in the same patient[67]. In melanoma cancer, CSC-like populations have been shown to not only be heterogenic but also have differential functional activity depending on their location. CSC located at the invasive tumor front were of mesenchymal phenotype and quiescent (CD24- CD4+), meanwhile the central CSC were highly proliferative and epithelial-like (ALDH+)[68]. For CD44 expression gives distinct cell features to the $CD133^+$ or $CD90^+$ CSC populations. Two functions of CD44 in CSC maintenance have been reported. CD44s, the standard isoform, regulates TGF-β-mediated epithelial-mesenchymal transition (EMT), maintaining the mesenchymal phenotype, and results in resistance to sorafenib in HCC[69]. Meanwhile in gastrointestinal cancer, CD44v protects CSCs against reactive oxygen species (ROS) by stabilizing xCT, which leads to a regulated redox status[70]. Additionally, Kim and colleagues, indicated that the subpopulation of CD13+ in CSCs play a role in resistance to chemotherapy and radiation therapy in HCC by increasing plasticity linked to the cellular "stemness"[71], indeed, the combination of CD13+CD166- population demonstrated higher expression of stemness-related genes than CD13+CD166+[72].

In another study in 2010, CD133+ and CD44+ cells exhibited preferential expression of some SC-associated genes and were more resistant to chemotherapeutic agents due to upregulation of the ATP-binding cassette (ABC) superfamily transporters ABCB1, ABCC1, and ABCG2. Those findings suggested that CD133+ CD44+ cells might represent an important subpopulation of CSCs cells, which allows for a better understanding of HCC initiation and progression and establishes a precise target for implementing more effective therapies[33]. Finally, it has been demonstrated that the patterns of markers have different functions in CSCs. Singh and coworkers indicated that signaling at the IGF-1R-AKT axis is important for functional heterogeneity in ovarian CSC populations, and they concluded that oscillatory IGF-1R-AKT signaling affects chemo-resistance during early and late stages. This result emphasizes the importance of inhibition of a given target during different stages: using only IGF-1R during early time points and only AKT inhibition at late time points will significantly affect ovarian CSC population[73].

The consensus is that CSC subpopulations exist. These subpopulations seem to have important functions, and they are able to inter-convert, depending on the cellular context. These CSC phenotypes can vary according to the different cancer entities. Further research regarding the functions of CSC subpopulations in HCC, which will probably emerge in the coming years, will be necessary for understanding tumor growth.

RELATIONSHIP BETWEEN LIVER REGENERATION AND CELL OF ORIGIN OF CSCs

The carcinogenic properties of some extrinsic factors might rely on their capacity to induce local tissue damage and proliferative repair to create an expanded population of cells that are susceptible to malignant transformation[74]. The liver has a unique ability to regenerate after damage[75]; however, the type of cells that participate in liver repair is a highly debated topic. Recent studies have shed light on this issue, allowing us to have a more comprehensive view of the very complex liver regeneration process. Therefore, to better understand which cells are candidates for the cell of origin of HCC, we will briefly describe the state of the art of liver regeneration in the next section.

The classical model of liver regeneration

The classical model establishes that under most circumstances, liver function is restored through replacement of damaged hepatocytes by self-duplication of the remaining hepatocytes. However, when hepatocyte proliferation is impaired, as it is under chronic or severe injury conditions, other cells may contribute to liver regeneration by giving rise to hepatocytes[76].

Initial historic studies in rats that have severe liver damage and a blockade of hepatocyte proliferation described the emergence of a short-lived, highly proliferating cell type that expresses both cholangiocyte- and hepatocyte-specific markers as well as the embryonic liver marker AFP[77]. These cells were designated oval cells mainly because of their morphology (they have oval nuclei and a high nuclear-to-cytoplasmic ratio) and of their ectopic emergence/expansion in the parenchymal region of injured livers, which often forms a cluster that has duct-like and/or cord-like structures[77]. Oval cells are proposed to originate from a quiescent, facultative stem cell that is anatomically located at the interface between bile ducts and hepatocytes in the Canals of Hering[78]. This phenomenon is named the "ductular reaction"[8,79]. Experiments labeling these transit-amplifying cells with (3H)-thymidine suggested that oval cells were responsible for rat liver repopulation by generating new hepatocytes[80,81], although lineage tracing studies are required to formally prove this finding. Since oval cells are only seen in rodents, we will use the term liver stem progenitor cells (LSPCs) for all species[82].

Histologic/clinical correlation of LSPCs in human diseases

Notably, in human liver diseases, a severe and progressive impairment of hepatocyte proliferative capability is common[79,83-86], and the ductular reaction is correlated with the progression of liver injury. In such scenarios, the LSPC pathway originating from the biliary compartment can theoretically be a mechanism for the repopulation and regeneration of cirrhotic livers in humans. Histological examination of liver buds present in cirrhotic livers with three-dimensional reconstruction revealed that intraseptal hepatocytes were connected mostly to the CK19+ ductules, implying that the hepatocytes are newly derived from putative LSPCs located in the ductules[87]. EpCAM+ hepatocytes in cirrhotic livers are suggested to come from CK19+ ductular cells because they have a telomere length between that of EpCAM- hepatocytes and bile ductular cells[88]. The degree of LSPC activation correlates with survival in severe human liver diseases, such as alcoholic hepatitis[89]. However, the clinical significance of LSPC propagation in patients during late stage cirrhosis is obscured by the lack of restoration of hepatocyte numbers and functional recovery[87,90].

Available murine models and labeling strategies

In murine models of liver injury, existing protocols do not cause as severe blockade of hepatocyte proliferation as that seen in rat or human chronic damage, thus limiting the induction of LSPC activation during parenchymal renewal[75]. Acute acetaminophen or carbon tetrachloride injury does not produce LSPC expansion, while alpha-naphthyl-isothiocyanate (ANIT), dietdiethoxycarbonyl-1,4-dihydro-colldine (DDC)-supplemented diet, a choline-deficient ethionine (CDE)-supplemented diet and a methionine choline-deficient diet supplemented with ethionine (MCDE) do produce LSPC expansion. Differences in the type, duration, and severity of liver injury between experimental models and human diseases may also suggest that the contribution of LSPCs to liver regeneration in human chronic diseases may exceed the extent of what is observed in mouse models. However, mice are the only species that have available the genetic lineage tracing tool[91].

In the absence of specific LSPC markers, researchers have turned to broader lineage markers to perform cell isolation or lineage tracing studies. These markers could be shared by cholangiocytes and putative LSPCs or expressed in an inducible population of LSPCs. The ability to reach strong conclusions about lineage is deeply dependent on the specificity of the tracing tools used[92], and we will discuss the specificity of each tool.

LSPCs role in liver regeneration

Labeling LSPCs inducible populations: In 2011, Furuyama *et al*[93] showed that SRY (sex determining region Y) box 9 (Sox9)+ cells contributed to the majority of new hepatocytes formed during homeostatic maintenance and during different kinds

of regeneration after liver injury. However, later, a new study demonstrated that the Cre driver strain used in the study marked not only the Sox9+ cholangiocytes but also the hepatocytes surrounding the bile duct[92,93], which was partially due to the re-expression of SOX9 after tamoxifen induction (see below)[93,94] and nonendogenous artificial promoter use[95].

Osteopontin (OPN)-marked cholangiocytes and LSPCs did not contribute significantly to hepatocytes under normal homeostatic conditions or after partial hepatectomy, DDC or carbon tetrachloride treatment (acute and chronic). However, in mice fed a CDE diet, 2.45% of hepatocytes were derived from OPN-marked cells after the mice were allowed to recover on normal chow (CDE-stop model)[95]. However, OPN is shown to be expressed in cell types other than cholangiocytes and LSPCs (*e.g.*, hepatic stellate cells (HSC) and inflammatory cells)[96,97]. This could be an important limitation of this study since recent studies suggest that HSC can act as progenitor cells in the injured liver[98,99].

HNF1β is expressed in cells that form the ductular reaction and in expanded LSPCs in human diseases and in animal models of liver injury. Lineage tracing demonstrated that mice fed with a CDE diet showed a small population of hepatocytes derived from HNF1β+ cells that were expanded to 1.86% of the total hepatocyte population after injury recovery[91].

In Cre-based inducible models, the inducer tamoxifen is usually administered as a pulse label before beginning the injury model, so that only pre-existing populations are marked. However, tamoxifen remains active in mouse livers for one to four weeks[78,100,101]. Hepatocytes have a propensity for expressing certain biliary markers, including OPN and Sox9, during stress[102-104]. The Sox9, OPN and HNF1β studies and the GFAP study (discussed below) were conducted by initiating liver injuries one week or less after induction with tamoxifen, which may have resulted in marker gene activation in injured hepatocytes that non-specifically expressed genes whose promoters were used to drive Cre. Therefore, these studies discuss the genes expressed by facultative LSPCs that are induced by liver injury; however, they cannot provide information about the cell of origin of these populations.

Forkhead box protein L1 (Foxl1) is a marker for LSPCs found in injured livers, and Foxl1-Cre-expressing cells can be isolated, expanded, and differentiated into both cholangiocyte and hepatocyte lineages *in vitro*[95,105]. By tracing Foxl1-expressing LSPCs, more than 50% of CK19+ cholangiocytes and 29% of HNF4α+ hepatocytes were marked by YFP in the livers of Foxl1-Cre mice that were fed a CDE diet after the recovery period[106]. However, at the hepatocellular and cholestatic levels, there is not a significant difference in the function of injured livers in which Foxl1 cells were ablated during the recovery phase and those that were not depleted. One caveat to the current model is the fact that it employed Foxl1-Cre mice, which is not an inducible system. Thus, it is definitely possible that pre-existing hepatocytes activated the Foxl1-Cre promoter sometime during the course of the CDE paradigm and became YFP+ by this route[106]. Following liver damage and similar to Foxl1, Lgr5 (leucine-rich-repeat-containing G protein-coupled receptor 5) marks a population of cells that proliferate, and differentiate into hepatocytes and/or ductal cells, upon damage caused by carbon tetrachloride, DDC, or MCDE, as shown by lineage tracing[107]. In both models, lineage tracing was induced after the liver injury and detected Lgr5 cells in hepatocytes and biliary ducts. Lgr5+ cells can differentiate towards cholangiocytes and hepatocytes (bipotentiality) *in vitro* and *in vivo* after transplantation in the FAH-/- mouse model. However, because this marker only appears after damage *in vivo*, the cell of origin from which these Lgr5+ cells arise is still unknown [107].

Labeling LSPCs preexisting populations: In contrast to the mass labeling of SOX9+ cells conducted by Furuyama[93], a study involving clonally traced SOX9+ cells was recently reported. Biliary-derived Sox9+ proliferative ducts insignificantly contributed to regeneration of the hepatocyte pool in several classic mouse oval cell injury models, including the CDE oval cell activation regimen (< 1%)[78]. To address the possibility of this clonal tracing strategy could have failed to mark hypothetical LSPCs, the authors created a chimera lineage experiment. In this model, all hepatocytes are ablated (> 99% in analyzed zones) and replaced by a transplantation of mTomato+ donor hepatocytes. Thus, this model system generally tests whether any non-parenchymal cell (NPC) has the capacity to differentiate into hepatocytes. This model further confirmed the scarce (< 1%) contribution of NPC precursors to the hepatocyte pool after CDE LSPC activation, while hepatocyte-derived progenitors give rise to hepatocytes at a much higher efficiency (> 60%)[78].

As a terminal biliary marker, CK19 appears to be an exception to the marker specificity problem; in contrast to Sox9 and OPN, CK19 is not expressed by hepatocytes upon injury. CK19-CreER mice are likely to represent a more specific and, hence, more reliable tool for assessing the contribution of cholangiocytes and atypical ductal cells to liver regeneration[92]. Under all injury-recovery circumstances (DDC, CDE, CCl_4 or ANIT), all YFP+ cells stained for the biliary marker CK19, but not the hepatocyte marker HNF4α (hepatocyte nuclear factor 4 alpha)[92]. Schuab *et al*[101] also used CK19 tracing but used the CDE-Stop model

that was reported by Español-Suñer *et al*[95], which produced 2.45% Osteopontin+ derived hepatocytes; however, with CK19 tracing, the frequency of fate-traced hepatocytes remained negligible[101].

Cells with slow proliferation kinetics that retain a nuclear label over long periods, called label-retaining cells (LRCs), represent multipotent stem cells in a number of adult tissues. To identify quiescent cells without the bias of using a marker, in 2017, Vill *et al*[108] induced the expression of histone 2B fusion protein (enhanced green fluorescent protein [H2B-EGFP]) in the liver cells of newborn pups and chased the label until the liver matured. The liver label-retaining cells (LLRCs) were clustered in portal areas in biliary ducts and expressed biliary and oval cell markers. In this model, the EGFP mark is diluted among the daughter cells when LLRCs proliferate, and this how proliferation is detected. Moreover, the LLRCs were induced to proliferate under general (CDE) and biliary (total bile duct ligation or DDC) injuries but not upon hepatocyte injury (acute CCl_4 damage and partial hepatectomy), and they formed colonies of cells bearing only biliary but not hepatocyte markers in culture. This study demonstrated for the first time that LLRCs established during normal liver morphogenesis act as unipotent biliary progenitor-like cells[108].

A severe hepatocyte depletion mouse model demonstrates a nonparenchymal source of hepatocytes: A novel mouse model was developed in which hepatocyte-specific inactivation of Mdm2 results in upregulation of p53 and induction of p53-mediated hepatocyte death and senescence in more than 99.96%. The extent of the ductular reaction in this model was significantly greater than that in the CDE diet, resulting in rapid activation of LSPCs throughout the liver, which can proliferate, differentiate into hepatocytes, and completely restore liver architecture and function. Loss of the reporter was seen over 6 months, indicating that there was progressive replacement of Mdm2-negative hepatocytes of parenchyma by LSPC-derived (Mdm2 intact, p53 low) hepatocytes. These data suggest the existence of a facultative and functionally significant LSPC population that does not contribute to homeostatic repair but that is activated in severe liver injury where hepatocyte senescence is widespread[109]. It will be interesting to see what results from lineage tracing experiments with available LSPC markers using this new model.

Hepatocyte role in liver regeneration

Experiments utilizing hepatocyte lineage tracing in mice have shown in various liver injury models that hepatocytes regenerate themselves without any significant contribution from LSPCs. Yanger *et al*[92] used the hepatocyte-specific promoter thyroid hormone-binding globulin (AAV8-TBG-Cre) to label hepatocytes. This transduction was highly specific, as all YFP+ cells were HNF4α+. Under DDC, CDE, CCl_4, or ANIT injury conditions, the percentage of labeled hepatocytes remained unchanged. As a control, a 2/3 partial hepatectomy was performed, which also showed no change in the YFP labeling index. Thus, using this sensitive labeling technique, the study failed to find evidence that hepatocytes arise from non-hepatocytes after recovery from multiple types of LSPC-inducing injuries[92]. The same was confirmed by lineage tracing using a hepatocyte-specific transthyretin promoter (AAV8-Ttr-Cre) that does not label cholangiocytes, HSC, macrophages, or endothelial cells. In this study, a CDE diet did not change the frequency (0.40% ± 0.23%) of non-fate-traced hepatocytes[101].

The key enzymes from various pathways and thus the corresponding metabolic capacities are found to be differentially expressed according to the zone[110]. Wnt signaling is active in perivenous hepatocytes[111] and has been shown to induce metabolic zonation of liver lobules[112]. Additionally, it has been shown that hepatocytes do not have equivalent replicative ability during homeostasis[113]. By lineage tracing of the Wnt-responsive gene Axin2 in mice, Wang *et al*[113] identified a population of proliferating and self-renewing cells adjacent to the central vein in a liver lobule. Axin2+ hepatocytes express the early liver progenitor marker Tbx3, and they are diploid; therefore, they differ from mature hepatocytes, which are mostly polyploid. The descendants of pericentral cells differentiate into Tbx3-negative, polyploid hepatocytes, and they can replace all hepatocytes on the liver lobule during homeostatic renewal. Adjacent central vein endothelial cells provide Wnt signals that maintain pericentral cells, thereby constituting the niche[113]. It has been postulated that these Axin2+ hepatocytes are a "unipotent stem cell-like" hepatocyte subpopulation[82]. Their role in regeneration upon liver damage remains to be determined[82].

Font-Burgada *et al*[114] recently described a pre-existing population of periportal hepatocytes that are found in the limiting plate around the bile duct and the portal vein of normal livers that contact the Canals of Hering. These cells express normal amounts of HNF4α and low amounts of Sox9 and other bile-duct-enriched genes (OPN, EpCAM, HNF1β), but they are negative for the ductal marker CK19. Because of this expression pattern, these cells were called hybrid hepatocytes (HybHPs), and they are a specialized type of hepatocytes that poorly express drug metabolizing genes. This and other characteristics protect HybHPs from toxic injury minimizing the probability that they will originate cancer (see below). These cells have a high regenerative potential because they undergo extensive proliferation and they replenish liver mass after chronic hepatocyte-depleting injuries, such as

repetitive CCl_4, producing fully differentiated and functional hepatocytes. A CDE diet leads to extensive liver damage with high mortality[115], and in these mice, most HybHPs are probably lost, and LSPC expansion is observed, suggesting that when HybHPs are not available, LSPC take control but do not originate new hepatocytes[114].

Further experiments in mice have shown that hepatocytes can change into a biliary ductular phenotype [104, 116] and then later re-differentiate into hepatocytes [117]. Acute activation of Hippo pathway signaling *in vivo* is sufficient to dedifferentiate adult hepatocytes into cells bearing progenitor characteristics, such as ductal markers panCK and CK19, and validated progenitor markers, such as SOX9, MIC1C3, and A6, at very high efficiencies. These hepatocyte-derived progenitor cells demonstrate self-renewal and engraftment capacity at the single-cell level. Hepatocyte-derived progenitors can redifferentiate into the hepatocyte lineage when normal Hippo pathway signaling is re-established *in vivo*[116]. Human and mouse hepatocytes can undergo reversible ductal metaplasia in response to DDC injury, expand as ducts, and subsequently contribute to restoration of the hepatocyte mass[117]. Likewise, after 6 weeks of cholestatic injury by DDC, most HybHPs changed by reducing cell and nuclear sizes and gaining strong expression of SOX9 and the ductal marker OPN. Ten percent of HybHPs lost HNF4α expression, and 2.5% of these cells were incorporated into bile ducts and showed strong CK19 expression. Suggesting that pre-existing ductal cells become oval cells during cholestatic injury but also imply that a small proportion of oval cells originate from trans-differentiating hepatocytes, probably identical to HybHPs[114].

Recently, Pu *et al*[118] described a new marker for a hepatocyte subpopulation named Mfsd2a. This marker is also expressed in periportal hepatocytes. During liver homeostasis, the periportal hepatocyte population decreases. Nevertheless, liver regeneration induced by partial hepatectomy and chronic liver injury significantly stimulates expansion of the Mfsd2aþ periportal hepatocytes, replacing the pericentral hepatocyte population. Mfsd2aþ hepatocytes regressed during cholestatic injury (bile duct ligation and DDC). However, Mfsd2a-CreER might not precisely target the same specific layer of hepatocytes that was targeted by the Sox9-CreER marker used by Font-Burgada[114]. Therefore, the combinatory use of both Sox9- and Mfsd2a-based recombinases could be valuable in understanding the functions of various periportal hepatocyte sub-populations in the future[118].

Evidence of HSC as LSPCs

Studies of fate tracing using a human glial fibrillary acidic protein (GFAP) promoter reported that HSC, a mesenchymal liver cell type that plays a significant role in liver fibrosis, are precursors of liver epithelial cells. Studies suggested that up to 24% of hepatocytes were derived from HSCs in mice that were fed the MCDE diet and had BDL injured livers[98,99,101,119]. However, the fact that GFAP marks CK19-expressing cholangiocytes makes it an unspecific marker[98,99,120]. When this tracing was performed with lecithin-retinol acyltransferase as a more specific HSC/myofibroblast marker[120] with BDL, MCDE diet, DDC diet, CCl_4, or 70% partial hepatectomy conditions, rare HNF4α+ hepatocytes were found that express the Cre reporter (at a frequency of 0.2 per 1000 cells). In 2014, Schuab[101] assessed the contribution of HSC/myofibroblasts to hepatocyte regeneration by performing fate tracing based on platelet-derived growth factor receptor beta (Pdgfrb) expression in mice, a marker that was recently shown to be specifically and efficiently expressed in HSC/myofibroblasts[121]. After mice were fed a CDE diet for 3 wk, the number of fate-traced hepatocytes was negligible in both injured and non-injured livers. These results suggest that HSC/myofibroblasts are not a source of new hepatocytes in CDE-diet-induced chronic liver injury and exclude that HSCs function as epithelial progenitors.

Kordes *et al*[122] transplanted GFP+ HSCs into wild-type rats of two liver injury models with LSPC expansion: partial hepatectomy in the presence of 2-acetylaminofluorene or retrorsine. Transplanted HSCs contributed to liver regeneration in recipient animals by forming approximately 10% to 14% of mesenchymal tissue, progenitor cells, hepatocytes, and cholangiocytes and elevated direct bilirubin levels in blood sera of GUNN rats, indicating recovery from the hepatic bilirubin-handling defect in these animals[122]. These authors did not specify the percentage of HSC-derived hepatocytes but suggest that this is evidence for the characterization of HSCs as LSPCs and for their contribution to tissue repair. However, it should be considered that enzymatic isolation of HSCs from their niche and transplantation into the regenerating liver generates a condition that can promote HSC reprograming in a way that does not resemble *in vivo* behavior (see below)[122].

Evidence of endothelial cells as LSPCs

There is only one report in which lineage tracing of liver sinusoidal endothelial cells (LSECs) was performed. This report used mice carrying Tie2-Cre or VE-cadherin-Cre constructs to trace LSECs in liver regeneration. Tie2-Cre is expressed in the vascular endothelium and VE-Cadherin is expressed in the endothelium of developing and quiescent vessels. Some YFP-positive liver sinusoidal endothelial cells were observed to convert into hepatocytes following a two-thirds partial hepatectomy, accounting for 1% of

all hepatocytes[123].

The dominant role of hepatocytes in hepatic regeneration

According to the majority of previously described LSPC results, when LSPC response is activated, LSPCs do not differentiate into hepatocytes, and the purpose of their expansion is unknown. One hypothesis is that oval cells could be in charge of rebuilding of the bile canaliculi network and liver polarity[114,124]. Inking the ductal tree in models of liver injury has showed that expanding LSPCs are extensions of the pre-existing ductal tree, which further challenges the role of LSPCs in hepatocyte regeneration[114].

No evidence has been provided to either prove or disprove the existence of resident LSPCs. In most of the reports, it seems that only a very low percentage of hepatocytes were traced from LSPCs and would not have a significant contribution to the liver regeneration. It is important to note that the cumulative effects of LSPC-to-hepatocyte differentiation at a constant low rate could hypothetically reach a functionally significant level over time in long-term chronic injury[92]. Therefore, the studies presented here do not rule out a functionally significant contribution of LSPCs in long-term liver injuries, such as chronic hepatitis, in humans.

We can conclude that most studies point towards a dominant role of hepatocytes in regeneration at all levels of liver damage. However, these studies also point to the fact that during severe or chronic damage, hepatocytes are the main source of facultative LSPCs since they must go through a dedifferentiation process to be able to fully reach their regenerative potential.

HCC CELL OF ORIGIN

In many experimental models of hepatic carcinogenesis, LSPC responses precede cancer development, something similar occurs when ductular reactions precede HCC in human cirrhotic livers[125]. Biliary/progenitor markers[126-128] are often linked with poor prognosis and stemness features[128,129]. These observations have led to the idea that progenitors or cholangiocytes might be the cell of origin of HCC[130,131].

LSPCs as the cell of origin of HCC

Very few studies have carried out lineage tracing LSPCs during hepatocarcinogenesis. HNF1β+ LSPCs were traced in mice that were induced to express the reporter 7 d before treatment with carcinogenic DEN at postnatal day 17. HNF1β+ cells were also traced in Mdr2KO animals, which are commonly used as genetic models for spontaneous, inflammation-induced HCC development[132-134], with extensive activation of LSPCs that could undergo malignant transformation. However, the identified tumors never expressed the reporter in either of the HCC models[132]. Another study traced Foxl1-positive cells simultaneously with exposure to diethylnitrosamine (DEN), followed by multiple injections of CCl_4. None of the tumor nodules expressed YFP, indicating that Foxl1-expressing cells are not the origin of hepatotoxin-induced liver tumors[131].

LSPCs were marked by tamoxifen-inducible OPN-iCreERT2 in several HCC models: chronic administration of DEN, DEN injection into 15-day-old animals, DEN and multiple doses of CCl_4. The last model listed mimics hepatocarcinogenesis in the setting of liver fibrosis[135]. The same study also marked K19-CreERT LSPCs in the DEN+CCl_4 HCC induction model. Reporter expression was absent in all tumors[7], confirming that in several genotoxic DEN-induced hepatocarcinogenesis models and in mice traced with various LSPCs markers, HCCs did not arise from LSPCs or cholangiocytes.

As we discussed in the previous section, most of the markers used to trace LPSCs present some grade of unspecificity because they can also be expressed by injured hepatocytes. However, because none of these markers produced HCC tumors that expressed the reporter gene, it is possible to conclude that LSPCs expressing such markers are not the cell of origin of HCC in these hepatocarcinogenic models (Table 2). These studies question the hypothesis that progenitors are the cell of origin of HCC. However, we should note that these studies did not exclude the potential role of LSPCs to tumorigenesis in other models of HCC. Hepatocytes are the ones that metabolically activate DEN, therefore LSPCs may not have been exposed to genotoxic stress in most of these models[131].

Hepatocytes as the cell of origin of HCC

HybHPs are highly proliferative during chronic liver injury, therefore they could be an alternative origin for HCC. This hypothesis was tested tracing HybHPs and ductal cells in three independent mouse models of HCC: DEN-induced HCC[136], MUP-uPA mice fed a high-fat diet (HFD)[137], and the STAM model of diabetes-promoted HCC[138], using the Sox9-CreERT;R26RYFP reporter. DEN is metabolically activated in pericentral/zone 3 hepatocytes (unlike HybHPs) and does not induce LSPCs expansion, however HFD, which induces liver damage and compensatory proliferation in both MUP-uPA and STAM mice, gives rise to extensive LSPC proliferation. Tumor nodules and hyperproliferative lesions were negative for YFP+ cells; suggesting that the source for HCC in these models is different form HybHPs or ductal cells. The authors propose that even with the high rate of proliferation that HybHPs

Table 2 Studies tracing possible cells of origin of hepatocellular carcinoma

Cell	Tracing gene promoter	HCC models in mice	Tumor positive to lineage-traced	Ref.
LSPCs	HNF1β	DEN 17 d postnatal Mdr2KO	NO	[132]
	Foxl1	DEN/CCl_4	NO	[131]
	OPN	DEN 15 d postnatal DEN/CCl_4	NO	[7]
	K19	DEN/CCl_4	NO	[7]
HybHPs + LSPCs	Sox9	DEN MUP-uPA/HFD STAM	NO	[114]
Hepatocytes (mature)	TBG	DEN/CCl_4 DEN/CDE DEN/DDC Mdr2KO	YES (approximately 100%)	[7,131]
HSC	Lrat	DEN/CCl_4 Mdr2KO	NO	[7]

HCC: Hepatocellular carcinoma; LSPC: Liver stem progenitor cell; HSC: Hepatic stellar cells.

present, they lack the metabolic properties needed to participate in HCC initiation. DEN is metabolically activated by CYP2E1, which is expressed only in fully differentiated pericentral zone 3 hepatocytes[139] but absent in HybHPs. The same applies for class 4 genes, which only are expressed by fully differentiated hepatocytes, are needed for de novo lipogenesis and ROS generation through fatty-acid oxidation[137] in HCC development that is dependent on HFD consumption[114].

The evaluation to determine if mature hepatocytes give rise to HCC was made by two groups using an AAV-TBG-Cre promoter to label both periportal and pericentral hepatocytes in a model of HCC induced by DEN, followed by multiple injections of CCl_4. The treatment led to the activation of ductular reactions and to the development of multiple nodules that had the morphology of HCC. Both groups found that 99.7% of tumors[7] and all tumors[131] were lineage-traced as hepatocyte-derived HCC, concluding that hepatocytes are the cell of origin of HCC in this model (Table 2). The HCC nodules in the first study were positive to progenitor and hepatoblast markers AFP and H19, respectively, and most of them expressed high levels of CD133[7]. Therefore, mature hepatocytes can give rise to tumor cells that are positive for biliary/progenitor markers, therefore the idea that progenitors are the cells of origin of HCC, cannot be supported by the expression of biliary/progenitor markers by HCC[131].

Considering the possibility that LSPC origin may only be evident in models of HCC that have higher LSPCs cell presence and turnover. Mu *et al*[7] also used the combination of DEN with CDE or DDC diets. Comparable to the DEN+CCl_4 model, DEN+CDE and DEN+DDC-induced tumors showed high expression of HCC, LSPCs and hepatoblast markers, but they were originated completely from AAV-TBG-Cre+ hepatocytes. Hepatocyte origin could be specific to the HCC models that are dependent on the metabolism of carcinogens in hepatocytes; this possibility was assessed by using the DEN-free model of HCC: Mdr2KO mice, in which all HCCs arose from AAV8-Tbg-Cre-labeled hepatocytes[7]. Confirmation of such results must be performed using other HCC models that do not require the metabolism of carcinogens. All these evidence strongly suggest that HCC is originated from differentiated hepatocytes (Table 2). Further research using alternative labeling strategies will be essential to identify the subpopulation of hepatocytes responsible of HCC origin, such as tracing Axin2+ hepatocytes[131]

HSC as the cell of origin of HCC

HSCs were labeled by Lrat-Cre in mice that underwent injury-driven hepatocarcinogenesis induced by DEN+CCl_4. The authors did not find any tumors derived from HSC Lrat-Cre-labeled cells. Comparable results were obtained in the Mdr2KO model, where no tumor cell was derived from Lrat-Cre-labeled HSCs and all fluorescent cells within tumors were desmin positive and HNF4α and cytokeratin negative[7].

CHARACTERIZING CCO WITH DIFFERENT ASSAYS RESULTS IN DIFFERENT CONCLUSIONS

Depending of which experiments researchers used to delineate the origin of tumor a cell, conclusions were drawn that are controversial. Previously, works have reported that different populations participate in acute and chronic liver damage and that the cellular response is strongly associated with the risk factors and severity of the lesion[140].

With respect to CCOs, it was demonstrated that "any" cell could switch their cellular program to give rise to CSCs after a strong oncogenic stimulus, but it is important to consider that in this work, there was a genetic manipulation of the cells that were transformed with transgenes that encode oncogenic H-Ras and SV40LT[141]. In 2011, Chang and colleagues indicated that oval cell lines transfected with the HBV x gene (HBx) and treated with aflatoxin B1 in vivo, are responsible of HCC. These intrahepatic tumors included HCC cell markers (HepParl, ALB, CK8 and AFP) and mesenchymal cell markers (Vimentin and SMA), which is a stem-like phenotype[142].

In 2016, Kaestner's group used genetic lineage tracing to demonstrate that hepatocellular adenomas (HCA) and HCC are derived from mature hepatocytes in one model that was induced by DEN/CCl4; however,

a disadvantage of these studies was that they yielded too few HCCs for the studies to be considered a good evaluation. Moreover, these HCCs did not correlate well with c-Myc, but other authors have reported that in the liver, transgenic overexpression of c-Myc in murine hepatocytes is sufficient to induce liver tumors[131]. Another study by Mu and colleagues concluded the same result, although they selected a different stem-cell marker promoter[7]. In this study, the research group obtained higher numbers of HCCs that showed good correlation with HCC markers that had been reported by other authors, such as CK19[11,143]. The fact that there was a difference in the number of HCCs obtained when both groups used the same system makes the assay questionable.

In 2016, another group also used the line tracing assay with CD133+ to describe the ability of SCs to display different tumors[74]. Some tumors, especially HCCs, are considered to have a tendency to evolve and grow for more than 30 years, which means they are not common in young populations[144]. Finally, the authors did not have a different evaluation of different the liver cancers that were obtained, such as HCC, HCA and ICC (intrahepatic cholangiocarcinoma).

The lineage tracing assay is used to assess the probable CCO during cancer initiation. During lineage tracing, a specific subpopulation is labeled using different cell-specific promoters *in vivo*. Like other techniques, there are perks and disadvantages accompanying lineage tracing. The first point with lineage tracing is that it only uses mice. Anatomically, there are important differences between human and rodent. The liver is our organ of interest, and in a mouse liver, there are four lobes, denoted as right, left, medial and caudal. A human liver is formed by only two lobes that are separated by the falciform ligament; therefore, the anatomical distribution differs between species, but until now, there was no report of how this fact could affect the development or growth of HCCs. In addition, there is a difference in isoform composition, expression and catalytic activities of drug metabolizing enzymes between animal species. For example, CYP2E1 is the only isoform that has no appreciable differences in expression and catalytic activity among several species. However, CYP2A expression in humans corresponds to the 2A6, 2A7 and 2A13 isoforms, but its expression in mice corresponds to 2A4, 2A5, 2A12, 2A22. Additionally, CYP2B, C and CYP3A have distinct patterns of expression. Another example is the marked differences in the metabolic patterns of testosterone and 7-hydroxycoumarin; the main testosterone metabolites that are observed in both species are 2β-OHT, 6β-OHT and androstenedione. Nevertheless, the last two are reported to have different metabolic rates, 7α-OHT is only detected in mice and rats. Moreover, in the human liver, it had been reported that the amount of 7-hydroxycoumarin sulphate was minimal and that it differs between other species, such as rat, monkey and mice. With that being said, mice tumors that are caused by the induction of specific CYPs may not be of human significance because of the well-known species differences between human and mouse CYP gene regulation[145]. In addition, somatic mouse cells have high telomerase activity and significantly longer telomeres than human cells, suggesting that, in most linages, mouse cells may never experience actual terminal differentiation. Although some expression patterns of HCC in rodents and humans are not closely related, there is a comparison of global gene expression in one study indicative of the reproduction of some molecular pathways and expression of molecular patterns. Therefore, these authors indicate the importance of the identification and use of animal models that allow for comparison and extrapolation of the results to their specific human cancers or their subclasses.

The second problem is using the combination of mouse tumor models and lineage tracing to elucidate the cell of origin. In general, the majority of human epithelial cancers, especially liver cancer, develop very slowly with decades of clonal evolution and accumulation of epigenetic and genetic alterations. It is well known that the diagnosis of HCC occurs in the late stage, generally when the disease is in the symptomatic phase. Thus, the initial point when mutations trigger carcinogenesis is not easy to elucidate. Nonetheless, in mouse models of human cancer, the promoter of the gene of interest is instantly turned on, leading to genetic defects that occur all at once in an entire population of cells. This phenomenon is fundamentally different from what happens in most human cancers, where there is a sequential acquisition of mutations. Ideally, a cancer model should recapitulate the natural course of the disease by introducing sporadic mutations at a low frequency. For example, studies involving line tracing of CD133(+) and (TBG)-Cre do not study or describe the mutations that are gained or appear during the development of HCC[56].

The third problem is that the labeling efficiency in lineage tracing studies is highly variable and is dependent on Cre- or reporter-driving promoters. The efficiency is usually low and the results are often subject to alternative interpretations. It also depends on the promoter, for endogenous promoters, activity in poor differentiated cells could be not enough to drive reporter expression, causing low efficiency and difficult interpretations. For example, when CreER is driven by the endogenous X promoter, this study will conclude that the cells that express X will regenerate into more X cells. However, the SCs can differentiate

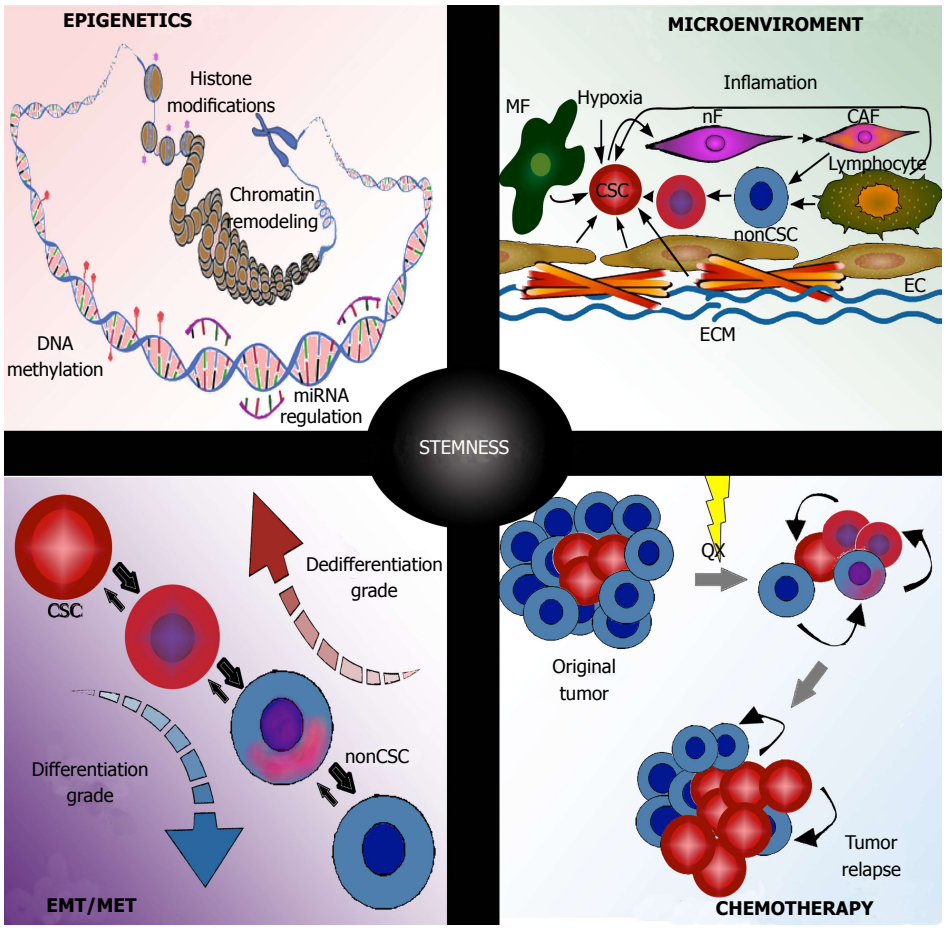

Figure 2 Stimuli that trigger the gain of stemness of non-cancer stem cells in hepatocellular carcinoma. The diagram includes the conditions that are reported to induce stemness: epigenetics (histone modifications, DNA methylation, Chromatin remodeling and miRNA regulation); microenvironment that includes hypoxia, inflammation and cellular populations participation (MF, nF, CAF, lymphocytes, CSC and non-CSC, ECM, EC); epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) and chemotherapy (QX: chemotherapy insult, *i.e.* carboplatin, curcumina and cisplatin). MF: Miofibroblast; nF: Normal fibroblast; CAF: Cancer associated fibroblast; CSC: Cancer stem cell; ECM: Extracellular matrix; EC: Endothelial cells.

into cells other than X cells, and then the no-X cells are not labeled because Cre activation is weak. This problem is conducive to inaccurate or erroneous conclusions, because only some differentiated cells will be tagged by the reporter. Similarly, exogenous or heterologous promoters randomly integrate into the genome and are regulated by different mechanisms than endogenous promoters, leading to expression patterns different from those of the endogenous gene. Due to read-through transcripts or regulatory elements at the insertion site, promoters may drive expression in tissues other than predicted[56].

Finally, all systems need to be optimized because the remaining construct has insertions that could cause effects; in the absence of an inducer could finish in spontaneous back ground recombination. In order to eliminate background recombination, AhcreERT can be used, since Cre transcription is controlled by tamoxifen binding and the Ah promoter. A transgene insertion of Cre recombinase under the control of a specific promoter may alter the function of the endogenous locus *via* activation or silencing. Sensitivity to Cre-mediated recombination of different LoxP-flanked target genes can be quite variable. Unexpected excision or incomplete incorporation of regulatory elements into the driver construct could occur. Lastly, the genetic background and variable maternal/paternal germline expression can also modify Cre activity. Therefore, it is important to optimize the animal models used in this assay[56].

The cell transplant study is another assay that allow know the possible CCO. However the main disadvantage present in this study is the limitation of only one population being selected. The study could not demonstrate the participation of other CSC subpopulations that could potentially participate in tumor development[146]. This study also did not evaluate the CSCs plasticity, which is the ability of CSCs to transition to non-CSCs or vice versa. Another crucial point is the immunophenotype of the animals used for this assay. In general, the assays are performed in immunodeficient mice, which is a different context

than real patient situations. In addition, there are big differences between the original tumor environment (niche), the proteins of the extracellular matrix, non-CSCs, and cells that cooperate within the tumor (immune cells, endothelial cells, *etc.*). One study demonstrated that when HCC cell lines are cultured under standard conditions *in vitro* and then injected subcutaneously into NMRI mice, they dedifferentiated into embryonic-like type cells. Thus, the behavior of CSCs from a distinct origin could probably condition the results[147].

In vitro, clonogenicity and multilineage differentiation have been extensively regarded as assays that demonstrate stem cell potential[148]. Some of the cellular markers that identify liver cells with clonogenic and multilineage potential are EpCAM, Lgr5, CD133, MIC1-1C3, Foxl1 OPN, Sox9 and CD24[76,94,107,149-153]. However, it has recently been suggested that removing liver cells from their *in vivo* environment could trigger the activation of a stem cell state because of damage to the tissue, similarly to what occurs during a regenerative response, and these plasticity properties may not be representative of *in vivo* biology[101,154,155]. Therefore, we mainly focused on discussing *in vivo* experiments, such as repopulation following transplantation and lineage tracing.

ARE CSCs A CELL STATE? OR IS THERE A DYNAMIC INTERCONVERSION EQUILIBRIUM BETWEEN CSCs AND NON-CSCs?

Currently, in respect to the CSC hypothesis, new data proposed that the added concept of plasticity ability explains how CSCs are maintained in the tumor. The proposal is that there is a dynamic interconversion between CSCs and non-CSCs in response to environmental injury, intercellular communication and cues from the tumor niche. These new concepts complicate the perspective of cancer, and it is still controversial because there is no agreement of how it could be validated *in vivo*. Altered conditions could trigger the gain of stemness, including EMT-MET, epigenetics, microenvironment and selective stimuli, such as chemotherapy (Figure 2).

The epithelial-mesenchymal transition (EMT) is defined as the process by which epithelial cells lose their cell polarity and cell-cell adhesion and gain migratory and invasive properties to become mesenchymal SCs. This condition is important, and it is present in numerous developmental processes; it has been described to be implicated in wound healing, fibrosis, and cancer in which non-CSCs gain SC properties and become CSCs. This is a phenomenon that demonstrates high cellular plasticity[156] and has also been seen in hepatocytes. When cells are in the transition to the SCs-like phenotype, some factors could be induced, such as Octamer 3/4 (Oct3/4), sex determining region Y-box 2, c-Myc, and Kruppel-like factor 4 (Klf4)[157]. Moreover, the signaling pathways related to stemness are activated, including the Wnt/β-catenin, Hedgehog, Notch, and TGF-β signaling systems. Targets, such as β-catenin, human telomerase reverse transcriptase, cyclin-dependent kinases, and Myb (from the myeloblastosis gene family), are also activated[46].

The link between EMT and the CSC phenotype has been studied broadly. It had been reported that cells undergoing EMT may resist toxic injuries and chemoradiation therapy, and a series of studies demonstrated that CSCs are more resistant to conventional therapies than differentiated cells. Fillmore and coworkers selected the cells with surface phenotype CD44+/CD24-/low/ESA+ to isolate CSCs from human breast cancer cell lines and demonstrated preferential resistance of CSCs to chemotherapy[158]. In another tumor model (pancreatic cancer), the cells that are rich for the "stem-cell-like" phenotype when they undergo EMT have chemoradiation-resistant characteristics. They also have increased migratory and invasive capacities *in vitro* and *in vivo*[159]. Many stimuli trigger the EMT, such as IL-6, by an inflammatory loop, which accelerates the transformation of normal LSPCs to metastatic CSCs. In addition, Malfettone A and coworkers demonstrated the induction of EMT in HCC cell lines *via* TGF-β stimulus, and that these lines could gain molecular markers and characteristics of CSCs such as invasiveness. The authors also concluded that the partial EMT phenotype confers a higher stemness potential than that of the full EMT[160]. In addition, experimental EMT and derived inflammatory cytokines, such as IL-6, IL-8, TGF-β, and TNF-α, can all promote the manifestation of CSC phenotypes and properties in non-CSCs[161].

Epigenetic is new to cell research that is defined as stable alterations or changes in gene expression that are not caused by changes in DNA sequence and are heritable across every round of cell replication. Changes in expression affect the cellular phenotype and/or differentiation state. One of the main control mechanisms of cellular developmental hierarchies is through epigenetic regulation of the genome. Until now, there were four important epigenetic mechanisms: chromatin remodeling, DNA methylation, histone modifications, and noncoding RNAs, including miRNAs[162].

For the first epigenetic mechanism, histone methylation occurs mainly on lysine and arginine, histone readers use such methylated residues as docking sites. In general, histone H3 lysine 4 (H3K4), histone H3 lysine 36 (H3K36), and histone H3 lysine

79 (H3K79) are associated with gene activation, whereas histone H3 lysine 9 (H3K9), histone H3 lysine 27 (H3K27) and histone H4 lysine 20 (H4K20) are associated with gene repression and heterochromatin formation. It was reported that in the absence of the UTX, one histone, H3K27 demethylase, induces epithelial-mesenchymal transition (EMT)-mediated breast CSC properties by increasing the expression of SNAIL, ZEB1 and ZEB2[163]. In head and neck squamous cell carcinoma (HNSCC), it was reported that the participation of G9, a histone methyltransferase that makes H3K9, triggers the transcriptional repression of E-cadherin, thus influencing the EMT, cell migration, tumorsphere formation and increased expression of CSC markers[164].

DNA methylation results in long-term gene silencing, which is performed by DNA methyltransferases (DNMTs), transfer a methyl group from S-adenosyl methionine to CpG dinucleotides at cytosine bases on gene promoters and regulatory regions. CpG are concentrated in short regions called "CpG islands". Rountree and colleagues showed that TGF-β regulated CD133 expression through inhibition of DNMT1 and DNMT3b and the subsequent demethylation of promoter-1. Another relation between DNA methylation and CSCs was found in breast and colon cancers. It was described that aberrant Wnt/β-catenin enhanced the methylation of promoters of various Wnt inhibitors, such as Wnt inhibitor factor-1 (WIF-1), AXIN2, Dickkopf-related protein (DKK1) and secreted frizzled-related protein 1 (SFRP-1)[165,166]. In HCC, it was described that methylation affects the expression of E-cadherin and some other markers related to the EMT-phenomenon that are highly related to the stemness hallmark. Genes that are commonly epigenetically altered in cancer cells through promoter CpG methylation often show a specific epigenetic regulation pattern in embryonic SCs, termed "bivalent chromatin". This bivalent state could explain the dynamic response to environmental changes, such as differentiation stimulus. Thus, these genes can easily switch between an active or repressed condition. One clear example is during the EMT, where TGF-β stimulus changes the E-cadherin expression state[167].

miRNAs are endogenous non-coding RNAs that have been identified as post-transcriptional regulators of gene expression. These RNAs function by targeting mRNAs for degradation or repressing mRNA translation by binding to the 3′- untranslated region of mRNAs. They have been reported to be involved in the control of self-renewal and the differentiation of embryonic SCs, and they are also implicated in the progression and tumorigenesis of various cancers. Some miRNAs, such as miR-181s, miR-130b, miR-150 and miR-121, have been suggested to be important in epigenetic regulation in liver CCO. In HCC patient samples, a correlation was found in the miR181 family to features of hepatic SCs and progenitor cells. In addition, functional studies showed that forced expression of miR-181s induced stemness in HCC cells with a significant enrichment of the EpCAM+ marker. miR-130b overexpression was found in HCC cells that had a CD133+ marker. Moreover, its overexpression induces high proliferation, self-renewability, tumor initiation and chemotherapy resistance abilities in HCC cells. Moreover, it seems that miR-150 negatively affects the proliferation of CSCs and spheroid formation in HCC cells, probably through modulation of the downstream target c-Myc [168]. Jung and colleagues have identified miR-122 as the regulator of a common network of genes that promote SC self-renewal and HCC proliferation. In human primary hepatocytes, miR-122 is expressed, but it was attenuated in HCC. Finally, miR-122 expression may be associated with the methylation status and RNAPII binding activity of the promoter region of this gene[169]. Currently, the importance of epigenetic turn on or off in cancer is not only for the gain of stemness in non-CSCs, but it is also considered to be important for the regulation of many functions, such as cell plasticity and drug resistance acquisition[170].

Development of resistance represents a major drawback in cancer treatment, indeed, there are a lot of reports of conversion of non-CSCs to CSCs by this way and the treatments include: irradiation (IR), radiochemotherapy and chemoteraphy. IR may cause changes in a tumor microenvironment that can affect invasion and metastasis. IR changes cellular metabolism, leading to EMT and CSC phenotypes. Additionally, in liver cancer, it was reported that EMT is responsible for enhancing the motility and invasiveness of cancer cells; a possible mechanisms for this is that it is mediated by the induction of ROS production, directly through extracellular water radiolysis, or indirectly through intracellular metabolic alterations and mitochondrial dysfunction[171]. Also it had been described that IR could activate stemness pathways in liver heterogeneous no-cancer stem cells by enhancing upregulation of the pluripotency genes Sox2 and Oct3/4, resulting in a high enrichment subpopulation with new abilities such as better spherogenesis and resistance to radiotheraphy[172]

It was also suggested that radiochemotherapy has a side effect of directly transforming non-CSCs into induced CSCs, which possibly contributes to tumor recurrence and metastasis[173]. For example, Vares and coworkers indicate that the combination of progesterone and irradiation could induce a higher cancerous phenotype in MCF-10A cells and increase the proportion of CSCs that give the cells a resistance phenotype[174]. Another study showed that combining

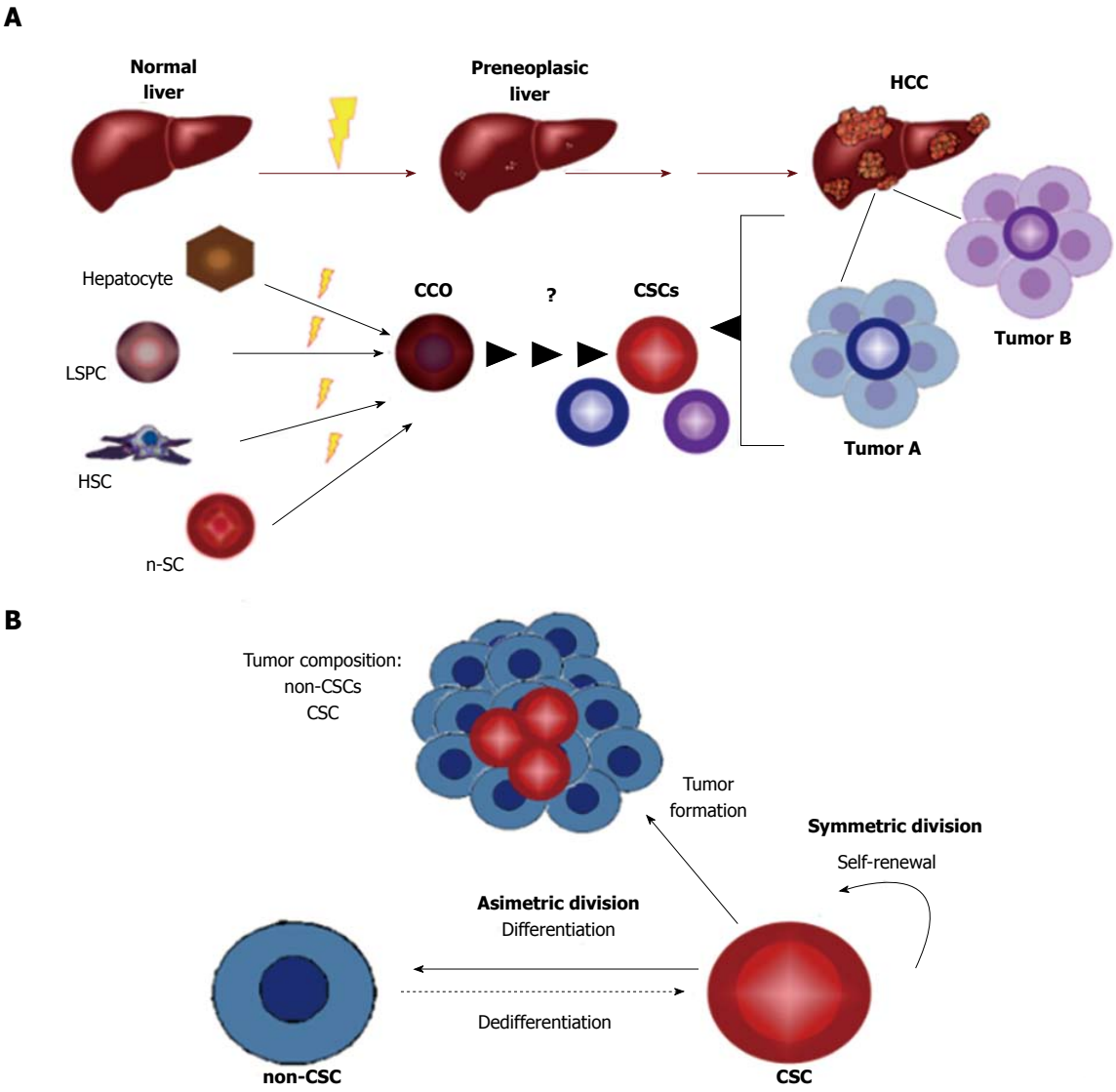

Figure 3 An integrated model of cancer cell of origin and cancer stem cell hypotheses in hepatocellular carcinoma. A: Different cells involved in adult liver regeneration are potential targets of malignant transformation when they received hard insults (Yellow ray, *i.e.* carcinogenic agent, partial hepatectomy). These cells could be considered CCO (Hepatocytes, LSPC, n-SC, HSC), however many studies point to the fact that during severe or chronic damage and consistently in the beginning of HCC, hepatocytes have the main role. The relationship between CCO and CSC is not clear until now. CSCs composition is heterogeneous in HCC tumors. B: The hierarchy hypothesis shows the tumor composition: CSCs and non-CSCs. This includes the plasticity model, which indicates the interconversion by differentiation/dedifferentiation balance between non-CSCs and CSCs. LSPC: Liver stem progenitor cell; n-SC: Normal stem cell; HSC: Hepatic stellar cells.

valproic acid (VA), a histone deacetylase inhibitor, and ionizing radiation can promote the dedifferentiation of ALDH- cells into ALDH+ cells. In ovarian cancer, it had been described that short-term, single treatments of chemotherapy cause an enrichment of CSC-like cells[175], and a similar result was seen in lung adenocarcinoma A549 cells that were exposed to a combination of paclitaxel and being cultivated in serum-free medium [176]. These data propose that non-CSCs can give rise to CSCs through dedifferentiation or induction. These new cells are termed induced-CSCs (iCSCs).

In the case of chemotherapy there are some studies that describe the gain of new iCSCs population after the treatment with anticancer-drugs. For example, Hu and coworkers describe in liver cancer cells treated with carboplatin, significantly increases the ability to form spheres, it seems that the treatment with carboplatin induce some key genes of stemness maintenance such as Oct4 and Sox2[177]. Another example is one study with curcumina, which has been demonstrated in some cancers that suppress cancer cell proliferation, invasion metastasis and angiogenesis, however it was demonstrated that although it induces arrest on glioma cells also induces changes in cell morphology from spindle to round with an overexpression of stem markers CD133 and Nestin. In addition, the round glioma cells show induction of the Sox2/4-Oct4 axis, hallmarks factors of stem cells[178]. Moreover Nör and colleagues discover that cisplatin enhances the fraction of CSCs in head and neck squamous cell carcinoma (HNSCC). The induced-

CSCs overexpress ALDH and CD44, also they report activation of the signaling pathways involved in the stemness *via* IL-6/STAT3[179].

Neighborhood impact on CSCs

It has been reported that pathophysiological changes in the liver during inflammation/regeneration could induce the initiation or promotion of liver cancers[180,181]. Some alterations of the hepatic tumor microenvironment are: increased lymphocyte infiltration, HSC activation and the expansion of LSPC, cell migration, release of free radicals, cytokines, chemokines, and high proliferation rate. These stromal activations induce signaling pathways and cause the accumulation of genetic and/or epigenetic changes. These conditions may cooperatively support and maintain liver CSCs. Thus, the cellular neighborhood is important. There is much evidence indicating that co-cultivation of normal fibroblast and tumor cells facilitates/increases the tumorigenicity of tumor cells. Nevertheless, more recent evidence suggests that CSCs could modify the microenvironment by transforming neighboring fibroblasts into cancer-associated fibroblasts (CAFs), which have increased proliferation rates and unique secretory factors compared with their normal counterparts[182].

In addition, hypoxia favors an increase in the CSC pool *via* HIF-1α and HIF-2α activation, and it also induces EMT by activating EMT-associated signaling pathways, transcription factors or repressors (SNAIL, ZEB1, TWIST and TCF3). Moreover, both hypoxia and reactive oxygen species (ROS) upregulate CSC stress *via* the ROS, TGF-β and TNF-α signaling pathways with the goal of enhancing cancer cell survival and maintaining cancer cell stemness[183]. Another study showed that myofibroblast-secreted factors restore CSC phenotypes in differentiated colon cancer cells *in vitro* and *in vivo*[184].

Tumor angiogenesis is important in providing the tumor mass with nutrients and oxygen and maintaining the CSC population. In one study, it has been shown that vessel-forming endothelial cells within the niche closely interact with Nestin+/CD133+ brain CSCs to keep them in a SC-like state. Another study was interested in the CXCR4/ CXCL12 axis, which forms a chemo attractant gradient that allows normal and CSCs to find their niche. Antagonists against CXCR4, such as Plerixafor (AMD3100) and small peptide CXCR4 inhibitors (T140, TC14012, TN14003), can block the stroma-CSC communication axis thereby inducing the cells to become vulnerable to cytotoxic drugs[185]. For all the evidence that was previously mentioned, some of the investigators were focused on affecting the CSCs niche to dissolve the tumor. However, next studies must consider the heterogeneity in subpopulations and that there might be several distinct CSC niches within one tumor, which may change over time depending on the tumor stage and therapy.

CONCLUSION

There is no doubt that there is controversy about cell interplay during the initiation, maintenance and dissemination of cancer cells and about how to explain the phenomenon of radio/chemotherapy resistance of tumors. The hypothesis of cancer stem cells (CSCs) give us the opportunity to look forward, but now, several new questions have been introduced, such as determining the relationship between OCC and CSC, determining if markers correctly characterize CSCs and determining whether there is a main or single CSC population. This suggests using operational definitions rather than phenotypically defined cells to distinguish the definitions of cancer cell of origin, cancer stem cell, tumor propagating cell and stem/progenitor cell. The controversy is increased if the tissue in which the cancer originates is considered. Hepatocellular cancer, the most prevalent liver cancer, is unique in respect to this contention due to the great regeneration capacity of liver and the controversial role of liver progenitor cells in liver regeneration and cancer initiation.

The studies discussed here question the hypothesis that progenitor cells are the cell of origin of HCC. However, we should note that current studies did not exclude the potential role of LSPCs to tumorigenesis in other HCC models, or human HCC, considering the discussed distance between murine models and the human disease. Mature hepatocytes are emerging as the cell of origin of HCC. Importantly, they can produce tumor cells that are positive for biliary/ progenitor markers. This indicates that, on its own, the expression of such markers by HCC does not support the hypothesis that progenitors are responsible for the origin of HCC (Table 2, Figure 3). Further research using specific labeling strategies will be necessary to identify which subpopulation of hepatocytes contributes to tumorigenesis, such as tracing Axin2+, or Mfsd2aþ hepatocytes.

It is worth noting that, even though some facultative populations (of unknown origin) were induced by liver damage schemes and had a variable degree of participation during liver parenchymal regeneration, including the Sox9, Foxl1, OPN, and HNF1β populations, they are apparently not the targets of malignant transformation during carcinogenesis. Perhaps this shows that an expanding population of cells is not enough to ensure malignant transformation, and other considerations should be made. As we discussed earlier, this could mean that certain metabolic firms are required to be susceptible to malignant transformation. Another probable reason is that such populations are only transiently amplified, thus they only express the lineage marker temporarily, preventing it from being

traced for the extended periods that are needed during HCC development.

As for the results presented by several research groups about the progression of cancer, it seems that two new considerations stand out. The first is that many subpopulations of CSC (and their progeny) have different phenotypes and functions and coexist within the tumor. The second is that the hypothetical interchange of cell populations that have certain CSC phenotypes and possible interchanges with non-CSCs leave us with the fact that the main characteristic of tumor cells is plasticity. Such great plasticity is an advantage when cancer cells are compromised in the face of certain challenges, such as hypoxia or chemotherapy. This leads to the proposition that a CSC-state is a description of a highly adaptable, dynamic intrinsic property of tumor cells instead of a static subpopulation of a tumor. This characteristic that there are many different phenotypic populations within the tumor makes tumors out smart therapeutic protocols that are directed at a single target. Thus, for future research and clinical intervention, it is important to consider more than one marker to identify/target different subpopulation of CSCs. A further avenue of research will be to uncover mechanisms that trigger the interconversion of cell populations that have different phenotypes within the same tumor. In this way, we could find new protocols that first tackle the ability of cancer cells to exhibit plasticity and then lock them into a single, or at least a few, defined subpopulations. Then, we could find therapeutic targets for the locked cell populations.

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