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Review

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Stemness features in liver cancer

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Abstract

Heterogeneity is a cardinal hallmark of cancer, including primary liver cancer (PLC), and occurs at different layers including putative cell-of-origin. Current evidence suggests that within cellular subpopulations in PLC there are stem-like cells, the cancer stem cells (CSCs). The CSC concept has been recently proposed as an explanation of such intra-tumor heterogeneity. According to this model, CSCs are responsible for tumor initiation, recurrence, metastasis as well as drug-resistance. However, although the CSC hypothesis is intriguing and supported by a large number of experimental studies, there are still open questions regarding the origin of putative CSCs. Since chemo-resistance and recurrence represent major issues in PLC treatment, the development of new therapeutic strategies is needed, for which a good understanding of tumor behavior and in particular of CSCs biology is an imperative prerequisite. In this review we summarize the regulatory pathways that support CSC features in PLC. Moreover, we highlight the key features of hepatic CSC, in terms of enhanced drug-resistance, increased metastatic potential and metabolic rearrangement. Knowledge of the molecular mechanisms underlying CSC biology may provide novel options for PLC combination therapies.

Keywords: Hepatocellular carcinoma, cholangiocarcinoma, cancer stem cells, tumor heterogeneity, drug-resistance

MULTIPLE CELLS-OF-ORIGIN OF PRIMARY LIVER CANCER

Primary liver cancer (PLC) is one of the most common cancers worldwide and the second leading cause of cancer-related mortality[1,2]. The major forms of PLC comprise hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA)[1,3-5]. HCC accounts for approximately 90% of all PLCs[1,3], while CCA is the

second most common form and accounts for about 5% of all PLCs[3-5]. HCC causes over 600,000 deaths worldwide annually, and its incidence and mortality are increasing at a fast rate[6-10]. On the other hand, CCA is characterized by a very poor prognosis, with a 5-years survival lower than 20%, and its incidence and worldwide mortality are also increasing[5,11-13]. The high mortality rate of CCA may depend on its non-specific or silent clinical features and the lack of specific markers that make it difficult to diagnose[14-16].

Many studies carried out in these last years have attempted to define which type of epithelial cell [hepatocytes, cholangiocytes, hepatic progenitor cells (HPCs) or all three] should be considered as the PLC cell of origin[17]. For a long time, HCC and CCA have been commonly accepted to derive from hepatocytes and cholangiocytes, respectively. Since mature hepatocytes and cholangiocytes have an enormous self-renewal capacity and longevity, they meet the requirements to be targets for oncogenesis[17-23]. Detailed analyses of a wide range of PLC tumor types have reported that a rare form of combined HCC-CCA (cHCC-CCA) has intermediate characteristics between HCC and intrahepatic CCA (iCCA), suggesting that they could share the same stem/progenitor cell origin[18-24]. In this regard, since most PLCs arise on the background of chronic liver disease in the presence of an extensive activation of the HPC compartment (the so-called ductular reaction), several studies suggested that PLCs can be derived from HPCs rather than from mature cell types[25]. HPCs situated in the canal of Hering physiologically act as a reserve cell compartment activated in case of liver damage or when mature hepatocytes and/or cholangiocytes replication is compromised. These cells are bipotential, and may differentiate into either hepatocytes or cholangiocytes[26-28]. During the differentiation in malignant cells, bipotential HPCs undergo maturation arrest and give rise to a spectrum of tumor phenotypes with both admixed hepatocellular and cholangiocellular features, such as cholangiolocellular carcinoma and cHCC-CCA[29-31]. Additionally, a new subtype of CCA-like HCC (CLHCC) has been discovered and characterized as HCC expressing CCA-like traits[32]. CLHCC co-express embryonic stem cell (ESC) traits and hepatoblast-like genomic signatures, suggesting a HPC origin. These lines of evidence provided important insight into the heterogeneous progression of PLCs, which imply a common evolutionary origin from cells at different developmental stages[31-33]. The hypothesis of a progenitor cell origin has been supported by new advancement in genome wide analysis. Indeed, it has been suggested that iCCA and HCC are closely related at molecular level[19,29,34,35], since both tumor types share common copy number variations[11,36].

Such phenotypic variability and presence of progenitor cell features in PLC can be explained in two ways: either the cell of origin is a progenitor cell with acquired genetic alterations or, alternatively, mature tumor cells de-differentiate acquiring progenitor cell features during carcinogenesis (de-differentiation theory[37-40]). Interestingly, new findings provide direct evidence that any cell in the hepatic lineage can be the cell of origin of PLC[41]. In this regard, it has been recently suggested the development of iCCA by lineage conversion of malignant hepatocytes, through a co-activation of both Notch and protein kinase B (AKT) signaling, contributes to the acquisition of stem/progenitor cell features[42,43]. In spite of the marked plasticity in the underlying cells of origin, current evidence suggests that most PLCs are derived from undifferentiated cells with stem-like capabilities[40].

UNDERSTANDING THE CONCEPT OF CANCER STEM CELL

Extensive clinical and pathobiological heterogeneity at the level of cellular morphologies, genetic fingerprints and responses to therapies is a cardinal hallmark of cancer, including PLC. Such tumor complexity may reflect the presence of different cell subtypes with distinct self-renewal and differentiation potentials[40,44-46]. The traditional view of cancer development is based on a stochastic model, which states that every malignant cell may undergo genetic and/or epigenetic alterations and clonally expand to initiate tumor growth. Thus, every cell within the tumor may be equally responsible for tumor initiation and progression[47-51]. Unlike the stochastic model, the hierchical or cancer stem cell (CSC) model may explain intra-tumor heterogeneity representing tumor as a hierchically organized tissue with CSCs at the apex in the pyramid and more committed and differentiated tumor cell types progressively down[47-50].

According to this model, CSCs represent a fraction of cells resident in the tumor endowed with stem-like features like the ability to self-renew and differentiate into heterogeneous tumor cell progeny as well as with the unresponsiveness to treatments[52,53], and represent the unit of selection within the tumor, while any other bulk tumor cells lead to clonal exhaustion[50]. More importantly, CSCs are thought to be a unique cellular subset responsible not only for tumor initiation but also for tumor growth maintenance, tumor recurrence and metastasis, showing intrinsic resistance to chemotherapeutic drugs compared to bulk tumor cells[52,54-56]. In this view, the existence of CSCs represent an entirely distinct dimension of intra-tumoral heterogeneity[57].

Interestingly, a third model has been recently proposed to explain the intra-tumor heterogeneity, the so-called "CSC plasticity model". According with this theory, tumor cells represent a very plastic and dynamic population, with the ability to continuously shift between non-CSC and CSC states, in response to intrinsic and extrinsic stimuli. In this view, the stochastic and the CSC model not only are not mutually exclusive, but can be integrated with each other, adding a new level of tumor complexity[58].

The idea that tumor initiation and progression are driven by stem-like cells is still a subject of debate, since the first time it was proposed[59] until today. While CSC existence has been confirmed in a growing range of hematologic and solid tumors (e.g., acute myeloid leukemia, pancreatic cancer, breast cancer, lung cancer, hepatocellular carcinoma, head and neck cancer, colon cancer, prostate cancer, melanoma, and glioblastoma), no agreement has yet been reached regarding the origin of putative CSCs[60]. Some reports have indicated that CSCs can originate from normal resident stem cells, due to their inherent self-renewal capacity and long life span that can allow them to accumulate oncogenic and epigenetic modifications, resulting in malignant transformation. Alternatively, CSCs may originate from more committed progenitor cells[47], or even from differentiated non-CSCs that re-acquire stem cell properties by de-differentiation or reprogramming processes[61,62]. Thus, tumor hierarchical organization does not imply that CSCs originated from normal stem cells, and the CSC model does not address the cell-of-origin, that represents the normal cell that acquires the first cancer-promoting mutation(s) and is not necessarily related to the CSC concept[63,64]. These considerations interconnect with the debate on the true nature of the cell-of-origin of PLC. While it has already been accepted that HCC progression is driven by CSCs[22,65-69], very few studies have indicated the presence of CSCs in CCA[70] (reviewed in[71]).

REGULATORY PATHWAYS INVOLVED IN PLC-ASSOCIATED STEMNESS

Many of the identified CSC regulatory pathways are also known to be involved in normal stem-cell maintenance as well as in self-renewal potential and pluripotency of embryonic stem cells[72-77]. Here, we will briefly review the key regulatory pathways that support stemness features in the context of PLC [Figure 1].

Wingless-type MMTV integration site family member (Wnt)/β-catenin pathway

Disruption of Wnt/β-catenin signaling results from both genetic and epigenetic changes in many tumors, including PLC. Wnt/β-catenin canonical signaling pathway appears to be involved in stemness maintenance in both embryonic and cancer stem cells[78,79]. Extracellular Wnt ligand binds to Frizzled cell surface receptors leading to increased cytoplasmic β-catenin levels, with the following induction of Wnt key target genes[31,55,80]. Notably, β-catenin is expressed in 58% of CCA, mutated in 8% of cases and it is considered an early determinant in CCA-progression[71]. In up to 90% of HCCs, the Wnt receptor FZD-7 is overexpressed, and 20%-40% of HCCs have unusual cytoplasmic and nuclear accumulation of β-catenin[81]. Moreover, in 25% of HCCs, β-catenin and Axin1 mutations are observed[69,81].

Notch signaling pathway

The Notch canonical signaling plays an important role in cell differentiation, proliferation and apoptosis, as well as in stem cell and HPCs maintenance[31,71,82,83]. Moreover, Notch signaling is implicated in bile duct morphogenesis (reviewed in[84]), and dysfunction in this pathway may result in reduced detoxification,

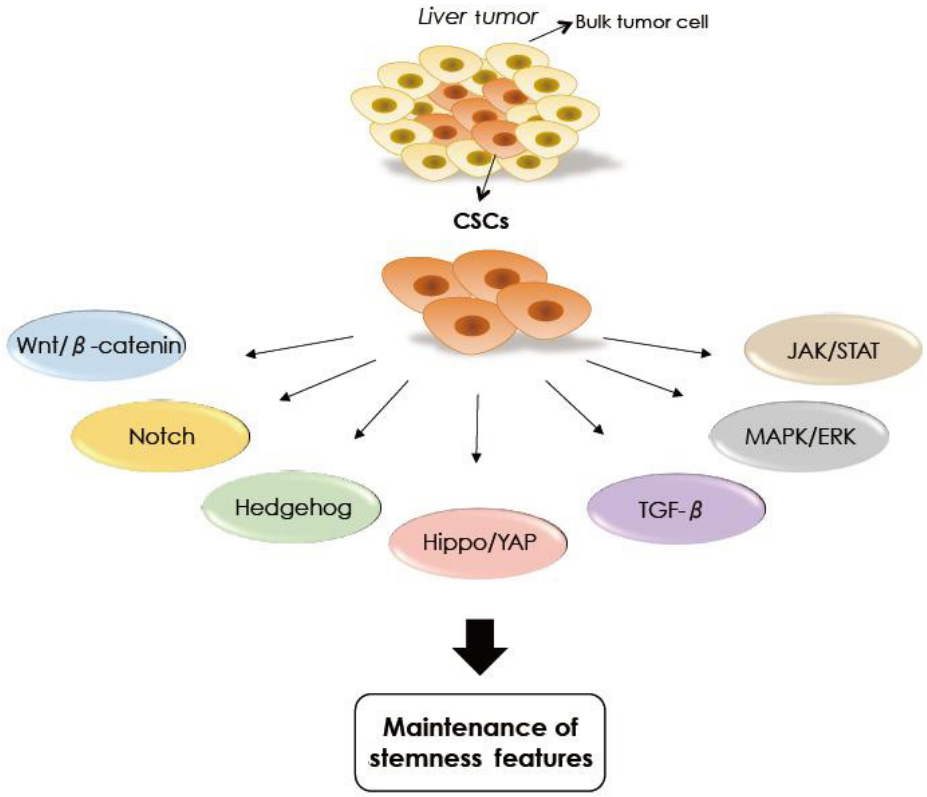

Figure 1. Regulatory pathways of liver cancer stem cells (CSCs). Primary liver cancers are heterogeneously composed by bulk tumor cells and CSCs. Liver CSCs are characterized by the activation of several molecular regulatory pathways that contribute to support the maintenance of CSC stemness features, including Wnt/β-catenin, Notch, Hedgehog, Hippo/Yes-associated protein (YAP), transforming growth factor-β (TGF-β), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signaling pathways

ultimately leading to liver damage and iCCA development. Interestingly, the expression of Notch receptors 1 and 3 correlates with CCA progression and poor survival[71], whereas overexpression of Notch receptors 1 and 4 in HCC exerts tumorigenic effect[85]. Moreover, in up to 30% of HCCs, nuclear expression of Notch 1 and 3 is associated with the presence of stem cell signatures, supporting the role of Notch in promoting the expansion of the CSC niche[81,86]. Since Notch signaling can contribute to either CCA or HCC, it has been suggested that this pathway could be deregulated in bipotential HPCs[82].

HEDGEHOG SIGNALING PATHWAY

The Hedgehog (Hh) pathway regulates embryonic development, cell differentiation, regeneration and stem cell biology. The aberrant activation of the Hh pathway has been reported in different malignancies[87], and its correlation with prognosis is well known[88]. In addition to HCC carcinogenesis and HPC proliferation, activation of Hh pathway promotes CCA proliferation[71,79]. Notably, Sonic Hh (Shh) is the predominant ligand in the liver and is overexpressed in over 60% of HCCs[31,69,81,89].

Hippo signaling pathway

The Hippo signaling cascade is an evolutionarily conserved pathway involved in organ development[90-92]. This pathway has been implicated in multiple events during tumor onset. Strong evidence indicates a significant role of Hippo signaling in regulating stem cells, including HPCs[93-95]. Yes-associated protein 1 (YAP1) is a primary effector of the Hippo cascade and is frequently expressed in HCC and cHCC-CCA mixed tumor types, which retain stemness-related features[94]. Furthermore, constitutive activation of YAP in bile ducts, in association with AKT, seems to be essential in inducing CCA in a murine biliary injury model[31,96].

Phosphatidyl inositol 3-kinase/AKT signaling

AKT plays a critical role in many human cancers, including HCC and CCA[3,97]. AKT signaling can be triggered downstream of tyrosine kinase receptors activation, phosphatidyl inositol 3-kinase (PI3K) constitutive activation or loss of phosphatase and tensin homolog (*PTEN*)[3]. PTEN deletion results in the proliferation of a CD133+ population[71,98]. PI3K signaling promotes stem-like properties of HCC cells and it is implicated in HCC chemo- and radio-resistance as well as in epithelial-to-mesenchymal transition (EMT) and metastasis[99-104]. Notably, the co-activation of *AKT* and neuroblastoma rat sarcoma viral oncogene homolog (*N-RAS*) oncogenes leads to development of cHCC-CCA-like liver tumors, through the expansion of HPCs or malignant conversion of hepatocyte into progenitor-like cells[42].

Mitogen-activated protein kinase/extracellular signal-regulated kinases signaling pathway

The mitogen-activated protein kinase (MAPK) cascade regulates many important cell function, such as proliferation, invasion and survival and is critical for HPCs proliferation[71]. Gain-of-function mutations of *KRAS* are some of the most frequent mutations observed in iCCA, defining a class of patients characterized by poor outcome and enriched in CCA stem like-cells and tumor recurrence predicting signatures. Moreover, these mutations are also detected in patients with primary sclerosing cholangitis, suggesting that this could be an early event that contributes to the malignant transformation of cholangiocytes[36]. It is known that the MAPK pathway is directly associated with HCC cell growth and tumor-initiating capability[105-107]. Moreover, the long non-coding RNA H19 is highly expressed in HCC cells, where it activates the MAPK/extracellular signal-regulated kinases signaling pathway, regulating oxidative stress and chemotherapy resistance of CD133+ HCC CSC[108].

Transforming growth factor-β signaling

The transforming growth factor-β (TGF-β) pathway plays a key role in self-renewal and maintenance of an undifferentiated stem cell state. Its disruption is implicated in CCA development through impairment of stem cell differentiation and deregulated proliferation of HPCs[98]. Nonetheless, the role of TGF-β in PLC development is still controversial. Indeed, TGF-β acts as a tumor suppressor early in tumor initiation, whereas at late stages it promotes tumor growth, metastasis and EMT. It has been demonstrated that TGF-β1/Snail activation induces EMT in CCA both *in vitro* and *in vivo*, and this is associated with a higher CCA aggressiveness[109]. Moreover, TGF-β is upregulated in 40% of HCCs[69,81,89], and it may promote HCC progression via regulatory T cells recruitment and subsequent creation of a tumor suitable microenvironment[110,111].

Janus kinase/signal transducers and activators of transcription signaling

Several lines of evidences highlight the central role of interleukin (IL)-6/signal transducers and activators of transcription 3 (STAT3) signaling in CCA. Binding of IL-6 to the gp130 receptor leads to Janus kinases (JAKs) (JAK1, JAK2 and TYK2) and STAT3 activation, inducing the transcription of target genes essential for cell growth, differentiation and proliferation (reviewed in[34,112]). STAT3 signaling is also involved in maintenance of CSC population[113-115] and EMT-triggering in diverse tumors, including PLC[116,117]. Increased IL-6 expression has been reported to drive CSCs expansion through STAT3 activation in HCC[118]. Moreover, a recent study has demonstrated that EMT+ metastatic CSCs can be generated in a $\beta 2SP^{+/-}$ mouse model of HCC, mainly due to overexpression of IL-6 in addition to the partial disruption of TGF-β signaling[119].

KEY FEATURES OF LIVER CSCS

Drug-resistance

A fundamental aspect contributing to poor PLC survival rate is the unresponsiveness to conventional therapies[11,12]. Currently, effective treatment is limited to surgical resection for both HCC and CCA, as well as liver transplantation for HCC. Unfortunately, 80% of HCC patients are diagnosed at an advanced tumor stage, which is not amenable to curative treatment[8-10]. Although other treatment procedures (e.g.,

cryosurgery, radiofrequency ablation and embolization) are also available, they are mostly palliative approaches and the treatment regime is shifting towards systemic chemotherapy[9]. Moreover, more than 70% of patients with early-stage HCC develop post-surgery recurrence[9,110]. Likewise, CCAs are generally asymptomatic in early stages and are usually diagnosed at an advanced unresectable stage. Moreover, although chemotherapy improves the patients' quality of life, it still remains only a palliative treatment[5,13,120]. Therefore, the majority of patients with unresectable CCA undergoes a rapid decline in clinical conditions and dies within 12 months of the onset of symptoms. Thus, PLC still remains a fatal disease, mainly due to frequent tumor recurrence and chemoresistance.

CSCs represent a peculiar sub-compartment of tumor cell population crucially involved in recurrence, metastasis as well as drug resistance[86,121] [Figure 2]. CSCs can escape drug-induced cell death through different intrinsic and external mechanisms. The intrinsic mechanisms consist of enhancement of DNA damage repair pathways, self-renewal ability of CSCs, high expression of drug efflux-related proteins and over activation of growth- and other stem-related pathway. The external mechanisms refer to the role of the tumor microenvironment (TME) on CSC drug resistance. This includes TME-derived EMT signals, hypoxia stimulation and angiogenesis trigger[122]. Consistently, increasing evidence suggests that sorafenib resistance in HCC correlates with the activation of EMT and enrichment of CSC traits[123-125].

Several CSC markers seem to be implicated in drug resistance, such as CD13, that protects PLC CSCs from apoptosis and ROS-dependent DNA damage induced by different chemotherapeutic drugs (e.g., 5-FU)[86]. The HCC epithelial cell adhesion molecule (EpCAM)+ CSCs also show chemo-resistance against genotoxic agents like 5-FU[9]. Next, CD133+ HCC CSCs exhibited chemo-resistance to fluorouracil and doxorubicin through AKT and Bcl-2 pathway activation. Furthermore, CD133+ CSC and CSC spheres isolated from HCC cell lines display enhanced resistance to a panel of chemotherapeutic drugs (e.g., paclitaxel, methotrexate, vinblastine, cisplatin, carboplatin, docetaxel, irinotecan, *etc.*)[126,127]. According to these data, we have recently demonstrated that CCA CSCs isolated by tumor sphere assay possess higher resistance to common chemotherapeutic agents[70]. Additionally, laminin-332 expression is fundamental for maintaining self-renewal abilities of hepatic CSCs and for inducing mTOR-associated resistance to doxorubicin and sorafenib. Laminin-332 not only protects hepatic cancer cells against chemotherapy but also stimulates simultaneously cell proliferation upon sorafenib exposure, and it has been hypothesized that while laminin-332 may induce quiescence in PLC in "normal" circumstances, under cellular stress (e.g., sorafenib treatment) it could stimulate PLC cells to react by enhancing their proliferation[17,86].

Metastatic activity

The spread of circulating tumor cells (CTCs) in the blood plays a major role in tumor recurrence and metastasis initiation. Nevertheless, only a subset of CTCs can survive in the bloodstream, migrate to distant sites and establish secondary tumors. Consistent with CSC-hypothesis, stem-like CTCs might represent a potential source for cancer relapse and metastasis[121,128] [Figure 2]. In fact, mature tumor cells have only a short blood circulation time and mostly die through natural apoptosis. CSCs, however, have shown to have significantly higher viability, enhanced homing ability into the bloodstream as well as higher distant metastasis initiation capability compared to other tumor cells[121,122,128]. According to the CSC-hypothesis, circulating CSCs (cCSCs) are particularly difficult to eradicate, with a consequent permanence of minimal residual disease and tumor recurrence[121,128].

Some putative markers has been proposed for identification of liver cCSCs. It has been demonstrated that CD90+ cCSCs express key stem-like genes (e.g., *BMI1*, *CD44*, *OCT4*, *WNT3A*, *STAT3* and *HIF-1α*) at very high levels, also when compared to tissue CD90+ CSCs[121,129,130]. Moreover, CD90+ CXCR4+ cCSCs are able to initiate tumor metastasis formation in transplanted mice, enhancing the metastasis initiating ability of CSCs[121,131]. Considering that intercellular adhesion molecule 1 (ICAM1) inhibition by shRNA results in reduced metastasis in mice, ICAM1 has been proposed as another cCSC marker in PLC patients[121,132]. An

explanation for the different metastatic activity observed between CSCs and other tumor cells might be the EMT status of CSCs, which enables them to have a prominent role in the metastasis and invasion[122]. Malignant cells undergo molecular changes typical of EMT, which represents a key stage of the metastatic multistep process, and eventually undergo a mesenchymal-to-epithelial transition (MET) to generate secondary tumors in target organs. Hence, CSCs mediate tumor metastasis by maintaining plasticity to transition between epithelial or mesenchymal states, and the EMT process represents the potential link between CSCs and circulating metastasis-initiating cells[121,133]. For example, in the CCA cell line TFK-1, TGF-β1 is able to induce not only EMT, but also CSC generation with a consequent decreased sensitivity to the chemotherapeutic agent 5-FU. Furthermore, the EMT-related overexpression of hepatic transmembrane 4L six family member 5 (TM4SF5) has a potential role in generating HCC cCSCs with metastatic properties through interaction with CD44[121,134]. In addition, HCC CSCs isolated by sphere assay are associated with an enhanced expression of the variant isoforms of CD44, which are related to CSC chemo-resistance, as well as with an increased frequency of intrahepatic metastasis when injected in the spleen of NOD-Rag1^{null} IL2rγ^{null} double mutant mice (NRG mice). Also in this case, enhancement of the EMT correlates to the metastatic potential and CSC state[135]. Another study has revealed that CD44 is associated with a mesenchymal phenotype in HCC cell lines, and knockdown of CD44 reverses EMT and inhibits lung metastasis of HCC cells in a murine model[136]. Another gene expression analysis of microarray data from 238 HCC cases has revealed an enriched EMT signature in CD90+ stem-like cells[137]. Finally, a recent study has found that CD44 protein levels are enhanced after TGF-β1 treatment and that interaction between CD44 and TGF-β1 induces EMT and CSC phenotypes through β-catenin signaling in HCC[138]. All these findings strengthen the hypothesis of an existing link between EMT and CSC cellular states in relation with the metastatic process.

Metabolic reprogramming

Starting from the pioneering work of Otto Warburg, several observations have indicated that tumor genetic alterations imply also cell metabolism reorganization[139,140]. In particular, it has been shown that tumor cells produce ATP via glycolysis and accumulate extracellular lactate even under normoxic conditions[140,141], and often present a limited or absent mitochondrial oxidative phosphorylation (OXPHOS)[140]. Although metabolic reprogramming is currently considered a hallmark of cancer, no consensus has been reached on the metabolic features of CSCs. which are very plastic and capable of either reside in a dormant state, or rapidly proliferate to replenish the tumor mass. A number of studies suggest that CSCs more strongly favor the glycolytic pathway compared to bulk tumor cells, while other studies report that mitochondrial oxidative metabolism is the prevalent source of energy for CSC (reviewed in[141]) [Figure 2]. However, even if investigation of PLC metabolism is still at its very beginning in comparison with other tumor systems, recent evidence has revealed the importance of the metabolic rearrangement in PLC CSCs. CD44+ CCA CSCs adapt their redox status regulation according to their needs and contribute to reactive oxygen species (ROS) defense promoting glutathione synthesis by way of xCT (a cysteine-glutamate transporter), resulting in evasion of cell death[142]. Moreover, CD133+ HCC CSCs are characterized by high glycolytic metabolism with concomitant overexpression of glycolytic genes and enhanced extracellular acidification rate, demonstrating that CD133+ cells are more glycolytic compared to CD133- cells. Further, CD133+ cells stemness features are significantly reduced when glycolysis is inhibited[143]. Extensive trascriptome and metabolome analysis of CD133+ HCC cells revealed the key role of MYC in the regulation of glycolytic metabolism in HCC CSCs[144].

There is also an increasing interest in lipid metabolism and specifically in alterations in lipid and cholesterol-associated pathways. It is well known that proliferating tumor cells require lipids and cholesterol, and they may increase the uptake of exogenous lipids and lipoproteins or hyper-activate metabolic pathways deputed to produce lipids and cholesterol. When specifically looking at the stem cell compartment, it has been demonstrated that stem-like cells rely on fatty acid oxidation (FAO) for the generation of ATP and NADH[145] [Figure 2]. Metabolism analysis has revealed that NAD+ concentrations

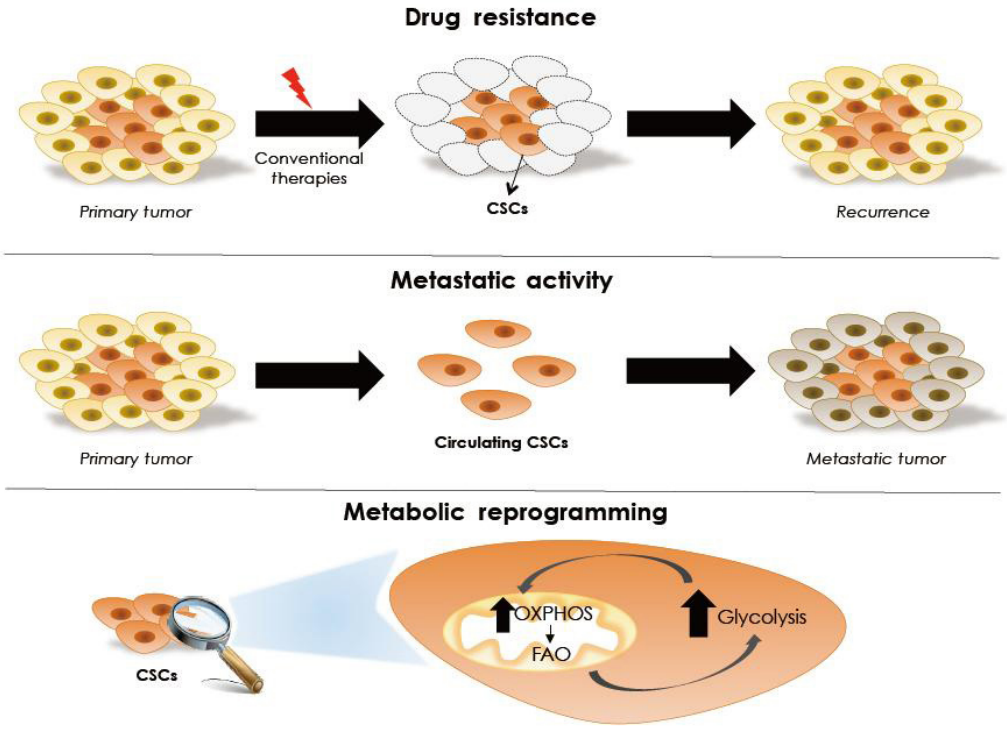

Figure 2. Key features of liver cancer stem cells (CSCs). Liver CSCs present some common key functional features. CSCs represent a peculiar sub-compartment of tumor cells crucially involved in drug resistance. Current therapeutic strategies for the treatment of hepatic cancer mostly focus on the inhibition of tumor growth, resulting in the death of only bulk tumor cells. CSCs are able to survive thanks to intrinsic and extrinsic mechanisms of chemo-resistance and subsequently they can give rise to primary liver cancer recurrence. Moreover, CSCs are the only cells endowed with metastatic potential. While mature tumor cells mostly die through natural apoptosis into blood circulation, CSCs, however, have a significantly higher viability, enhanced homing ability into the bloodstream as circulating CSCs as well as higher distant metastasis initiation capability. Next, CSCs are characterized by metabolic changes, but, no consensus has been reached on the metabolic features of CSCs, which are very plastic and so able to modify their metabolic features according to specific needs. Then, CSCs can exhibit enhanced glycolytic activity as well as increased mitochondrial oxidative phosphorylation (OXPHOS) with subsequent increased fatty acid oxidation, depending on tumor context. All these features make CSCs particularly hard to eradicate. FAO: fatty acid oxidation

are increased in CD133+ cells, and this is directly correlated with SIRT1-dependent enhanced FAO[144]. In HCC, genome-wide transcriptional profiling and Ingenuity pathway analysis have suggested NANOG to be the connecting point between FAO and stem-like features, because of its simultaneous OXPHOS repression and FAO activation actions[145]. Moreover, it has been observed that stearoyl-CoA desaturase 1 (SCD1), a central enzyme involved in the conversion of saturated fatty acids into monounsaturated fatty acids (MUFAs), regulates liver CSCs[146]. In addition, enhanced activation of SCD1 and the consequent production of MUFAs appear to be a potential hallmark of CSCs[141].

All these findings prompt metabolic plasticity as a central force that enables CSCs to modify their replicative capabilities according to specific needs [Figure 2]. Further, emerging evidence suggests that CSCs may adopt specific metabolic phenotypes based on their location within tumor mass[147].

CONCLUSIONS AND CLINICAL IMPLICATIONS

Unresponsiveness to current conventional therapies remains one of the major challenges in PLC. Current therapeutic strategies for the treatment of hepatic cancer mostly focus on the inhibition of tumor growth, with unsatisfactory results. Future treatments are likely to target CSCs and their specialized niche. In this view, it is imperative to decipher the molecular mechanism behind chemoresistance of PLC cells and especially of CSCs, with the objective to develop novel therapeutic strategies targeting features, markers or signaling pathways essentials for CSC biology.

Since CSCs are characterized by metabolic changes, drugs that inhibit OXPHOS have been studied as potential anticancer agents. Metformin, which interfere with OXPHOS by inhibiting NADH-coenzyme Q oxidoreductase (complex I), is a key example and has been shown to be particularly cytotoxic for CSCs, as well as for cells with mutations in OXPHOS complex I[148,149]. Despite metabolic studies in the field of liver CSC are still at an early stage, the dual inhibition of glycolytic and mitochondrial energy pathways may represent a promising superior therapeutic approach to effective eradicate heterogeneous liver CSCs and to overcome therapeutic resistance.

Moreover, since EMT pathway and CSC features seem to be intimately linked, improving our understanding of these cellular states may help to develop novel therapies. The plasticity of CSCs further suggests that simultaneously targeting CSCs existing in both epithelial and mesenchymal states rather than either state alone is needed to achieve complete tumor eradication[150]. Hence, future investigations in this direction are imperative.

It is important to underline that the development of CSC-specific therapeutic strategies imply the presence of a common recognized method for isolation and subsequent characterization of liver CSCs. During the last decade a large number of studies have aimed to identify liver CSCs and several attempts have been made to enrich liver CSCs. Common strategies for PLC CSC enrichment, varied from the widely used classical antigenic approach that relies on surface CSC markers detection (e.g., CD133[46,56,151-154], CD44[71,153-155], OV6[156], CD90[129,130,157], EpCAM[22,68,71,158], CD13[159], CD24[153,154,160], CD47[161]) to functional techniques including side population (SP) analysis[65,162-165], Aldefluor assay[166-169] and tumor-sphere formation[65,67,70,170,171]. In all different published studies, enriched PLC CSC subsets have been then tested in immune-deficient mice for the *in vivo* tumorigenic potential[22,56,65,67,68,129,130,151,152,155,156,159-162,166,170].

One important challenge in developing new therapeutic strategies is the dynamic and plastic behavior of tumor cells, especially of CSC. As it's well known, a central role in the regulation of cancer cell plasticity is played not only by genetic alterations, but also by epigenetic changes, including DNA methylation, histone modifications and non-coding RNA (ncRNA) activity[58]. By acting at transcriptional, post-transcriptional and translational level, ncRNAs represent key regulators of CSCs by modulating several biological processes including asymmetric division, unresponsiveness to treatments and EMT, thus affecting tumor progression and recurrence[58]. In addition, recent studies also suggest that similar to normal stem cells, CSCs seem to reside in specialized microenvironment ("CSC-niche")[46,50,70,172], whose signals can support self-renewal and drug-resistance features and, thereby, may influence the plasticity of CSCs[173-177]. Therefore, targeting only CSCs may not be enough, and continued development of therapies targeting CSCs and their microenvironment in combination with chemotherapy may be essential to improve the outcomes of PLC patients.

DECLARATIONS

Authors' contributions

Analysis of publications and drafting of the manuscript: Correnti M, Booijink R, Di Maira G
Critically revised the manuscript: Raggi C, Marra F
Read and approved the final manuscript: All authors

Availability of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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