Epigenetic regulation of cancer stem cells in liver cancer: Current concepts and clinical implications

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Introduction

Two general models of carcinogenesis exist: the stochastic and hierarchic cancer model. According to the traditional clonal evolution model, tumor formation is the consequence of accumulating random genetic events in any normal differentiated cell whereas the cancer stem cell (CSC) model postulates that a single CSC gives rise to a hierarchic organization within a tumor [1,2]. Even though the concept of a rare group of cells being responsible for tumor initiation in vitro and in vivo is not new, the CSC model remained hypothetical until compelling evidence has emerged in the last decade [3–9]. The stochastic and hierarchic cancer models were thought to be mutually exclusive, although current findings favor a likelihood of the complementary co-existence based on the assumption that cancer is a genetically generated disease that is maintained and tightly regulated by epigenetic changes (Fig. 1). Similar to the phenotypic diversity of normal adult tissues that is generated by tissue specific stem cells, the CSC model posits that at the apex of tumor formation is a stem-like cell (commonly referred to as CSC or tumor-initiating cell) that is responsible for the heterogeneity observed within the clonally derived tumors including liver cancer [9,1,10,2]. Despite functional similarities with the adult tissue stem cells, including the fundamental properties of self-renewal and differentiation capacity, the term CSC does not consider the origin of these cells [11].

The CSC model predicts several possible scenarios of how cancer stem cells and tumor heterogeneity may originate [9,12], including (i) differentiation arrest of adult tissue stem cell and/or progenitor cell, (ii) dedifferentiation of mature cell, and (iii) transdifferentiation of a stem cell from a different tissue, e.g. bone marrow. (For a more detailed discussion of the potential origin of CSC, we refer to recent reviews [13–15].) The relative contribution of each scenario may vary depending on factors, such as type of cancer, microenvironment, the contributing mutagen(s), and/or a combination of these factors [13].

Notably, the concept of a hierarchic tumor organization has important clinical implications that include diagnosis, prevention, and most importantly therapy [16]. Thus, defining CSC-specific biomarkers may contribute to early diagnosis while identification of cell of origin ("cell-at-risk") is required for effective reduction of the CSC numbers. Classical therapeutic regimens target predominantly the proliferating cells, which are unlikely to be CSCs. Similarly, new generation therapies (e.g. sorafenib) seem not to target the CSC as evidenced by frequent tumor relapse and resistance after therapy [17–22]. The eradication of tumors with hierarchic organization would require the development of new therapies directed towards the CSCs. This implies a detailed understanding of the fundamental CSC properties, such as self-renewal, differentiation, chemoresistance, and, most importantly, unraveling the underlying regulatory pathways and molecular, genetic, and epigenetic mechanisms responsible for tumor initiation, seeding of metastasis, and local recurrence which are currently attributed to the CSC [12,23]. This review focuses on the existing evidence for the role of CSC in liver cancer and...
provides an overview of the current approaches for the prospective isolation and regulation of CSC. The clinical implications of the CSC model for the management of human HCC as well as critical issues and questions in the field of liver CSC are also addressed.

Over the last decade, there is increasing understanding of the hierarchic organization in hepatocellular cancers with the cancer stem cells (CSCs) responsible for tumor initiation, generation of metastasis, and relapse after therapy.

The cancer stem cells (CSCs) rest on the apex of tumor formation and share functional properties ascribed to the normal tissue stem cells, including self-renewal, proliferation, and differentiation capacity thereby leading to tumor heterogeneity.

Currently, isolation of liver CSCs relies on their antigenic (e.g., CD133, CD90, EpCAM) or functional (e.g., Side- Population, ALDH1-Activity, Sphere formation and Asymmetric Cell Division) properties.

Eradication of tumors with hierarchic organization would require the development of new therapies specifically directed against the CSCs and their regulatory mechanisms.

Aberrant gene expression in CSCs is linked to genetic and epigenetic deregulation of key signaling pathways controlling stem cell maintenance, self-renewal and pluripotency, such as WNT/β-Catenin, TGF-β, Hedgehog, and MYC.

Activation of these pathways in the CSC reflects the clinical behavior of the tumors and makes CSCs the prime target for efficient CSC eradication.

Identification of cancer stem cells in liver cancer

Cancer stem cells are defined by (1) self-renewing capacity; (2) differentiation capacity; and (3) tumor-initiating capacity. Additionally, the seeding of metastasis and tumor relapse are attributed to CSC [12]. A description of the basic properties and respective experimental assessment criteria are provided in Table 1.

Two general approaches for the prospective isolation of CSCs are based on their immunogenic and functional properties. The antigenic approach utilizes a variety of cell surface markers whereas functional isolation relies on the surrogate characteristics, such as anchorage independent growth, chemo-resistance, self-renewal, asymmetric division, and pluripotency. Functional approaches are particularly useful when the specific CSC markers have not been defined as is the case for most CSCs. Given the plasticity of the CSC, it is unlikely that CSCs can be defined by a single marker or functional property. Therefore, a combination of functional and antigenic approaches seems to be the most appropriate for identification and isolation of CSCs.

**Key points**
- Over the last decade, there is increasing understanding of the hierarchic organization in hepatocellular cancers with the cancer stem cells (CSCs) responsible for tumor initiation, generation of metastasis, and relapse after therapy.
- The cancer stem cells (CSCs) rest on the apex of tumor formation and share functional properties ascribed to the normal tissue stem cells, including self-renewal, proliferation, and differentiation capacity thereby leading to tumor heterogeneity.
- Currently, isolation of liver CSCs relies on their antigenic (e.g., CD133, CD90, EpCAM) or functional (e.g., Side- Population, ALDH1-Activity, Sphere formation and Asymmetric Cell Division) properties.
- Eradication of tumors with hierarchic organization would require the development of new therapies specifically directed against the CSCs and their regulatory mechanisms.
- Aberrant gene expression in CSCs is linked to genetic and epigenetic deregulation of key signaling pathways controlling stem cell maintenance, self-renewal and pluripotency, such as WNT/β-Catenin, TGF-β, Hedgehog, and MYC.
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**Table 1. Cancer stem cell properties.**

<table>
<thead>
<tr>
<th>Property</th>
<th>Definition</th>
<th>Assay</th>
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<tbody>
<tr>
<td>Self-renewal</td>
<td>The ability to undergo symmetric division and thereby indefinitely replenish itself</td>
<td>Re-plating assays, Serial transplantations</td>
</tr>
<tr>
<td>Differentiation capacity</td>
<td>The ability to undergo asymmetric division and thereby recapitulate all tumor cell types</td>
<td>Differentiation assays <em>in vitro</em> Transplantation</td>
</tr>
<tr>
<td>Tumor initiation/metastasis</td>
<td>The ability to propagate tumor when transplanted into the proper environment</td>
<td>Sphere formation Invasion assays Transplantation</td>
</tr>
<tr>
<td>Relapse</td>
<td>The property of resistance to different therapies and the ability to relapse</td>
<td>Chemo/radio-resistance assays</td>
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**Antigenic markers**

A variety of markers have been successfully used to enrich for a cancer stem cell fraction from different tumors including HCC.
The CSC properties of EpCAM+ cells were further validated in studies using primary liver tumor samples. It has been shown that compared to the CD90+CD44+ type, including a higher metastatic and self-renewing capacity glycoprotein CD44, produced an even more aggressive phenotype in anchorage independent growth experiments than EpCAM- cells.

EpCAM (epithelial cell adhesion molecule) (TACSTD1, ESA) from two HCC subtypes [34,38]. EpCAM+ cells isolated from patients with HCC were reported to convert the ALDH substrate BAAA (BODIPY-aminooacetate)-aminoacetaldehyde). The ALDEFLOUR assay is based on the ability of ALDH expressed family of enzymes which play a role in proliferation, differentiation, and survival by supporting conversion of retinol to retinoic acid. The ALDEFLUOR-approach (ALDH-activity) can be identified by flow cytometry[44]. The combination of this approach with the CD133 staining revealed a considerable overlap between the ALDH+ and CD133+ cells, but not vice versa. The combination of both approaches may potentially facilitate isolation of a more “homogeneous” CSC fraction.

Sphere formation

Another non-immunological approach adopted from the isolation of hematopoietic stem/progenitor cells is based on aldehyde dehydrogenase (ALDH) activity [41]. ALDH belongs to a ubiquitously expressed family of enzymes which play a role in proliferation, differentiation, and survival by supporting conversion of retinol to retinoic acid. The ALDEFLUOR assay is based on the high activity of the ADLH approach with the CD133 staining revealed a considerable overlap between the ALDH+ and CD133+ cells, but not vice versa. The combination of both approaches may potentially facilitate isolation of a more “homogeneous” CSC fraction.

**EpCAM (epithelial cell adhesion molecule) (TACSTD1, ESA)**

The surface antigen EpCAM is increasingly recognized as a specific cancer stem cell marker for a variety of tumors [32–37]. Recently, Yamashita et al. demonstrated the utility of EpCAM for classification of HCC patients and later confirmed the differential expression of AFP and EpCAM in tumor specimens derived from two HCC subtypes [34,38]. EpCAM+ cells isolated from two HCC cell lines displayed CSC-like characteristics, were highly tumorigenic in vivo and formed significantly more spheres in anchorage independent growth experiments than EpCAM- cells. The CSC properties of EpCAM+ cells were further validated in studies using primary liver tumor samples. It has been shown that activation of Wnt/β-catenin signaling increased the EpCAM+ cell population whereas the blockage of EpCAM caused decrease in tumorigenicity of these cells. It is important that the results of CD90 and EpCAM studies obtained on cancer cell lines were reproduced in fresh human HCC specimens thus providing direct evidence for the significance of putative cancer stem cells in human HCC patients.
favors stem cell propagation and are performed under clonal conditions [46]. Alternatively, a matrix-like medium (e.g., Matrigel) can be used for investigating three dimensional growth. The contribution of the sphere formation assay in the study of CSCs and their role in liver cancer is unclear and needs further evaluation [47,48]. Unpublished observations from our laboratory show increased representation of CSC markers (CD133, SP) in liver cancer spheres as compared with monolayer cultures.

**Asymmetric division**

CSCs, similar to normal adult stem cells, are thought to maintain themselves through symmetric and asymmetric cell divisions. Most recent studies are focusing on the identification of liver and lung CSCs by qualitatively and quantitatively assessing the co-segregation of template DNA during mitosis, resembling the process described in different normal tissues (fibroblast, muscle, mammary, intestinal, and neural cells) (Avital et al., personal communication, [49]). The demonstration that CD133-positivity co-segregated with the template DNA, while differentiation markers were passed to the opposing daughter cell, suggested that the fate of lung CSCs may be regulated during the cell division process. More results from this promising method can be expected.

In summary, the current methods used for isolating CSCs from primary liver tumors and established tumor cell lines allow for enrichment of cells displaying CSC properties including self-renewal, multipotency, and extensive proliferation capacity. Consistent with the CSC hypothesis and in agreement with observations from other tumor types, the CSC-enriched fractions comprised only a minority of the liver tumor. Importantly, gene expression patterns and antigenic characteristics of the isolated CSCs were vastly different suggesting that the heterogeneity of human liver cancer may be related to heterogeneity of liver CSCs.

**Caveats and unresolved questions in the isolation of CSCs**

The current methods used for CSC isolation have important deficiencies which may contribute to the inconsistencies in results across different studies. Thus, CSC isolation by a single marker, although attractive, may be not sufficiently discriminatory [33]. Also, it is frequently not clear what these markers really “mark”. Antibody and/or dye dependent toxicity as well as cross-reactivity may negatively impact the results regardless of using the markers for a negative [50,51] or positive selection [52]. Additionally, stochastic activation of antigenic markers by experimental conditions and/or epigenetic events may impact the validity of these approaches [53–56]. Functional isolation using sphere-formation also does not provide definite information on which cells are actually propagated and how heterogeneous they are. Even though an increased expression of CSC markers in the tumor spheres is commonly observed, it is not always associated with enhancement of tumor-initiated ability in vivo. These discrepancies might cause over-interpretation and significantly impair the reproducibility of the results [48,57,58]. In addition, the xenotransplantation animal models frequently used for testing CSC tumor-initiating ability in vivo also pose problems. Although the ability to generate tumors in an inappropriate environment is consistent with CSC hypothesis, there is a concern that the use of different immune compromised mouse models as well as various sites of transplantation and different ways of application (e.g., with or without Matrigel, hormone pellets, etc.) may significantly impact the outcome and experimental reproducibility [59–61].

Another fundamental question relates to the primary source of CSC isolation. Currently, CSCs are isolated from primary cancers, primary tumor xenografts, and established tumor-derived cell lines. Both primary tumor cells and cancer cell lines are shown to possess a distinct tumor hierarchy based on the expression of surface markers and other commonly accepted CSC criteria. It remains to be determined whether the clonally derived cancer cell lines which are maintained for decades under culture conditions retain the clinical characteristics of CSCs [48]. Nevertheless, the validity of using cancer cell lines in CSC research has been demonstrated in the past [30,62,63]. Thus, most of the markers which are currently used for isolating CSCs from primary tumor samples were established and adapted from cancer cell lines. More importantly, mechanistic studies are difficult to conduct on primary cells, since the current technology used for molecular profiling of CSCs demands in vitro expansion to obtain a sufficient amount of nucleic acids and proteins from a minute CSC population. Another problem involves the use of heterogeneous and not clonally derived cells from primary tissues for lineage tracking and differentiation experiments. In particular, single cell assays are particularly compromised and difficult to reproduce. In these cases, clonally derived cell populations may help to ensure the reproducibility of the data. At the end, the clinical relevance of the in vitro findings should be demonstrated and validated in primary tumor samples. This could involve immunohistochemistry and/or gene expression analysis of tumor samples. As outlined in many studies on CSC, a combination of different approaches seems to be optimal for critical evaluation of the clinical relevance of the experimental findings.

**Regulation of cancer stem cells in hepatocarcinogenesis**

Considerable advances in the field of "omics" over the last decade have unraveled key molecular mechanisms of hepatocarcinogenesis [38,64–68]. Interestingly, many of the identified regulatory pathways are known to be involved in stem cell maintenance as well as self-renewal and pluripotency, including WNT/β-Catenin, TGF-β, MET, Hedgehog, MYC, p53, EGF, etc. The disruption of these functionally overlapping pathways showed a frequent association with prognosis in liver cancer [14,69–74]. Moreover, several studies have correlated the clinical course of HCC patients with cellular origin of tumors [66,75,75,76]. Finally, a strong association with bad prognosis was found in HCCs characterized by hepatic progenitor cell origin [38,75].

Growing evidence indicates that many if not all signaling pathways identified in liver cancer are also active in the prospectively isolated liver CSC [14,15] supporting the idea that molecular heterogeneity of HCC originates in the CSC compartment. Therefore, the common pathways could serve as novel prognostic biomarkers and represent targets for the development of new therapeutic strategies to specifically eradicate CSCs.

**WNT/β-catenin**

WNT signaling has been studied intensively in embryonic development. The response of cells to WNT signaling is both tissue-
and content-dependent and involves survival, proliferation, and change in cell fate. Disruption of WNT signaling results from both genetic and epigenetic changes and is associated with a range of diseases and is frequently found in many cancers, especially colon cancer and HCC [77–79]. Disrupted WNT signaling by mutational and non-mutational events (e.g. cross-talk with other signaling pathways such as TGF-β) is observed in around one third of all HCCs which emphasizes the importance of this pathway for hepatocarcinogenesis [80]. Indeed, activation of WNT signaling has been demonstrated in different prospectively isolated CSCs [81,82]. In the liver, elevated expression of WNT and its downstream mediators was reported in CD133+ and EpCAM+ liver CSCs [25,34,38]. Additionally, gene expression profiling of SP cells from two different HCC cell lines showed activation of this pathway [41,42]. However, the specific targeting of this pathway is problematic due to the multiplicity of the involved and highly conserved proteins and pleiotropic ways of activation [18,77,83]. Nevertheless, RNAi mediated knockdown of β-catenin resulted in decreased proliferation, colony formation, migration, and drug resistance of lung cancer stem cells (Table 2). The knockdown of the downstream target EpCAM in liver CSC had a similar effect [34,82].

MYC

The proto-oncogene MYC is involved in the regulation of 15% of all genes. MYC is activated by diverse stimuli including WNT, Hedgehog, and MAPK/ERK. MYC effects on target genes are mediated by various genetic and epigenetic mechanisms, including DNA-methylation and chromatin remodeling [84,85]. MYC has a broad impact on almost all cellular processes, such as proliferation, apoptosis and differentiation, and is involved in maintaining stem cell pluripotency [86]. Overexpression and structural modifications of the MYC gene are frequent in many cancers [68,87]. Recent studies confirmed the crucial role of MYC in both murine and human hepatocarcinogenesis, particularly in the process of malignant transformation [74,88,73]. High levels of MYC were found in the SP of colon cancer cells [89,90] whereas knockdown of c-Myc by lentiviral shRNA significantly reduced proliferation concomitantly with cell cycle arrest and increased apoptosis. The role of MYC in the prospectively isolated liver CSC has not been systematically assessed and needs further evaluation. The dual oncogenic and pro-apoptotic functions of MYC indicate that therapeutic targeting of MYC should be conducted with caution [91].

TGF-β

The members of TGF-β family are under intensive investigation due to their importance both for stem cells and cancer [92,93]. TGF-β is also found to have a crucial role in maintaining the CSCs in different tumors including liver cancer [15,93,94,92,95]. Mishra and colleagues showed that lack of responsiveness to the TGF-β signaling pathway in liver stem cells led to the generation of liver CSCs and that disrupted TGF-β signaling is observed in the potential CSC from human HCCs. The results were confirmed in the EpCAM+ CSCs isolated from liver cancer cell lines [34,95]. Recently, it was demonstrated that the targeting of this pathway using indirect modulation of IL6/STAT3 appeared to be effective for eradication of CSC [96] as also discussed in the recent reviews on this topic [15,94,92].

Notch

The evolutionary conserved NOTCH pathway is involved in many developmental processes, including differentiation, fate decision (e.g. epithelial–mesenchymal–transition), proliferation, apoptosis, and cell adhesion [97]. In the liver, it is involved in development by coordinating biliary epithelial cell differentiation and morphogenesis [98]. Disrupted NOTCH signaling is recognized in a growing number of cancers including liver cancer [97,99]. The role of NOTCH has been also demonstrated in putative CSCs isolated from liver and other cancers [25,100]. Increased expression of genes involved in this pathway has been shown in CD133+ liver cancer cells as compared to the CD133− counterpart. Inhibitors of the NOTCH pathway are currently under investigation in clinical trials for solid tumors although the role in liver cancer remains unclear [101].

Hedgehog

Hedgehog signaling is crucial for many cellular processes involved in cell and stem cell biology. Activation of this pathway is observed in variety of human cancers, including basal cell carcinomas (BCCs), medulloblastomas, leukemia, gastrointestinal, lung, ovarian, breast, prostate, and hepatobiliary malignancies [102,103]. Activation of the Hedgehog pathway has been demonstrated in CD44+CD24−EpCAM+ pancreatic CSCs, particularly at the invasive front of tumors [37,104]. The high expression of genes involved in this pathway was further established in highly tumorigenic CD133+ liver CSCs [25]. Discovery of the specific inhibitors of the hedgehog pathway seems promising for targeting liver CSC.

BMI

BMI1 is a part of the polycomb group genes (PcG) that are highly conserved throughout evolution. BMI acts as an epigenetic chromatin modifier and is known for its contribution to embryonic and stem cell self-renewal program [105]. It is frequently overexpressed in different cancer types and disruption of BMI1 signaling has been linked to the activation of the hedgehog pathway in some cancers, e.g. medulloblastoma [106,107]. Further, BMI1 upregulation is associated with malignant transformation and acquisition of the malignant phenotype in HCC [108]. Aberrant BMI1 expression is reported in many CSC populations and it has been shown to have a critical role in maintaining and propa-

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Table 2. CSC signaling in liver cancer.

<table>
<thead>
<tr>
<th>Signaling</th>
<th>CSC marker</th>
<th>Inhibitors (under investigation)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>WNT/β-Catenin</td>
<td>EpCAM, CD133, SP</td>
<td>RNAi, mAB</td>
<td>[25,38,42]</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Human HCC, EpCAM</td>
<td>NSC74859 targeting IL6/STAT3</td>
<td>[34,38,95,96]</td>
</tr>
<tr>
<td>NOTCH</td>
<td>CD133</td>
<td>γ-secretase inhibitors, mAB</td>
<td>[25]</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>CD133</td>
<td>Cyclopamine, mAB</td>
<td>[25]</td>
</tr>
<tr>
<td>Pluripotency</td>
<td>EpCAM, CD133, SP, human HCC</td>
<td>RNAi</td>
<td>[25,34,42]</td>
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</tbody>
</table>

RNAi, RNA interference machinery; mAB, monoclonal antibody.

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increasingly recognized as one of the fundamental mechanisms for not surprising that abnormal epigenetic regulation is the somatic tissues while the DNA remains the same. It is there-
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of the epigenetic mechanisms is best illustrated by the fact that
ished the tumorigenicity of SP cells[25,43,109]. BMI1 silencing completely abol-
expression in CD133+ liver CSCs. The role of BMI1 in liver CSC
constituted by the signals from their microenviron-
chymal transition appears to play a pivotal role[23]. Several
ascription of CSC and self-renewal
Cancer stem cells and metastasis
The incidence of metastases impacts the prognosis of most can-
ers for human HCC[95]. Furthermore, prospectively isolated
CSC and self-renewal
Epigenetic regulation of CSC
Epigenetics can be defined as modifications of the DNA and/or
associated proteins other than DNA sequence variation carrying
information content during cell divisions[110]. The importance
of the epigenetic mechanisms is best illustrated by the fact that
the epigenome undergoes profound changes from the zygote to the somatic tissues while the DNA remains the same. It is there-
known that DNA methylation changes are
the early events in carcinogenesis leading to allelic imbalances and cancer progression[112]. The early occurrence of DNA meth-
ylation changes during carcinogenesis starting from the preneo-
plastic stage implies that epigenetic changes may arise in stem cell compartments either preceding and/or predisposing to genetic changes and whereby leading to tumor heterogeneity.
This notion is supported by a recent study suggesting that DNA methylation changes may shift the functional balance from stem cell homeostasis to cancer[113]. Consistent with this, epigenetic profiling pointed to a stem cell origin in some cancers[114,115].
The importance of epigenetics has been also recognized in liver cancer[20]. Changes in the DNA methylation patterns, including global hypomethylation and promoter hypermethylation, were established as frequent and early events during human liver oncogenesis[116]. Furthermore, we recently demonstrated that these changes strongly correlate with the biological behavior of the tumors and the clinical outcome of cancer patients[117]. Notably, patients with tumors displaying progenitor cell properties presented the worse prognosis[75].

Our own work on epigenetically modified SP cells using a DNMT1-inhibitor zebularine provides further evidence for the significance of epigenetic mechanisms for liver CSCs([118]; own unpublished data). We observed that inhibition of DNA methylation in human liver cancer derived cell lines resulted in increase of highly tumorigenic cells within the SP fraction. Furthermore, global transcriptomic analysis of the SP cells allowed generation of a common SP-gene expression signature capable of predicting the clinical outcome (survival and recurrence) of HCC patients.

MicroRNA
MicroRNAs (miRs) are small non-coding RNAs involved in the posttranscriptional control of hundreds of target genes, and are recognized as key regulators in numerous cellular processes including differentiation and pluripotency. Aberrant expression of this class of molecules considerably contributes to cancer development, progression, and generation of metastasis[119].

In liver carcinogenesis, miRs have been found to have both tumor suppressive (miR-122, miR-26, miR-223) and oncogenic (miR-221, miR-222) activity[120–124]. Recently, a crucial role of miRs has been established in regulating CSC suggesting the possibility of miR-directed CSC eradication[125–128]. Currently there is only one study focusing on miRs in liver CSCs which suggests that miR-181 may be an attractive target for therapy by decreasing EpCAM+ liver CSCs[129]. More results are expected from this emerging and exciting field in CSC research.

Cancer stem cells and metastasis
The transcription factors OCT4, SOX2, KLF4 and MYC are well known for their role in mediating and sustaining adult and embryonal stem cell self-renewal and pluripotency[86]. In addition, emerging evidence emphasizes misregulation of these genes in many cancers[130,131]. Several recent investigations have used global approaches to compare the transcriptional programs operating in embryonic stem cells (ESC) and adult tissue stem cells in the context of different cancers[132–134]. Despite different experimental approaches, activation of ESC-like gene sets was consistently observed in aggressive human epithelial cancers, including liver cancer[133,134]. Interestingly, the ESC signature published by Ben-Porath and colleagues showed no association with tumor-initiating CD44+/CD24− breast CSC[134]. In contrast, the MYC oncogene identified in the ESC-like module by Wong et al. had an essential impact on both tumor initiation and reac-
tivation of the ESC-like module in normal and cancer cells[133]. These data highlight the importance of the MYC gene in both normal and cancer stem cell biology.

Activation of OCT4 in putative liver CSCs with misregulated TGF-β signaling confirmed the importance of pluripotency mark-
ers for human HCC[95]. Furthermore, prospectively isolated EpCAM+ and CD133+ cells also exhibited activation of pluripoten-
ciety-associated genes[25,34]. Together, these data suggest that similar albeit not identical processes controlling self-renewal and pluripotency in stem cells are also present in the CSC. More work is needed to understand the mechanisms and regulatory pathways involved in CSC self-renewal.
identified in most of the investigated HCC patients but not in cirrhotic or disease free control patients.

Clinical implications and future challenges in CSC research

Implicit in the CSC hypothesis is the assumption that cancer initiation, local recurrence, metastasis, and therapy resistance are the fundamental domains of the CSC. Consequently, CSCs should be the principal target of therapy. However, the traditional cancer therapies which primarily target rapidly dividing and most likely well differentiated tumor cells, would fail to eliminate CSCs. This may explain the disappointing results of current cancer therapies (chemotherapy, irradiation, and immunotherapy), which most often lead to tumor relapse [148]. It is therefore important to emphasize the necessity to explore the susceptibility of CSCs to existing therapies in combination with the disruption of key “stemness” pathways controlling self-renewal, pluripotency, radio-chemoresistance, and neo-angiogenesis [11].

Other novel and important directions for effective therapy may include disruption of the tumor niche essential for CSC homeostasis [149–151] or depletion of CSCs by forced differentiation. The latter approach has been shown to be promising against brain tumors and leukemia, using BMP and retinoic acids as differentiating agents, respectively [152–154]. Another comprehensive study employed high-throughput screening to validate the targeting of differentiation as a specific therapy against breast CSCs. The highest efficiency was achieved by a compound that increased differentiation of tumor cells [155]. Experimental evidence also exists suggesting that differentiation forced by transduction of liver enriched transcription factor HNF4α might be effective against liver cancer [156]. Hepatoma cells transduced with HNF4α displayed a decrease in “stemness” gene expression and a reduced number of CD133+ and CD90+ as well as a dramatic reduction of tumorigenicity and metastatic potential. However, more work is needed for the accurate assessment of the power of this approach to exclude inadequate differentiation of non-target cells [15].

Direct targeting of CSC-specific surface markers, such as CD133, CD90, EpCAM and CD44, may be a powerful therapeutic approach to specifically eliminate liver CSC. However, the prognostic relevance of the available surface markers remains controversial in histopathological evaluation of HCC as well as other cancers [157–159]. Nevertheless, RNAi targeting of EpCAM significantly decreased the CSC pool and reduced both the tumorigenicity and invasive capacity of liver CSCs [34]. Since EpCAM expression is a downstream target of WNT/β-catenin, these results may have implications for development of novel target therapies. Finally, there is experimental evidence that reveals that targeting of pluripotency genes involving OCT4 and NANOG could be potentially useful for the specific eradication of CSCs [160–162]. Importantly, all of these transcription factors also mediate “stemness” of the normal stem cells. Therefore, the effects of their targeting should be critically evaluated. A therapeutic strategy utilizing a combination of traditional anticancer therapy eradicating the bulk of tumor cells with specific CSC targets may be the most promising approach.

In summary, accumulating evidence suggests a hierarchic organization of liver cancer. Over the last years, substantial progress has been made in the prospective isolation and identification of key signaling pathways which define the unique properties of liver CSCs, including WNT, TGF-β, NOTCH, and Hedgehog. Current results strongly indicate the advantage of targeting CSCs to improve the limited efficiency of existing therapies. More work, particularly utilizing integrative whole genomic and epigenomic approaches, is needed to advance our knowledge on CSCs and develop effective anti-CSC therapy.

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