

Cite this: *J. Mater. Chem. B*, 2025, 13, 13184

## Engineered nanomedicine targets liver cancer stem cells to treat liver cancer disease

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Liver cancer stem cells (LCSCs) are a population of cells with self-renewal and self-differentiation capacities, widely recognized as critical for hepatocellular carcinoma (HCC) development. Accordingly, eliminating LCSCs is considered a viable strategy for HCC treatment. However, conventional chemotherapy and radiation therapy struggle to eradicate LCSCs, underscoring the critical need for LCSC-targeted therapies. Nanotechnology offers unique advantages for LCSC targeting *via* the selective delivery of drugs to tumor sites. Various engineered nanomedicines—including polymeric nanoparticles, biological nanomaterials, and inorganic nanoparticles—have been developed for their elimination. This article primarily reviews the biological concepts and biomarkers of LCSCs. In addition, it summarizes various strategies for targeting LCSCs using engineered nanomedicines.

Received 28th May 2025,  
Accepted 8th September 2025

DOI: 10.1039/d5tb01278c

rsc.li/materials-b

### 1. Introduction

Liver cancer is one of the six major cancers in the world and has a high mortality rate. To address the urgent need for effective prevention and treatment, it is essential to dissect the key pathological cascades leading to hepatocellular carcinoma (HCC)—the most prevalent subtype of liver cancer, accounting for over 80% of all liver cancer cases. Among these cascades, chronic liver injury stands out as a pivotal precursor event, and its gradual, progressive pathological process is tightly intertwined with the malignant transformation of normal hepatocytes into cancerous cells. Studies have shown that infections with the hepatitis B virus (HBV) and the hepatitis C virus (HCV), non-alcoholic steatohepatitis (NASH), and chronic alcohol exposure can induce recurrent injury–repair cycles within the hepatic parenchyma. Unlike acute liver damage that resolves with timely intervention, these factors exert long-term, cumulative stress on the liver: HBV/HCV persistently replicates in hepatocytes to disrupt cellular homeostasis, NASH-related lipid accumulation causes continuous lipotoxicity, and ethanol metabolites (*e.g.*, acetaldehyde) directly damage liver cell membranes and DNA. Such sustained harm forces the hepatic parenchyma into repeated “injury–repair cycles”—a process where damaged cells are cleared, but incomplete repair (due to persistent stress) fails to restore the liver’s normal structure. This process is driven by the persistent activation of inflammatory

pathways, which subsequently activate hepatic stellate cells and promote the progression of hepatic fibrosis. The progression of hepatic fibrosis to cirrhosis increases the risk of HCC development by 20–40-fold.<sup>1–3</sup> At the molecular level, oxidative stress triggered by chronic liver injury can activate proto-oncogenes *via* the accumulation of oxidative DNA damage, while inhibiting the function of tumor suppressor genes, thereby driving abnormal hepatocyte proliferation.<sup>4,5</sup> Furthermore, clinical data reveal that approximately 70–80% of HCC patients have an underlying cirrhosis, with the annual incidence of HCC in cirrhotic patients ranging from 2% to 8%. Among these, the risk of developing HCC is further elevated in patients with metabolic syndrome (*e.g.*, diabetes and obesity), indicating that the synergistic effect of metabolic disorders and chronic liver injury is a critical driver of HCC development.<sup>5,6</sup> This cascade reaction from chronic injury to malignant transformation provides a pathological microenvironment for the generation of liver cancer stem cells (LCSCs), and the survival and expansion of LCSCs further exacerbate HCC progression and treatment resistance.

Among primary liver cancers, hepatocellular carcinoma (HCC) accounts for 90% of all liver cancer cases.<sup>7</sup> Despite conventional treatments, including chemotherapy, arterial embolization, surgical resection, and radiofrequency ablation, the recurrence rate of HCC remains alarmingly high at 70%.<sup>8</sup> Studies have shown that hepatocellular carcinoma originates from hepatic stem cells with endogenous and exogenous origins in human liver tissue, whose endogenous origin is oogenic cells located at the smallest end of the intrahepatic bile duct.<sup>9,10</sup> This population of cancer cells with stem cell properties is called liver cancer stem cells (LCSCs), which possess many biomarkers such as EpCAM, CD44, CD133,

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CD90, CD24, *etc.* LCSCs can mutate genes, disrupt epigenetics, block signaling pathways, and alter the tumor microenvironment. They play a key role in the self-renewal, tumorigenesis, metastasis, recurrence, and drug resistance of cancer cells in HCC.<sup>11</sup> Therefore, the LCSC theory may offer novel strategies for tumor diagnosis, therapeutic intervention, and prevention.

An increasing number of drugs, such as sorafenib and doxorubicin, have been demonstrated to be effective in eliminating or inhibiting HCC. However, these drugs tend to cause resistance in tumor cells under long-term administration. In most cases, HCC cells with resistance exhibit a significant mesenchymal phenotype and stem cell profile,<sup>12</sup> indicating that targeted elimination of tumor cells with this profile represents a more promising strategy for HCC therapy. Consequently, there is a great need to develop novel drugs or drug delivery systems that directly target LCSCs.

Nanotechnology has demonstrated significant potential for applications in cancer detection, prevention, and therapeutic intervention.<sup>13</sup> In recent years, the application of nanopreparations in cancer therapy has been further expanded, demonstrating the potential of multifunctional synergistic treatment. Nanoscale covalent organic frameworks (NCOFs) exhibit characteristics that include low density, a large specific surface area, and tunable pore size, which enable high drug loading capacities and precise release mechanisms through controllable synthesis. Their well-defined periodic structures can carry therapeutic agents (*e.g.*, chemotherapeutic drugs, photosensitizers) and release these active molecules upon light irradiation in photodynamic therapy, thereby effectively enhancing local drug concentration in tumors.<sup>14</sup> Metal-based nanoparticles such as platinum–iron (FePt) nanoparticles can decompose hydrogen peroxide in the tumor microenvironment to generate oxygen by mimicking peroxidase activity. This process not only alleviates hypoxia but also enables the ultrasound-triggered generation of singlet oxygen, enhancing the killing effect of sonodynamic therapy on deep tumors with good biocompatibility.<sup>15,16</sup> Beyond these single-structure nanopreparations (*e.g.*, NCOFs and FePt nanoparticles) that target specific therapeutic needs (*e.g.*, photodynamic therapy for superficial tumors, sonodynamic therapy for deep tumors), composite nanosystems—by integrating multiple functional components—have emerged as a more powerful approach to achieve “1 + 1 > 2” synergistic anti-tumor effects. Notably, they address the limitations of single-modal therapy (*e.g.*, insufficient tumor killing, easy drug resistance) by combining different treatment mechanisms. For instance, composite nanosystems, such as metal–organic frameworks (MOFs) wrapped with MnO<sub>2</sub> nanosheets, can co-deliver doxorubicin (DOX) and DNzyme. Under intracellular stimulation, these systems release Mn<sup>2+</sup>, which not only acts as a DNzyme activator to silence anti-apoptotic genes (*e.g.*, survivin) but also generates reactive oxygen species (ROS) through Fenton-like reactions. This achieves a synergistic combination of chemotherapy, gene therapy, and chemodynamic therapy.<sup>17</sup> In addition, redox-responsive nanogels (*e.g.*, hyaluronic acid–lipoic acid conjugates) accumulate in tumors *via* CD44-mediated active targeting. Upon entering a

high-glutathione environment, they disintegrate to release protein drugs (*e.g.*, cytochrome *c*). Their pH-sensitive components also promote endosomal escape, thereby significantly improving intracellular delivery efficiency.<sup>18</sup> Through structural design, these nanopreparations achieve targeted delivery, microenvironment-responsive release, and integration of multimodal therapies, providing new strategies to overcome tumor heterogeneity and therapeutic resistance.

Nanoscale drug delivery systems could have selective tumor targeting function, high drug loading capacity, and smart drug releasing ability.<sup>19</sup> The ability to target LCSCs by designing and modifying these nanocarriers has gradually become an upsurge. These engineered nanomedicine types include polymer nanomaterials such as poly(lactide-*co*-glycolide), polyethylene glycol, PLGA–PEG (poly(lactide-*co*-glycolide)-*block*-poly(ethylene glycol)) copolymer, PEG–polylysine (polyethylene glycol–polylysine) copolymer, liposomes, *etc.*<sup>20</sup> Meanwhile, natural biomaterials have also attracted attention due to their unique functional properties, such as exosomes, monoclonal antibodies, and aptamers, all of which have applications in targeting LCSCs.<sup>21</sup> Inorganic metal nanoparticles are easily scalable and very stable, and many are also used in the treatment of LCSCs, such as metal nanoparticles (gold, silver, zinc, oxide), silica nanoparticles (SiNPs), arsenic trioxide nanoparticles (AtoNPs), and nanodiamonds (NDs).<sup>22</sup>

In this review, we mainly outline the origin and biological properties of LCSCs and summarize the drug delivery systems capable of targeting LCSCs.

## 2. LCSC theory

### 2.1. Concept of cancer stem cells

Stem cells are a population of cells with self-renewal capacity and pluripotent differentiation potential, playing a central role in maintaining tissue homeostasis, facilitating tissue repair, and driving disease pathogenesis. Based on their differentiation potential, stem cells can be classified into totipotent stem cells (*e.g.*, zygotes), pluripotent stem cells (*e.g.*, embryonic stem cells), and multipotent stem cells (*e.g.*, adult stem cells). Among these categories, adult stem cells are present in various tissues, including the bone marrow, liver, and brain, and their primary function is to maintain tissue dynamic balance, replenishing damaged cells through proliferation and differentiation upon tissue injury.<sup>23,24</sup>

Hematopoietic stem cells (HSCs) represent the most well-studied model of normal stem cells, responsible for the lifelong maintenance of hematopoiesis. Their homeostasis is tightly regulated by intrinsic molecular mechanisms (such as gene regulation and epigenetic modifications) and extrinsic niche signals (including cytokines and niche interactions).<sup>25,26</sup>

Recent studies demonstrate that the transcription factor Nynrin maintains hematopoietic stem cell (HSC) homeostasis by transcriptionally repressing Ppif to reduce cyclophilin D (CypD) levels. This repression inhibits mitochondrial permeability transition pore (mPTP) opening, thereby stabilizing



membrane potential and reducing reactive oxygen species (ROS) production. Nynrin deficiency leads to excessive mPTP opening, mitochondrial swelling, and elevated ROS, which collectively impair the radiation resistance and self-renewal capacity of HSCs. In contrast, Nynrin overexpression not only alleviates radiation-induced damage but also prolongs mouse survival. These findings reveal a central regulatory role of the Nynrin–Ppif–mPTP axis, providing a novel target for ameliorating hematopoietic toxicity during cancer therapy.<sup>25</sup>

Additionally, spliceosome-mediated alternative splicing regulates HSC homeostasis by increasing proteomic diversity, with the ATP-dependent RNA helicase DHX16 acting as a key spliceosomal component. Dhx16 knockout in the hematopoietic system results in depletion of HSCs and progenitor cells, bone marrow failure, and rapid death in mice. Mechanistically, Dhx16-deficient HSCs exhibit multiple abnormalities, including impaired quiescence, G2-M phase arrest, reduced protein synthesis, abnormal ribosome assembly, increased apoptosis, and diminished self-renewal capacity. Multi-omics analyses show that Dhx16 deficiency causes retention of intron 4 in *Emg1* mRNA, leading to decreased EMG1 expression, which in turn disrupts ribosome assembly, induces nucleolar stress, and activates the p53 pathway—this cascade is a key contributor to HSC exhaustion. Overexpression of *Emg1* in Dhx16-deficient HSCs partially rescues their function, suggesting that *Emg1* may serve as a potential target for ribosome-related hematopoietic disorders.<sup>26</sup>

Unlike normal stem cells, cancer stem cells (CSCs) are a subpopulation within tumors that exhibit stem cell properties, with their core characteristics including self-renewal, multi-directional differentiation potential, chemoresistance, and tumor-initiating capacity.<sup>27,28</sup>

The term “cancer stem cells” (CSCs), also known as tumor-initiating cells, was first proposed by Sajiyo Makino. He identified a distinct subpopulation of cells characterized by chemotherapy resistance and a unique genetic profile differing from the bulk tumor cells.<sup>29</sup> CSCs are a subpopulation of tumor cells capable of driving tumorigenesis and contributing to disease recurrence. At the point of tumor initiation, CSCs are derived from differentiated cells or adult tissue-resident stem cells. The theory that CSCs have a greater capacity for self-renewal, metastatic spread, and treatment resistance is supported by a growing body of evidence.<sup>30</sup> CSCs were initially found in acute myeloid leukemia (AML) and subsequently isolated from various hematologic malignancies and solid tumors, which are believed to form the clonal core of tumors.<sup>31,32</sup> Currently, two hypotheses explain the origin of CSCs: the stochastic model and the stratified model.<sup>33,34</sup> The stochastic model proposes that any cell exposed to a group of mutant cells is likely to self-renew and differentiate, resulting in tumor heterogeneity. In contrast, the stratified model posits that somatic mutations selectively target stem and progenitor cells in hierarchically organized tissues, generating self-renewing cancer stem cells and their differentiated progeny. In conclusion, CSCs are cancer cell populations with self-renewing stem cell properties and the ability to produce differentiated progeny.<sup>35</sup>

Furthermore, autophagy, as a conserved mechanism for cellular homeostasis regulation, exerts dual roles in both normal stem cells and cancer stem cells (CSCs). In normal stem cells, autophagy sustains their survival by clearing damaged organelles and maintaining metabolic balance.<sup>28</sup> In CSCs, autophagy not only mitigates oxidative stress through mitophagy to adapt to the hypoxic microenvironment but also enhances their self-renewal and tumor progression capabilities *via* bidirectional regulation. This functional divergence reflects the plasticity of molecular regulatory networks in stem cells under physiological and pathological conditions.<sup>36,37</sup>

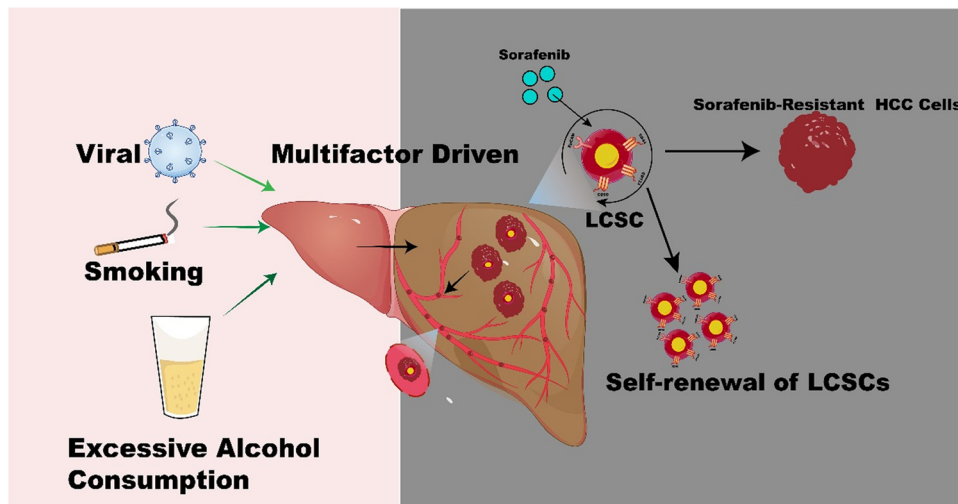
## 2.2. Link between hepatocellular carcinoma and liver cancer stem cells

Hepatocellular carcinoma (HCC) contains a subpopulation of cancer stem cells (CSCs) termed liver cancer stem cells (LCSCs). They are poorly differentiated cancer cells with specific characteristics. These characteristics are similar to those of normal stem cells (NSCs) and are capable of growth, invasion, and regeneration.<sup>38</sup> HCC is a multifactorial disease with the main causes being viral infections, excessive alcohol consumption or smoking, and nonalcoholic steatohepatitis.<sup>39</sup> HCC is a tumor with an abundant blood supply, and one of the reasons for its recurrence is the migration of tumor cells into the blood vessels. Although both LCSCs and mature cancer cells can migrate into the bloodstream, LCSCs exhibit a longer circulating capacity, which significantly promotes primary tumor growth and metastasis of secondary tumors. Therefore, LCSCs are a source of HCC recurrence<sup>40</sup> [Fig. 1].

LCSCs have been implicated in the development of secondary drug resistance in HCC. This malignancy exhibits considerable heterogeneity across morphological features, cellular behaviors, therapeutic responses, and clinical outcomes.<sup>41</sup> Traditionally, tumor heterogeneity in HCC has been attributed to clonal evolution-driven genetic mutations, which are regarded as a major contributor to drug resistance.<sup>42</sup> LCSCs are typical models of tumor heterogeneity that promote the phenotypic diversity of HCC cancer cells. Sorafenib is a first-generation drug that is effective in the treatment of patients with advanced HCC, but sorafenib resistance also poses a great obstacle to the treatment of HCC.<sup>43,44</sup> With long-term exposure to sorafenib, LCSCs undergo clonal evolution and become resistant to sorafenib. Therefore, LCSCs' tumor heterogeneity model is also a potential mechanism for the development of acquired sorafenib resistance in HCC patients.

Autophagy was initially characterized in 1963 by biochemist Christian de Duve, who observed the degradation of mitochondria and other intracellular components in rat liver following glucagon administration.<sup>45</sup> Autophagy has a dual role in HCC: on the one hand, autophagy exerts oncogenic spark activity by removing damaged mitochondria, abnormal proteins, protein aggregates, and oncogenic proteins;<sup>46</sup> on the other hand, autophagy can also promote tumor growth by allowing cancer cells to adapt to hypoxic and nutrient-deficient environments and by reducing metabolic stress. Autophagy is essential for maintaining LCSC viability. Mitophagy, the selective autophagy





**Fig. 1** Schematic diagram of the link between hepatocellular carcinoma and liver cancer stem cells. As a multifactorial disease, HCC primarily arises from viral infections, chronic alcohol abuse, and smoking. LCSCs exhibit enhanced circulatory capacity compared to differentiated tumor cells, facilitating their intravasation into the vasculature. This process significantly contributes to both primary tumor progression and distant metastasis, consequently increasing the risk of HCC recurrence. With long-term exposure to sorafenib, LCSCs undergo clonal evolution and become resistant to sorafenib.

of damaged mitochondria, mitigates the hostile tumor microenvironment in HCC by reducing oxidative stress, thereby enabling LCSC survival under hypoxic conditions. Furthermore, LCSCs reciprocally promote HCC progression.<sup>47</sup> These findings demonstrate a bidirectional regulatory relationship between autophagy and LCSC maintenance, including both survival and self-renewal capabilities.

### 3. Biological properties of LCSCs

The previous section clarified the definition, origin hypotheses of cancer stem cells (CSCs), and their relationship with tumor progression, with a focus on elaborating that LCSCs, as a specific subset of CSCs in HCC, are closely associated with tumor heterogeneity, drug resistance, and recurrence. On this basis, an in-depth analysis of the biological properties of LCSCs (including structural features, core functions, and specific roles in tumor progression) is a prerequisite for understanding the mechanisms underlying the maintenance of their “stemness” and developing targeted strategies. This section will systematically elaborate on the structural characteristics, self-renewal, differentiation potential, and other core properties of LCSCs, laying a foundation for subsequent discussions on their targeted biomarkers and microenvironmental regulation.

#### 3.1. Core biological properties of liver cancer stem cells (LCSCs)

**3.1.1. Self-renewal capacity.** LCSCs maintain a stable population and generate proliferative daughter cells through asymmetric division, which forms the basis for their long-term survival and ability to drive tumor progression. This process is regulated by signaling pathways such as Wnt/ $\beta$ -catenin, Notch, and Hedgehog, and the aberrant activation of these pathways can enhance their self-renewal potential.<sup>48–50</sup>

**Multidirectional differentiation potential:** They can differentiate into hepatocellular carcinoma cells with different phenotypes, contributing to tumor heterogeneity. During differentiation, phenotypic markers undergo dynamic changes (*e.g.*, shifting from high expression of stem cell markers such as CD133 and EpCAM to mature hepatocyte markers), which increases the difficulty of treatment.<sup>49,51,52</sup>

**3.1.2. Expression of phenotypic markers.** LCSCs express specific markers, including CD133, EpCAM, and CD44. Among these, CD133<sup>+</sup> cells exhibit stronger tumorigenic ability; EpCAM is highly expressed in certain subpopulations and is associated with tumor invasion and metastasis.<sup>53–55</sup> Additionally, LCSCs exhibit an intrinsic drug-resistant phenotype: LCSCs possess intrinsic resistance to radiotherapy and chemotherapy, which is related to the high expression of ABC transporters. They can also adapt to stress through metabolic regulation, such as enhanced glycolysis, further strengthening drug resistance.<sup>56,57</sup>

#### 3.2. The role of LCSCs in tumor initiation

As the “seed cells” for hepatocellular carcinoma (HCC), liver cancer stem cells (LCSCs) play a key role in tumor initiation. Under the influence of carcinogenic factors such as HBV/HCV infection, liver stem cells or differentiated hepatocytes can acquire gene mutations and epigenetic modifications that confer an LCSC phenotype. These transformed cells then form clonal populations through self-renewal, gradually developing into precancerous lesions and eventually primary liver cancer.<sup>58–60</sup> Meanwhile, LCSCs maintain the stability of their own population through asymmetric division and continuously generate differentiated tumor cells, providing sustained impetus for tumor growth.<sup>58,61,62</sup> Their multidirectional differentiation potential leads to differences in phenotype, function, and drug sensitivity among daughter cells, driving high heterogeneity of tumor tissues and serving as



the basis for tumors to adapt to the microenvironment and evade treatment.<sup>63,64</sup>

### 3.3. The role of LCSCs in therapeutic resistance

Liver cancer stem cells (LCSCs) are a major cause of therapeutic resistance through multiple mechanisms. First, they highly express ABC transporters (*e.g.*, ABCG2, MDR1), which actively efflux chemotherapeutic agents like sorafenib, thereby reducing intracellular drug concentrations.<sup>65</sup> Second, their quiescence (G0 phase) renders them insensitive to proliferation-dependent chemotherapeutics, allowing them to re-enter the cell cycle post-treatment.<sup>66</sup> Furthermore, LCSCs often exhibit an epithelial-mesenchymal transition (EMT) phenotype, which enhances drug resistance through the activation of pathways such as TGF- $\beta$ /Smad.<sup>67</sup> Finally, their metabolic reprogramming towards glycolysis not only reduces oxidative stress but also provides biosynthetic precursors for nucleic acids and lipids. This supports their survival in nutrient-deficient microenvironments and further strengthens their drug-resistant phenotype.<sup>68</sup>

### 3.4. The role of LCSCs in tumor recurrence

Liver cancer stem cells (LCSCs) drive tumor recurrence through multiple mechanisms, with core ones including: activating signaling pathways such as STAT3 to maintain stemness;<sup>69</sup> interacting with M2-like macrophages *via* the TGF $\beta$ -PD-L1 axis to inhibit CD8+ T cell function and evade immune surveillance;<sup>70</sup> relying on glycolysis, glutamine metabolism, *etc.*, for energy supply; strengthening stem cell characteristics through epigenetic regulation such as the interaction between SCARB2 and MYC; evading killing through dormancy or metabolic remodeling after treatment; and reactivating upon microenvironmental stimulation to trigger recurrence.<sup>63,71</sup> Therefore, targeting their stemness maintenance, immune interaction, and metabolism-dependent pathways is key to preventing recurrence.

## 4. Biomarkers of LCSCs

The preceding discussion has highlighted the unique structural, metabolic, and functional characteristics of LCSCs, particularly their properties of self-renewal, multi-lineage differentiation, and drug resistance, which render them a critical target for HCC therapy. The specific expression of these characteristics depends on signature molecules present on the cell surface—biomarkers that serve not only as important tools for identifying and isolating LCSCs but also as core targets for targeted therapy. This section will focus on the major biomarkers of LCSCs, such as EpCAM, CD44, and CD133, analyzing their expression patterns and clinical significance to provide a molecular basis for the subsequent design of targeted delivery systems. Tumors comprise heterogeneous cell populations, with LCSCs representing only a minor subset. This rarity complicates LCSC isolation and consequently restricts their investigation. Nevertheless, LCSCs can be identified through specific surface markers, such as EpCAM, CD44, CD133, CD90, and CD24.<sup>11,72</sup>

### 4.1. EpCAM

EpCAM is an epithelial adhesion molecule belonging to the family of type I transmembrane proteins that mediate cell-to-cell contact, signal to the nucleus, and regulate gene transcription. It is considered to be one of the most strongly and frequently expressed cancer antigens.<sup>73–75</sup> EpCAM is expressed not only in normal hepatocytes but also in embryonic liver tissue, bile duct epithelium, and proliferating bile ducts in cirrhotic livers. Consequently, it has been recognized as a marker for hepatic progenitor cells in adult liver tissue.<sup>76</sup> EpCAM participates in diverse physiological processes, including intercellular adhesion, proliferation and migration of hepatocytes, cell cycle regulation, signal transduction, cellular differentiation, metastatic progression, regenerative organogenesis, and tumorigenesis.<sup>11</sup> EpCAM expression in HCC is associated with elevated levels of methemoglobin. The survival rates of EpCAM-positive patients are lower than those of EpCAM-negative patients in the first three years.<sup>77</sup> Yamashita *et al.* stratified HCC patients based on EpCAM and alpha-fetoprotein (AFP) expression profiles. The EpCAM+ AFP+ subgroup predominantly comprised younger patients with advanced disease, whereas the EpCAM-AFP- subgroup mainly consisted of elderly patients at early TNM stages. These findings indicate that EpCAM+ AFP+ HCC exhibits liver stem/progenitor cell properties, while EpCAM- AFP- HCC displays mature hepatocyte characteristics.<sup>78</sup> Several studies have shown that EpCAM+ CD45- cells trigger tumor formation, whereas EpCAM- CD45- cells do not, supporting the hypothesis that EpCAM+ contributes to LCSCs' properties and promotes tumor growth, which indicates EpCAM is a reliable stem cell marker for HCC.<sup>79</sup>

### 4.2. CD44

CD44 is a hyaluronan receptor that has been recognized as a stem cell marker for a variety of cancers, including breast, colon, prostate, and bladder cancers. Normally expressed on the cell surface of mammalian cells, including monocytes and neutrophils, CD44 plays an important role in intercellular adhesion and cell-extracellular matrix interactions.<sup>80,81</sup> CD44-positive (CD44+) cancer cells exhibit characteristics of CSCs; however, the precise role and clinical significance of CD44 in HCC remain unclear. Tumor-associated macrophages (TAMs), immune cells commonly found in the solid tumor microenvironment, produce IL-6, which subsequently activate signal transduction and STAT3, promoting the expansion of the CD44+ population and tumor formation.<sup>72,82</sup> The knockdown of CD44 in CD44-expressing HCC cells leads to a sustained decrease in LCSCs and enhances chemosensitivity. In HCC patients and cell lines, CD44 overexpression is predominantly regulated by transforming growth factor-beta (TGF- $\beta$ ) and promotes a TGF- $\beta$ -induced mesenchymal phenotype *via* AKT/GSK-3 $\beta$ / $\beta$ -catenin signaling.<sup>83,84</sup> A meta-analysis by Luo *et al.* found that CD44 expression was positively associated with a higher TNM stage and poorer overall survival (OS) in HCC patients. This trend suggests that CD44 expression helps to monitor the development of HCC.<sup>85</sup>



### 4.3. CD133

CD133 is a glycoprotein with five transmembrane structural domains and two larger extracellular glycosylation chains. It is abundantly expressed in the cytoplasm and nucleus of tumor tissues from HCC, brain cancer, pancreatic cancer, prostate cancer, and colon cancer.<sup>11</sup> CD133 is involved in various molecular mechanisms, including self-renewal, multispectral differentiation, tumorigenesis, and treatment resistance. It was found that CD133+ HCC cells make up more than 50% of the population in HCC cells.<sup>86</sup> The knock-down of CD133 in HCC cells could lead to a reduction in tumorigenicity and an increase in cell cycle, while high CD133 expression was found to be associated with poor prognosis in clinical HCC patients. Elevated CD133 expression correlates significantly with reduced overall survival and increased recurrence rates, indicating its potential utility as a prognostic biomarker in clinical settings.<sup>72,87</sup> Furthermore, CD133 expression showed a significant positive correlation with hepatitis C virus (HCV) infection, while remaining undetectable in normal liver tissues.<sup>88,89</sup> Epithelial-mesenchymal transition (EMT) drives increased TGF- $\beta$  expression in adjacent endothelial cells after hepatitis B virus antigen (HBx) infection of HCC cells, which further leads to enhanced CD133 expression.<sup>90</sup> CD133+ HCC cells isolated from human HCC cell lines and xenograft mouse models increased chemoresistance to adriamycin (DOX) and fluorouracil (5FU) through activation of the Akt/PKB and Bcl-2 pathways.<sup>91</sup>

### 4.4. CD90

CD90 (Thy-1), a glycosylphosphatidylinositol (GPI)-anchored cell surface protein, is expressed in diverse cell types, including T lymphocytes, thymocytes, endothelial cells, astrocytes, and fibroblasts.<sup>92-94</sup> CD90 has been demonstrated to be associated with the tumorigenic and metastatic capacity of various HCC cell lines and is now considered a reliable marker for LCSCs. Sukowati *et al.* found that HCC CD90 levels were significantly higher in HCC compared to cirrhosis or normal liver tissue.<sup>95</sup> The gene expression profiling of sorted cells revealed a significant presence of CD90+ in primary HCC. CD90+ cells have a vascular endothelial cell profile, and their presence is associated with a high incidence of distant organ metastasis.<sup>96</sup> Circulating tumor stem cell (CTSC) populations in circulating tumor cells (CTCs) are critical for metastatic tumor formation at distant sites. Compared to CD133+ CD90+, CD90+ CXCR4+ better promotes tumorsphere formation *in vitro*, tumor development in primary and secondary three-transplantation experiments, and distal metastatic tumors after subcutaneous transplantation.<sup>97</sup>

### 4.5. CD24

CD24 is a highly glycosylated protein with a small protein core linked to a glycosyl-phosphatidylinositol anchor through the plasma membrane. In cancer, CD24 is a regulator of cell migration, invasion, and proliferation. It can be expressed in stem/progenitor cells and a variety of human malignancies,

such as HCC, breast cancer, and renal cell carcinoma.<sup>98</sup> It has been shown that CD24 is associated with cell metastasis, differentiation, self-renewal, and chemoresistance in HCC. For example, CD24 expression was positively correlated with metastasis of HCC cell lines MHCC97H and HCCLM3, and increased CD24 populations implied that these cells underwent proliferation, migration, and invasion. In addition, CD24 expression was associated with tumor number, size, vascular invasion, encapsulation, and differentiation. CD24 expression showed significant positive correlations with both proliferating cell nuclear antigen (PCNA) and  $\beta$ -catenin levels, and was associated with adverse clinicopathological characteristics.<sup>99</sup> It was found that CD24 overexpression led to increased PP2A protein production and induced inactivation of the mTOR/AKT pathway, which increased autophagy levels, demonstrating that CD24 can regulate sorafenib resistance by activating autophagy in HCC. These findings suggest that combining autophagy modulation with CD24-targeted therapy may represent a potential therapeutic strategy for HCC.<sup>100</sup>

The following table shows examples of biomarkers of LCSCs and the characteristics of marker-positive LCSCs [Table 1].

### 4.6. The clinical significance of these biomarkers

In hepatocellular carcinoma (HCC) research, biomarkers such as EpCAM, CD44, CD133, CD90, and CD245 play critical and distinct clinical roles, with studies on epithelial cell adhesion molecules (EpCAM) being relatively extensive. Research has shown that EpCAM can be detected in 17.5% of HCC cases, but not in normal control groups. Its positive expression is associated with higher serum levels of alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA), and is significantly correlated with distant metastasis, lymph node metastasis, and portal vein thrombosis.<sup>113</sup> Another study demonstrated that after EpCAM gene silencing in cells combined with chemotherapy, the expression of the drug resistance marker ABCG2 was reduced, and the ability to form colonies and spheroids was decreased. These findings suggest that EpCAM-targeted therapy could enhance chemotherapy sensitivity in HCC patients.<sup>114</sup>

CD44 exists in multiple isoforms, and its role in liver cancer stem cells is relatively complex. Studies have found that in hepatitis C virus-positive HCC patients, the expression of CD44 variant 9 (CD44v9) is associated with adverse clinicopathological features, including younger age, poorer histological differentiation, elevated alkaline phosphatase levels, and reduced overall and recurrence-free survival.<sup>115</sup> Knocking out the CD44 gene in Huh7 hepatocellular carcinoma (HCC) cells that exclusively express the standard isoform (CD44s) has been shown to reduce spheroid formation, enhance chemosensitivity, and downregulate the expression of stemness markers, including CD133 and EpCAM.<sup>83</sup> In addition, it has been found that CD44v6 can serve as an ideal surface marker for liver cancer stem cells, and CD44v6+ HCC cells express higher levels of Met and possess self-renewal and tumor growth capabilities.<sup>103</sup>

CD133 is one of the more clearly studied markers for liver cancer stem cells. CD133+ cells in HCC have been confirmed to



Table 1 Biomarkers of LCSCs

Marker	Source of LCSCs	Characteristics of marker-positive LCSCs	Therapeutic agent	Functional mechanism	Ref.
EpCAM	Huh7, Hepa1-6	Tumorigenesis, high recurrence frequency		Upregulation of CEACAM1 evades NK cell killing (killing rate ↓).	73
	HepG2, Huh7, Hepa1-6	Tumorigenesis, self-renewal, transfer	Histone lysine demethylase 4D		101
CD44	Huh7	Tumorigenesis, self-renewal	KLF4		102
	Huh7, SMMC-7721, MHCC97-H and HepG2	Mobility/invasive		CD44v6 maintains liver cancer stem cell stemness <i>via</i> the HGF/Met/cJun/Nanog axis.	103, 104
CD133	SNU-368, SNU-354	Mobility, tumorigenesis			84
	Huh7, PLC/PRF/5	Proliferation, mobility/invasive	Sialic acid binds immunoglobulin-like lectin-15		105
CD90	Huh7	Recurrence of cancer, chemical resistance		CD133+ cells highly express pyruvate kinase 2 (PKM2↑), sustaining spheroid formation.	106, 107
	Hep3B, Huh7, HEK293T	Self-renewal, proliferation and differentiation, tumorigenicity, and chemical resistance	CD133-apt-Dox		108
	Human HCC specimens	Proliferation, tumorigenesis, self-renewal, tumorigenicity			109
CD90	The liver cancer cell lines LO2, hepG2, Huh7, SK-Hep1, LM3 and MHCC-97L	Tumorigenesis, mobility, inflammation		Chemotherapy-induced CD90+ cell autophagy activation (LC3-II↑) mediates doxorubicin resistance.	110,111
	Huh7, MHCC 97L and SK-Hep-1	Cell apoptosis, adhesion, mobility, sphere-forming abilities, and fibrosis			112
CD24	Huh7, Hep3B	Chemical resistance, autophagy	Sorafenib		100

be involved in metastasis, tumorigenesis, tumor recurrence, and treatment resistance.<sup>106</sup> According to the studies, CD133+ liver cancer stem cells exhibit a distinct metabolic phenotype with enhanced glycolysis. Targeting this metabolic pathway inhibits their stem cell properties and counteracts resistance to the common HCC drug sorafenib.<sup>116</sup>

CD90 has also been identified as a marker for liver cancer stem cells. Research indicates that the high expression of CD90 in HCC tissues is associated with venous invasion in patients. CD90+ cells from HCC cell lines possess enhanced tumorigenic, chemoresistant, invasive, and metastatic capabilities, alongside activation of the Notch pathway.<sup>117</sup> By promoting spheroid formation and upregulating CD133 expression, CD90 activity suggests that targeting the CD90-integrin-mTOR/AMPK-CD133 axis is a viable cancer treatment strategy.<sup>118</sup>

As for CD245, although there are fewer studies compared to other markers, it is also associated with the stem-like properties of liver cancer stem cells. Some studies suggest that its expression may be related to the self-renewal and differentiation abilities of liver cancer stem cells and could affect the prognosis of HCC patients. For example, in certain HCC cell lines, changes in CD245 expression levels are accompanied by alterations in the expression of other stem cell-related genes, indicating its role in the regulatory network of liver cancer stem cells. However, its specific clinical significance still requires more research to clarify.

In summary, these biomarkers (EpCAM, CD44, CD133, CD90, and CD245) in liver cancer stem cells have diverse clinical significance, including predicting prognosis, metastasis, and treatment response, and are expected to provide guidance for personalized treatment strategies in HCC patients.

## 5. The LCSC microenvironment

Similar to normal stem cells, which are tightly regulated by their specialized microenvironment, LCSCs dynamically interact with and are modulated by various components of their tumor microenvironment. Furthermore, LCSCs possess the capacity to evade immune surveillance and elimination, akin to other malignant cells. Through the regulation of specific immunomodulatory proteins, LCSCs can create an immunosuppressive microenvironment that promotes tumor cell survival.<sup>119</sup> Tumor-associated macrophages (TAMs) may have anti-inflammatory and pro-tumor (M1 phenotype) or pro-inflammatory and anti-tumor (M2 phenotype) states, depending on their strengths of interaction with other immune cells and cytokine expression. It has been shown that TAMs could induce activation of signal transducer and activator of transcription 3 (STAT3) *via* IL-6 and stimulate cytokine production that allows the proliferation of LCSCs, leading to a positive feedback loop that promotes LCSC self-renewal<sup>82</sup> [Fig. 2]. Cancer-associated fibroblasts (CAF) are the main component of tumor stromal cells. They displayed higher mRNA expression of TGFB1 and FAP after co-culturing with Huh7 and JHH-6 human HCC cells compared with non-tumor fibroblasts (NTF). These phenomena suggest that CAF and HCC interact and play a role in the maintenance and progression of liver disease.<sup>120</sup> In a hypoxic tumor microenvironment, the rate of tumor growth further affects the proportion of LCSCs in HCC. Under hypoxic conditions, hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) directly activates the transcription of artemin (ARTN). Hypoxia-induced ARTN subsequently promotes the expansion of LCSC populations *via* AKT signaling<sup>121,122</sup> [Fig. 2].



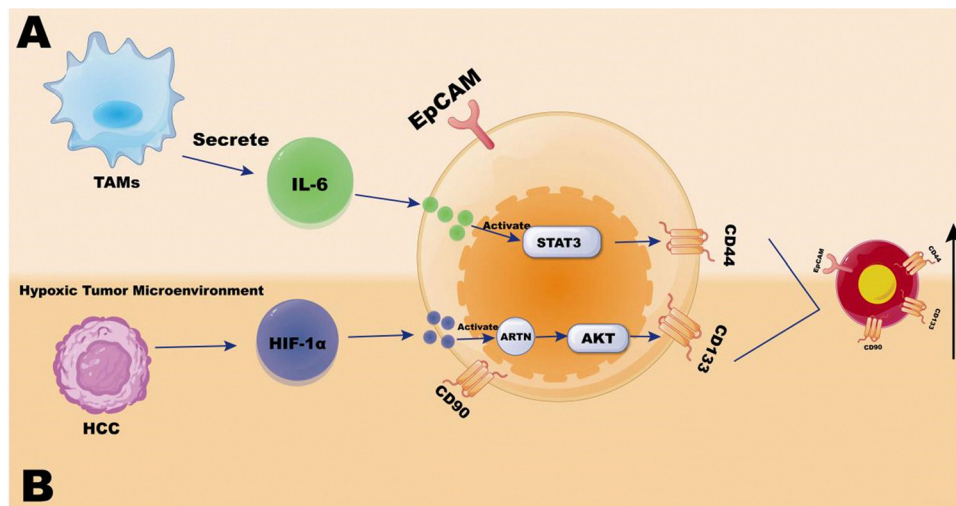


Fig. 2 Schematic diagram of the LCSC microenvironment. (A) TAMs can stimulate cytokine production through IL-6-induced activation of STAT3, which leads to the proliferation of LCSCs, thus forming a positive feedback loop that promotes the self-renewal of LCSCs. (B) Under hypoxic conditions, hypoxia-inducible HIF-1 $\alpha$  directly activates the transcription of ARTN. Hypoxia-induced ARTN subsequently promotes the expansion of LCSC populations via AKT signaling.

## 6. Targeting strategies for LCSCs

LCSCs do not exist in isolation; their maintenance of stemness, proliferative capacity, and development of chemoresistance are dynamically regulated by the tumor microenvironment (TME), which includes immune cells, cancer-associated fibroblasts, and hypoxic niches. This section will explore the composition of the LCSC microenvironment and its impact on LCSC properties, which reveal the interactions between the microenvironment, biomarker expression, and stemness maintenance to provide a basis for developing targeted strategies that take into account both the cells themselves and their microenvironment.

Despite the effectiveness of conventional therapies in eliminating large numbers of tumour cells, liver cancer patients continue to suffer from recurrence due to the presence of LCSCs. Nanotechnology offers new solutions for cancer treatment by engineering nanomedicines that can navigate the body in unique ways; therefore, it has been recognized as one of the most promising tools for the diagnosis and treatment of liver cancer.<sup>123</sup> As LCSCs undergo asymmetric division to form new stem cells or differentiated cells that repopulate tumour tissue, engineered nanomedicines with high bioavailability and low cytotoxicity can be designed to achieve the elimination of LCSCs by exposing them to high concentrations of the drug. Polymeric nanomaterials, bio-nanomaterials, and inorganic nanomaterials have all been used in the treatment of liver cancer in the last few decades.

Engineered nanomedicines target tumors in two main ways: passive targeting and active targeting.

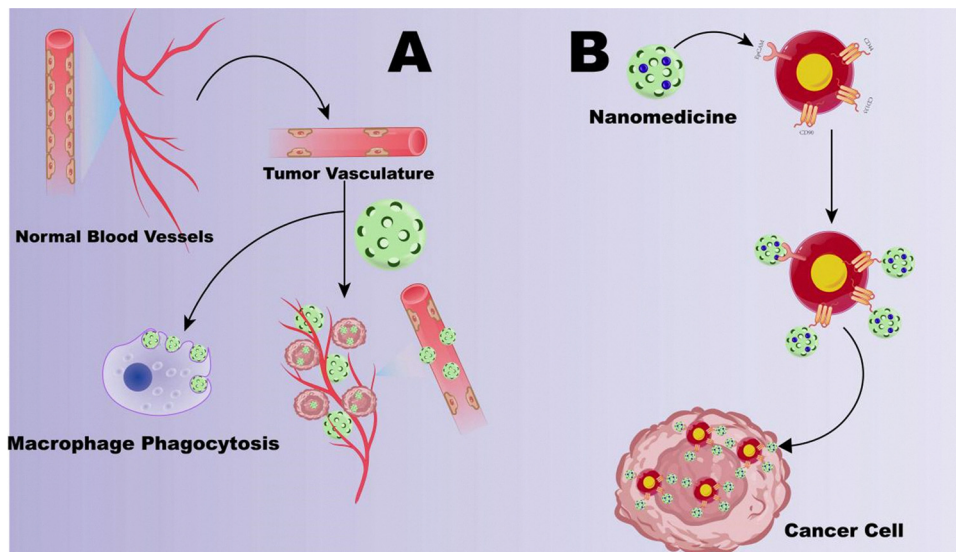
1. Passive targeting refers to the transfer of drug-carrying particles into the body or phagocytosis by macrophages, depending on the difference in vascular density and permeability between tumor tissue and normal tissue. Under pathological conditions, vascular endothelial growth factor (VEGF) is

overexpressed, and abnormal tumor angiogenesis occurs, resulting in large gaps between endothelial cells with pore sizes of 10–1000 nm.<sup>124</sup> The large gaps and poor lymphatic drainage in the tissue result in increased permeability and retention (EPR) effects, which allow nanomedicines to enter the tumor system.<sup>125</sup> The size, surface charge, and hydrophilic–hydrophobic properties of nanoparticles are core factors regulating the EPR effect. For instance, polyethylene glycol (PEG) modification can reduce the clearance of nanoparticles by the reticuloendothelial system (RES), prolong their blood circulation time, and thus enhance passive accumulation at tumor sites.<sup>126</sup>

2. Active targeting predominantly depends on specific ligand–receptor interactions to achieve selective cellular delivery. Typically, nanomedicines bind to biomarkers on the surface of LCSCs and the drug is then delivered to the cancer cells. This approach reduces cytotoxicity and the uptake of the drug by normal cells<sup>127</sup> [Fig. 3].

Liposomes modified with anti-CD133 monoclonal antibodies can significantly increase the cellular uptake rate of nanoparticles by recognizing CD133 receptors on the surface of LCSCs. In *in vitro* experiments, the killing efficiency against CD133+ LCSCs was 2.8 times higher than that of the unmodified group.<sup>128</sup> Similarly, aiming at another specific surface marker of LCSCs, nanodiamond carriers modified with CD44 aptamers can bind with high affinity to CD44 receptors on the surface of LCSCs, avoiding uptake by normal hepatocytes and exhibiting better tumor suppression effects in *in vivo* experiments.<sup>129</sup> Additionally, the synergistic application of passive and active targeting can further improve therapeutic efficacy. For example, studies have shown that PEGylation (to prolong circulation time, passive targeting) combined with folic acid or antibody fragments (active





**Fig. 3** Schematic diagram of targeting strategies for LCSCs. Engineered nanomedicines utilize two primary targeting strategies: passive and active targeting. (A) Passive targeting capitalizes on the pathological feature. VEGF overexpression-induced abnormal angiogenesis in tumors widens interendothelial gaps and, combined with impaired lymphatic drainage, enhances vascular permeability. This leads to an enhanced permeability and retention (EPR) effect, enabling nanomedicines to accumulate preferentially in tumor tissues *via* extravasation or macrophage-mediated uptake. (B) Active targeting involves specific ligand–receptor interactions for selective drug delivery. Ligand-functionalized nanomedicines target overexpressed surface biomarkers on LCSCs, ensuring precise tumor cell targeting and minimizing off-target cytotoxicity by reducing nonspecific uptake in healthy cells.

targeting) can increase the accumulation of nanoparticles at tumor sites.<sup>128,130</sup>

### 6.1. Engineered polymeric nanomedicines

Polymeric nanoparticles are sub-micron-sized colloidal particles that have shown their unique superiority in cancer treatment. Through complex surface modifications, polymeric nanoparticles could gain a specific ability to target tumors and deliver drugs to the tumor site, prolonging drug retention time and reducing cytotoxicity to normal cells. Recently, polymeric nanoparticles have shown outstanding advantages in the targeted treatment of LCSCs. Certain biocompatible polymers are used in constructing smart drug delivery systems for both LCSC and HCC targeting, such as poly(lactide-*co*-glycolide), polyethylene glycol, PLGA-PEG copolymer, PEG-polylysine copolymer, hyaluronic acid, and liposomes.

**6.1.1. Poly(lactide-*co*-glycolide) (PLGA).** Biodegradable polymers can be controlled and targeted in drug delivery.<sup>131</sup> PLGA (poly(lactic-*co*-glycolic acid)), a biodegradable and biocompatible copolymer, is commonly synthesized *via* two methods: (1) ring-opening polymerization (ROP) of lactide and glycolide and (2) polycondensation of lactic acid and glycolic acid monomers.<sup>132</sup> Due to its favorable properties, PLGA has been widely employed in biomedical applications and serves as a predominant carrier for drug-loaded nanoparticle preparation.<sup>133</sup> It is also known as a “smart polymer” because it stimulates sensitive behavior. Disulfiram (DS) is an anti-alcoholic drug that has shown very strong cytotoxicity in many cancer types. A PLGA-encapsulated DS system, DS-PLGA, was designed by Wang *et al.* The system significantly inhibited the population of CD133+ LCSCs upon

binding to copper. Moreover, its synergistic action with fluorouracil (5-FU) and sorafenib reduced the drug resistance of HCC.<sup>134</sup>

Such PLGA-based drug delivery systems (including DS-PLGA) show great potential in achieving precise control and targeting effects in the field of drug delivery. There are various strategies and mechanisms for achieving targeting. From the perspective of active targeting, numerous studies have focused on modifying targeting ligands on the surface of PLGA carriers to achieve specific recognition. By conjugating the cyclo(1,12)-ICAM-1-targeting cyclic peptide with polyethylene glycol-modified PLGA nanoparticles, these nanoparticles can rapidly bind to human umbilical vein endothelial cells (HUVECs) with upregulated intercellular adhesion molecule-1 (ICAM-1) expression after treatment with the proinflammatory cytokine interferon- $\gamma$ . This binding can be effectively blocked by free peptides, indicating that it is mediated by the specific interaction between the surface peptide and ICAM-1.<sup>135</sup> Modifying folic acid (FOA) onto PLGA-coated mesoporous silica nanoparticles (MSNs) enables the system (PLGA-FOA-MSNs) to target pancreatic cancer cells. Moreover, under the acidic pH (pH 6.8) of the tumor microenvironment, it can effectively control the release of the drug capecitabine. In *in vitro* MTT assays on pancreatic cancer cell lines Panc1 and MiaPaCa-2, the IC50 values of this system were 146.37  $\mu\text{g ml}^{-1}$  and 105.90  $\mu\text{g ml}^{-1}$ , respectively, demonstrating favorable targeted therapeutic effects.<sup>136</sup>

In terms of passive targeting, the high EPR effect of tumors is widely utilized. Tumor tissues have larger gaps between vascular endothelial cells and lack lymphatic drainage,



allowing PLGA nanoparticles (typically 10–200 nm in size) to penetrate through the vessel walls and accumulate in tumor sites. Nanoparticulate drug delivery systems benefitting from the EPR effect of tumor tissues can increase drug accumulation in tumors. However, this effect is limited to highly vascularized tumors and often fails to work in poorly perfused or hypoxic tumor regions.<sup>137</sup> Environment-responsive targeting cleverly leverages the special microenvironment of diseased sites to enhance targeting. Taking research related to the “PLGA-TK-PEG-COOH structure” as an example, by modifying the carboxyl group at the end of the PEG chain to conjugate targeting ligands, the PLGA-TK-PEG-COOH copolymer can achieve targeted delivery to specific cells or tissues. It contains reactive oxygen species (ROS)-sensitive ketal-thiol (TK) groups, which can trigger drug release in oxidative environments such as tumor tissues, significantly improving the selectivity of treatment. Studies indicate that the drug release kinetics from PLGA nanoparticles can be modulated by adjusting the lactic acid to glycolic acid (LA:GA) ratio. Furthermore, under the acidic conditions of the tumor microenvironment (TME), specially engineered PLGA carriers can undergo structural changes that enhance their binding affinity for tumor cells.<sup>138</sup> In summary, PLGA achieves targeting through multiple approaches such as active ligand modification, utilization of physiological characteristics, and response to microenvironments. The combined application of multiple strategies can further improve targeting accuracy and therapeutic effects, indicating extremely broad application prospects in the biomedical field, especially in drug delivery.

**6.1.2. Polyethylene glycol (PEG).** Since the 1950s, PEGs have been widely developed as separation and purification aids, embedded substrates, antifreeze agents, medical device lubricants, food additives, and carriers in dermatological applications, suppositories, injections, tablets, and pills.<sup>139</sup> PEG is a hydrophilic polymer that is widely used in the biomedical field due to its good water solubility, non-toxicity and non-immunogenicity. In a study, salinomycin (SAL) was loaded into PEG-ceramide nanomicelles to target LCSCs, and the results showed that the nanomicelles exhibited excellent cytotoxicity and enhanced apoptosis-inducing activity against LCSCs and HCC cells.<sup>140</sup>

In numerous studies, PEG modification has been widely applied in the biomedical field, especially its significant effects in targeting LCSCs. For example, PEGylated PLGA nanoparticles (PEGylated-PLGA-NPs) co-loaded with gemcitabine (Gem) and anti-miR-21 were prepared *via* a water-in-oil-in-water (w/o/w) double emulsion method. Experiments demonstrated that the cellular uptake of these nanoparticles in human hepatocellular carcinoma cells (Hep 3B and HepG2) increased in a time-dependent manner. Consistently, cell viability analysis indicated that the co-loaded nanoparticles exhibited a more significant inhibitory effect on hepatocellular carcinoma (HCC) cell proliferation than nanoparticles loaded with either Gem or anti-miR-21 alone at the same concentration. And this inhibitory effect was dose-dependent, with cell proliferation decreasing significantly as nanoparticle

concentration increased, leading to a marked improvement in therapeutic efficacy.<sup>141</sup>

Another experiment developed a folate-targeted, PEG-modified amphiphilic cyclodextrin nanoparticle for the safe, efficient, and specific delivery of melarsoprol (Mel) in the treatment of liver cancer. This targeted nanoformulation achieved cell-specific uptake in hepatocellular carcinoma cells, effectively exerting cytotoxicity, inducing apoptosis, and inhibiting cell migration.<sup>142</sup> Moreover, in an orthotopic tumor mouse model, the targeted nanoformulation significantly prolonged the survival time of mice without causing obvious signs of toxicity.

Studies have shown that a novel siRNA delivery system, cRGD-PSH-NP, based on modified polyethyleneimine (PSH) and DSPE-PEG2000-cRGD, was constructed. This system, loaded with survivin siRNA (cRGD-PSH-NP/S), exhibited superior gene-silencing and anti-tumor activities in HepG2 cells *in vitro* compared with unmodified nanoparticles. In *in vivo* experiments, after HepG2 tumor-bearing nude mice were treated with cRGD-PSH-NP/S, the tumor inhibition rate reached values as high as 74.71% without inducing obvious toxicity.<sup>143</sup>

**6.1.3. PLGA-PEG copolymer.** The development of PLGA-PEG copolymers has significantly enhanced drug delivery efficacy. Through structural modifications, these carriers can be engineered for multifunctional applications, including targeted delivery, stimulus-responsive release, and combination drug therapy. Owing to their biodegradable properties, PLGA-PEG copolymers are expected to play an increasingly important role in nanomedicine, potentially offering novel therapeutic strategies for disease treatment. Notably, core-shell nanostructures self-assembled from PLGA-PEG copolymers have demonstrated potential for delivering therapeutic agents to cancer stem cells (CSCs). This is particularly relevant as ABC transporter proteins – ubiquitous membrane-bound proteins – are closely associated with multidrug resistance (MDR) in CSCs.<sup>144</sup> This characteristic enables LCSCs to develop a MDR phenotype, significantly decreasing the intracellular retention of chemotherapeutic agents and resulting in poor treatment efficacy. Elacridar (ELC), an ABC transporter inhibitor, has been shown in several studies to effectively suppress ABC transporter activity, thereby enhancing the therapeutic performance of chemotherapeutic drugs.<sup>145</sup> Doxorubicin (DOX) is widely used in the treatment of advanced liver cancer. Chen *et al.* used poly(lactide-co-glycolide) (PLGA)/d-alpha-tocopherol polyethylene glycol 1000 succinate (TPGS) nanoparticles to achieve a synergistic combination of DOX and ELC. Based on *in vitro* studies and *in vivo* results in HepG2 xenograft mouse models, an optimal DOX/ELC ratio of 1 : 1 is used for optimal tumour targeting and inhibition of tumour growth. This design could overcome the MDR of LCSCs<sup>146</sup> [Fig. 4].

**6.1.4. PEG-polylysine copolymer.** The PEG-polylysine copolymer is an amphiphilic block copolymer composed of a hydrophilic polyethylene glycol (PEG) segment and a cationic polylysine chain. This unique structure enables versatile biomedical applications, including drug delivery and gene transfection, by leveraging PEG's stealth properties and polylysine's nucleic acid-binding capacity.<sup>147</sup> PEG-polylysine copolymers



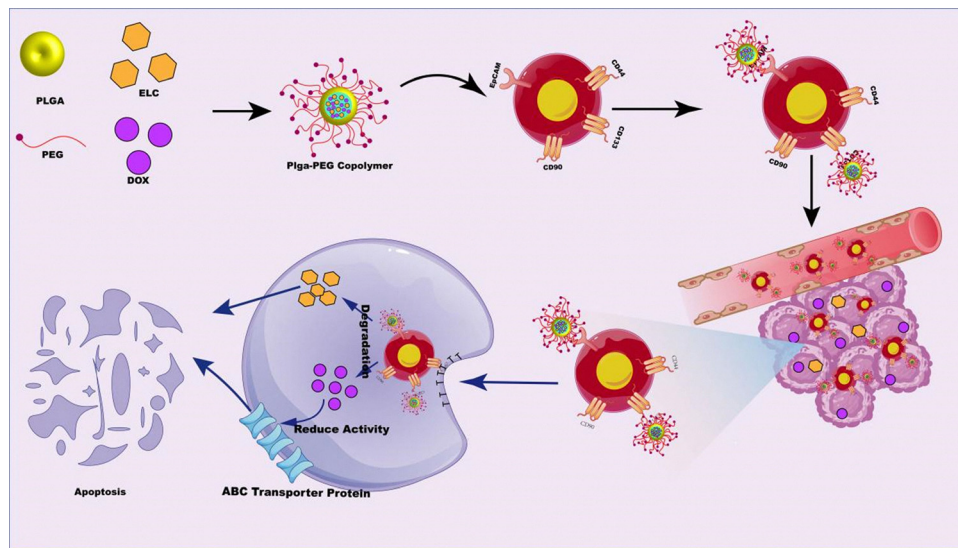


Fig. 4 Schematic diagram of the mechanism of targeting LCSCs by PLGA-PEG copolymer core-shell nanoparticles. The core of the PLGA-PEG copolymer is loaded with DOX and ELC, which bind to LCSCs and enter the cells via endocytosis. The acidic environment triggers the degradation of PLGA, releasing DOX and ELC, which inhibits the activity of ABC transporter proteins highly expressed on the surface of LCSCs and enhances the retention of DOX in the cells to induce apoptosis of the tumour cells.

are also used as drug delivery systems in anti-LCSCs. Amino-peptidase N (APN)/CD13 reduces reactive oxygen species (ROS) levels in LCSCs, inhibits their activity, and can sensitise LCSCs to chemotherapeutic agents.<sup>148</sup> Ubenimex competitively inhibits the APN/CD13 protease, and higher concentrations of ubenimex can lead to the death of LCSCs.<sup>149,150</sup> Therefore, one study used ubenimex as a therapeutic agent and developed a polyethylene glycol-polylysine block copolymer – ubenimex, which showed good anti-LCSC effects. In *in vivo* experiments, this preparation was more effective compared to polyethylene glycol-block-polylysine or phosphate-buffered saline.<sup>151</sup>

**6.1.5. Hyaluronic acid (HA).** HA is an anionic, non-sulphated glycosaminoglycan found in connective, epithelial, and neural tissues, which is also an important component of the ecological niche of stem cells and CSCs.<sup>152</sup> Especially in malignant tumours, HA concentrations are high. Li *et al.* found that HA mediates CD44 to promote tumour development. HA-based multilayers were used to create continuous changes in surface properties, and they simulated the ecological niches of LCSCs. Colony formation was observed on a series of poly-(allylamine hydrochloride) PAH/HA multilayers, which showed that the expression of CD133/CD44 double-positive LCSCs was upregulated to approximately 70% after 7 days of incubation.<sup>153</sup> In a later study, they demonstrated that dual-frequency low-intensity ultrasound (LIUS) induced colony differentiation in LCSCs and observed a decrease in the percentage of CD133/CD44 double-positive LCSCs and a significant difference in LCSC-related proteins after 5 cycles of LIUS stimulation.<sup>154</sup>

Polymer nanoparticles are ideal vehicles for targeting LCSCs due to their biodegradability, biocompatibility, and stability. However, nanoparticle aggregation and toxic reactions are not a negligible problem, so there is still a long way to go to bring polymeric nanoparticles to the clinic.<sup>155</sup>

**6.1.6. Liposomes.** Liposomes have a biofilm-like structure, possess superior biocompatibility, and are increasingly valuable in drug development. Hybrid liposomes (HLs) were obtained by Kosuke *et al.* using ultrasound treatment for their preparation. *In vitro* studies demonstrated that HLs induced apoptosis in HepG2 cells by activating caspase-3 and, more notably, significantly reduced the CD133(+)/EpcAM (+) LCSC subpopulations. Notably, DOX acting on HepG2 cells showed an increase in the number of LCSC subpopulations, exhibiting chemoresistance, and conversely, the number of LCSC subpopulations decreased in a dose-dependent manner in response to HLs, suggesting that HLs selectively inhibit LCSC subpopulations in HepG2 cells.<sup>156</sup> Recently, there has been a trend towards loading precursor drugs in a way that stimulates a response drug delivery system.<sup>157</sup> It has been shown that liposome-binding peptides can promote drug accumulation at tumour sites and enhance the sustained effect of drugs on tumours.<sup>158</sup> Wang *et al.* developed a redox-triggered dual-targeted liposome, CEP-LP@S/D, to co-deliver DOX and SAL to liver cancer sites. This system is equipped with a ligand, CEP, that binds to CD133 and EpcAM targeting peptides, allowing selective targeting of CD133+ EpcAM+ LCSCs. Upon arrival at LCSCs, CEP-LP@S/D liposomes undergo cytoplasmic endocytosis in which high concentrations of glutathione (GSH) break their disulfide bonds, thereby degrading the liposomes and releasing the drug.<sup>128</sup> A research team developed an ultrasound-responsive doxorubicin liposome (LID) that achieves targeted delivery through surface modification with the photosensitizer indocyanine green (ICG), combined with local ultrasound to trigger drug release. In the HepG2 tumor-bearing mouse model, LID combined with ultrasound treatment reduced tumor volume by 72%, and the proportion of CD133+ LCSCs decreased from 18% to 4.1%. Mechanistically,



LID activates the host cGAS–STING pathway by inducing mitochondrial DNA release to enhance anti-tumor immune responses, while significantly reducing cardiotoxicity.<sup>159</sup>

#### 6.1.7. Effects of different surface modifications on carrier targeting ability, drug release properties, and *in vivo* behavior.

In recent years, numerous studies have focused on surface modification of nanoparticles such as poly(lactic-*co*-glycolic acid) (PLGA), polyethylene glycol (PEG), hyaluronic acid (HA), and liposomes to enhance the selective uptake by liver cancer stem cells (LCSCs) and achieve controlled drug loading.

For PLGA nanoparticles, research has shown that coating PLGA nanoparticles with materials like PEG results in modified nanoparticles exhibiting different properties from unmodified ones in terms of stability and interactions with cells.<sup>160</sup> In the context of targeting LCSCs, similar surface modification strategies are equally applicable. For example, in another cancer therapy-related study, surface-modified PLGA nanoparticles could improve the efficiency of drug delivery to cancer cells. They can be designed to carry drugs and undergo specific interactions with LCSCs. Some surface-modified PLGA nanoparticles are capable of protecting the encapsulated drugs from premature release in the bloodstream, releasing them in a controlled manner only when approaching LCSCs. This is achieved through the design of polymer structures and the selection of surface-modified ligands.

As a hydrophilic polymer, PEG has been widely used in the modification of nanoparticles. PEG modification can improve the stability of nanoparticles, extend their circulation half-life, and enhance their ability to penetrate cells. When applied to nanoparticles targeting LCSCs, PEG modification can reduce non-specific adsorption of nanoparticles to normal cells, increase their circulation time *in vivo*, and promote their accumulation at tumor sites. For instance, PEG-modified nanoparticles can avoid rapid clearance by the immune system, thereby increasing the chance of reaching LCSCs. Meanwhile, PEG can be further conjugated with targeting ligands to achieve more specific delivery to LCSCs.<sup>161,162</sup>

Hyaluronic acid (HA) also shows great potential in modifying nanoparticles targeting LCSCs. Studies have demonstrated that HA-coated hybrid nanoparticles can target breast cancer stem cells through the interaction between HA and CD44 receptors on the cell membrane. Similarly, for LCSCs that also overexpress CD44 receptors on their surface, HA-modified nanoparticles can specifically recognize and bind to LCSCs.<sup>163</sup> As described, HA-based nanocarriers can be designed to respond to the unique microenvironment of liver cancer cells (such as the presence of hyaluronidase), enabling site-specific and controlled drug release, which is beneficial for the selective killing of LCSCs.<sup>164</sup>

Surface-modified liposomes can also play a role in targeting LCSCs. Although some current studies are not directly related to LCSCs, the general principle holds that surface-modified liposomes can improve both drug encapsulation efficiency and delivery specificity. By modifying the surface of liposomes with ligands (such as certain peptides or antibodies) that have affinity for LCSCs, liposomes can selectively deliver drugs to

LCSCs. They can encapsulate drugs in their lipid bilayer structure and control drug release through the design of lipid composition and surface modification. For example, pH-sensitive liposome modifications can trigger drug release in the acidic microenvironment of tumor tissues where LCSCs reside.

In summary, surface modification of nanoparticles such as PLGA, PEG, HA, and liposomes can effectively enhance the selective uptake of LCSCs and achieve controlled drug loading, providing new strategies and methods for liver cancer therapy targeting LCSCs.

## 6.2. Engineered biological nanomedicines

Biological nanomaterials are usually biocompatible, biodegradable, non-toxic, and non-immunogenic, and are abundant in nature. Engineered biomaterials have functional groups that can be easily modified to bind to drug molecules. In addition, they have specific target sites that help to deliver signals to the target. Thus, engineered bio-nanomaterials are attractive and promising as drug carriers. In recent years, bio-nanomaterials have been used extensively in the treatment of LCSCs, and they can act as both drugs and carrier materials, such as exosomes, monoclonal antibodies, and aptamers.

**6.2.1. Exosomes.** Most human cells produce a lipid membrane-encapsulated vesicle that can be secreted outside the cell and is called an extracellular vesicle.<sup>165</sup> Depending on the biogenesis pathway and diameter, extracellular vesicles can be subdivided into microvesicles, exosomes, and apoptotic vesicles.<sup>166</sup> The production, secretion, transport, uptake, and release of exosomes are regulated by specific signals and are an energy-consuming mechanism to maintain intracellular homeostasis. A growing number of laboratory and clinical studies have shown that abnormal secretion and function of exosomes are associated with the development of malignant tumors and that exosomes also play a huge role in tumor therapy.<sup>167</sup>

Regorafenib is a multi-kinase inhibitor for second-line targeted therapy in HCC.<sup>168</sup> RAB27A is a Rab GTPase that controls the release of exosomes from LCSCs.<sup>169</sup> Huang *et al.* demonstrated that RAB27A regulates exosome secretion in LCSCs, thereby maintaining their stem-like properties and resistance to regorafenib treatment.<sup>170</sup> In addition, in non-CSCs, exosomes secreted by LCSCs upregulated the expression of Nanog, which is a key factor contributing to the self-renewal of LCSCs. Therefore, RAB27A expression in HCC tissue is closely associated with Nanog, and LCSCs release exosomes in an RAB27A-dependent manner to induce Nanog expression and regorafenib resistance in differentiated cells. These findings suggest a potential therapeutic strategy for HCC by targeting exosome-mediated signaling among LCSC subpopulations.<sup>170</sup>

Mesenchymal stem cells (MSCs) are often used as a source of cell therapy because of their powerful immunosuppressive and regenerative functions, and their unique tumourphilic properties make them excellent candidates for targeting cancer cells.<sup>171–173</sup> Gu *et al.* investigated the role of MSC-derived exosomes on LCSCs. The results showed that the proliferation, migration, invasion, angiogenesis, and self-renewal capacity of



LCSCs treated with exosomes were significantly reduced. Furthermore, exosomes suppressed the malignant behavior of LCSCs through the C5orf66-AS1/miR-127-3p/DUSP1/ERK signaling axis.<sup>174</sup>

Although exosomes are closely associated with LCSCs, they also have unique advantages as natural carriers. With their biocompatibility, low immunogenicity, long cycle time, and high loading capacity, exosomes are now used as nanocarriers for drug and gene delivery.<sup>175</sup> In LCSCs, EpCAM expression is transcriptionally regulated by Wnt/ $\beta$ -catenin signalling, and inhibition of Wnt/ $\beta$ -catenin signalling has the potential to eliminate EpCAM+ LCSCs.<sup>175</sup> In a new therapeutic approach, RNA can be used to identify a target and then regulate gene expression.<sup>176,177</sup> In one study, synthetic oligonucleotide RNA aptamers that bind specifically to EpCAM and small interfering RNA (siRNA) targeting  $\beta$ -catenin were loaded into exosomes to develop a therapeutic biological nanoparticle.<sup>178</sup> *In vitro* and *in vivo* results show that this bio-nanoparticle has a good targeting effect on LCSCs. Thus, it seems that the application of RNA nanotechnology to design exosome carriers could provide a strategy for cellular biomarker-mediated targeting therapy.<sup>178</sup>

**6.2.2. Monoclonal antibodies (mAbs).** Monoclonal antibodies are highly homogeneous antibodies produced by a single B cell and directed against only one specific antigenic epitope. Monoclonal antibodies (mAbs) exhibit high target antigen specificity and low toxicity, making them promising therapeutic agents for CSC eradication. For decades, several mAbs have been successfully used in the treatment of cancer in clinical patients, such as VEGF antagonists bevacizumab for colorectal cancer, rituximab for lymphoma, ramucirumab for HCC, and so on.<sup>179</sup> The mechanism based on mAbs targeting CSCs mainly uses the host's immune system to activate humoral and cellular immunity to eliminate target cells, for example, antibody-dependent cellular cytotoxicity (ADCC). mAbs targeting CSCs and cancer cells constitute a complex therapeutic approach that is largely supported by the host immune system.<sup>180</sup>

Biomarkers that are highly expressed in LCSCs but absent or at very low levels in normal cells can be excellent target antigens for mAbs.<sup>181</sup> In a preclinical study, EpCAM/CD3 bispecific antibodies induced strong peripheral monocyte-dependent toxicity *in vivo* and *in vitro*, which in turn eliminated cancer cells in HCC.<sup>150</sup> Huang *et al.* found that cytokine-induced killing (CIK) cells conjugated with anti-CD3/anti-CD133 bispecific antibodies were able to effectively target and kill CD133+ LCSCs *in vitro* and *in vivo*.<sup>182</sup> However, this mAb therapy for LCSCs needs to be validated in numerous preclinical and clinical trials [Table 2].

**6.2.3. Aptamers.** Aptamers are short single-stranded oligonucleotides that fold into a unique tertiary structure.<sup>183</sup> Aptamers exhibit high affinity for their homologous targets and can bind specifically to them; this is why they are also known as chemical antibodies. Aptamers, which are 15–20 times smaller than antibodies, possess several advantages over conventional antibodies, including enhanced tumor penetration and

diffusion capacity due to their small molecular size.<sup>184</sup> Aptamers are frequently engineered into various therapeutic platforms, including aptamer–drug conjugates, aptamer-functionalized nanoparticles, and aptamer-modified liposomal nanocarriers, for applications in gene therapy, immunotherapy, and biotherapeutics. Although adenovirus serotype 5 expressing PTEN (Ad5-PTEN) demonstrates potent antitumor activity against hepatocellular carcinoma, its clinical application is constrained by inherent immunogenicity and nonspecific toxicity.<sup>185</sup> Xiao *et al.* prepared EpDT3-PEG-Ad5-PTEN (EPAP) by linking the RNA aptamer (EpDT3) to Ad5-PTEN with PEG by simple chemical synthesis.<sup>186</sup> This system reduced the non-specific toxicity of Ad5-PTEN and was more stable in human serum. EPAP was precisely targeted to LCSCs *via* EpCAM and could induce apoptosis in LCSCs, which had a significant inhibitory effect on the proliferation of cancer cells.<sup>187</sup> Aptamer-coupled drugs can improve the efficiency of chemotherapeutic drugs targeting tumours and prolong the drug's residence time. The CD133–apt–Dox system was designed by associating an RNA aptamer targeting CD133 with DOX.<sup>108</sup> CD133–apt–Dox inhibited the expression of several genes in LCSCs, prevented their metastasis and differentiation, and suppressed autophagy in HCC cells. The aptamer delivery system provides a valuable platform for targeting LCSCs.<sup>108</sup>

Although biological nanomaterials exhibit advantageous properties, including excellent biocompatibility, biodegradability, and low toxicity, their separation and purification currently pose significant technical challenges. In addition, exosomes carry biomolecules that stimulate tumor growth and metastasis, and more research is needed to identify and remove them. The pharmacokinetics of aptamers and antibodies are suboptimal, and selection techniques are complex.<sup>188</sup> Some of these obstacles need to be overcome through advances in RNA chemistry, biology, bioinformatics, manufacturing, and nanotechnology.

### 6.3. Engineered inorganic nanomedicines

Inorganic nanoparticles are nanocarriers synthesized from metallic and semi-metallic materials. Due to the advantages of easily scalable synthesis, targeted molecular modification, and high stability, inorganic nanoparticles are now being studied as carriers for delivering chemotherapeutic drugs. Inorganic nanoparticles, such as metal nanoparticles (gold, silver, zinc oxide), silicon dioxide nanoparticles (SiNPs), arsenic trioxide nanoparticles (AtoNPs), and nanodiamonds (NDs), are widely studied in the treatment of LCSCs.

**6.3.1. Gold nanorods.** Gold nanorods (GNRs) have a special geometry that is valuable in imaging, therapeutic, and biosensing applications.<sup>189</sup> GNRs also hold promise as carrier materials for a wide range of applications in cancer therapy. Erica *et al.* developed a drug delivery system, Adr/GNRs@PMS-antiEpCAM, that specifically targets EpCAM+ LCSCs by loading Adriamycin (Adr) and GNRs onto a PLGA-*b*-PEG copolymer vector with EpCAM antibody modification on the surface.<sup>190</sup> *In vitro* data show that Adr/GNRs@PMS-antiEpCAM effectively kills spheroid-assay-enriched LCSCs. In addition, the increased local Adr



Table 2 Clinical trials of different monoclonal antibodies in the treatment of liver cancer

Study title	NCT number	Interventions	Conditions	Phases
Floxuridine and dexamethasone as a hepatic arterial infusion and bevacizumab in treating patients with primary liver cancer that cannot be removed by surgery	NCT00410956	Biological: cixutumumab Procedure: computed tomography contrast-enhanced magnetic resonance imaging	Adult primary hepatocellular carcinoma advanced adult primary liver cancer localized unresectable adult primary liver cancer recurrent adult primary liver cancer	Phase 2
Dose finding study of AVE1642 in patients with advanced or metastatic liver carcinoma	NCT00791544	Drug: AVE1642 Sorafenib	Liver carcinoma	Phase 1 Phase 2
Bevacizumab and erlotinib in treating patients with advanced liver cancer	NCT00365391	Biological: bevacizumab Drug: erlotinib hydrochloride	Adult primary hepatocellular carcinoma Advanced adult primary liver cancer Localized unresectable adult primary liver cancer Recurrent adult primary liver cancer	Phase 2
Cixutumumab and sorafenib tosylate in treating patients with advanced liver cancer	NCT00365391	Biological: cixutumumab Other: laboratory bio-marker analysis Drug: sorafenib tosylate	Adult hepatocellular carcinoma Advanced adult hepatocellular carcinoma Localized non-resectable adult liver carcinoma Recurrent adult liver carcinoma	Phase 1
Study of IMC-1121B (ramucirumab) in participants with liver cancer who have not previously been treated with chemotherapy IRX-2, cyclophosphamide, and nivolumab in treating patients with recurrent or metastatic and refractory liver cancer	NCT00627042	Biological: cixutumumab Biological: ramucirumab (IMC-1121B)	Hepatocellular carcinoma	Phase 2
	NCT03655002	Drug: cyclophosphamide Biological: cytokine-based biologic agent IRX-2 nivolumab	Recurrent hepatocellular carcinoma Refractory liver carcinoma Stage IV/stage IVA/stage IVB hepatocellular carcinoma AJCC v8	Phase 1
Atezolizumab and bevacizumab before surgery for the treatment of resectable liver cancer	NCT04721132	Biological: atezolizumab bevacizumab Procedure: therapeutic conventional surgery	Resectable hepatocellular carcinoma Stage I hepatocellular carcinoma AJCC v8 Stage IA/stage IB/stage II/hepatocellular carcinoma AJCC v8	Phase 2
Sorafenib tosylate and pembrolizumab in treating patients with advanced or metastatic liver cancer	NCT03211416	Drug: cyclophosphamide Biological: cytokine-based biologic agent IRX-2 nivolumab	Recurrent hepatocellular carcinoma Refractory liver carcinoma Stage IV/stage IVA/stage IVB hepatocellular carcinoma AJCC v8	Phase 1
Pembrolizumab with or without elbasvir/grazoprevir and ribavirin in treating patients with advanced refractory liver cancer	NCT02940496	Drug: elbasvir/grazoprevir Ribavirin Other: laboratory bio-marker analysis Biological: pembrolizumab	Stage B/stage C hepatocellular carcinoma Refractory liver cancer Stage III hepatocellular carcinoma AJCC v7	Phase 2
BO-112 and pembrolizumab for the treatment of PD-1/PD-L1 refractory liver cancer	NCT04777708	Biological: nanoplexed poly I: C BO-112 Pembrolizumab	Advanced hepatocellular carcinoma BCLC stage B/BCLC stage C hepatocellular carcinoma Refractory hepatocellular carcinoma Metastatic hepatocellular carcinoma	Early Phase 1
BMS-986205 and nivolumab as first or second line therapy in treating patients with liver cancer	NCT03695250	Drug: IDO1 inhibitor BMS-986205 Biological: nivolumab	Stage III hepatocellular carcinoma AJCC v8	Phase 2
Lenvatinib combined pembrolizumab in advanced hepatobiliary tumors	NCT 03895970	Lenvatinib plus pembrolizumab	Liver neoplasm malignant primary Cholangiocarcinoma Biomarker	Phase 2
HAIC in combination with PD-1 inhibitors and lenvatinib for high tumor burden advanced HCC (CHANCE2416)	NCT 06631326	Procedure: hepatic artery infusion chemotherapy Drug: lenvatinib + PD-1 monoclonal antibody	HCC – hepatocellular carcinoma HCC – hepatocellular carcinoma BCLC stage C hepatocellular carcinoma lenvatinib PD-1 inhibitors	Phase 1
Zanzalintinib (XL-092) plus durvalumab and tremelimumab in unresectable hepatocellular carcinoma (ZENOBIA) (ZENOBIA)	NCT06698250	Drug: zanzalintinib Durvalumab Tremelimumab	Hepatocellular carcinoma	Phase 2
A study evaluating the efficacy and safety of multiple immunotherapy-based treatment combinations in patients with advanced liver cancers (Morpheus-liver) (Morpheus-liver)	NCT04524871	Drug: atezolizumab bevacizumab 15 mg kg <sup>-1</sup> tiragolumab Tocilizumab TPST-1120 Tobemstomig 2100 mg Bevacizumab 10 mg kg <sup>-1</sup> Tobemstomig 600 mg Tobemstomig 1200 mg	Advanced liver cancers	Phase 1 Phase 2



Table 2 (continued)

Study title	NCT number	Interventions	Conditions	Phases
SBRT + PD-1 monoclonal antibody in unresectable colorectal liver metastases (SPARKLE-L)	NCT06794086	ADG126 IO-108 1800 mg NKT2152	Colorectal cancer, liver metastases	Phase 3
		Drug: IO-108 1200 mg Radiation: stereotactic body radiation therapy Drug: PD-1 monoclonal antibody chemotherapy		

concentration in this system will increase the efficacy of tumour cell killing.<sup>190</sup> Studies have shown that CD133-targeted gold nanoparticles (Au-PEG-CD133-CB-839) can co-deliver the glutaminase inhibitor telaglenastat (CB-839) and chemotherapeutic drugs. In a human hepatocellular carcinoma PDX model, after tail vein injection of this formulation, the concentration of CB-839 at the tumor site was 6.8 times that of the free drug, and the sphere formation rate of LCSCs decreased from 38% to 7%. After 4 weeks of treatment, the tumor weight was reduced by 59% compared to the control group, with no obvious liver or kidney function damage observed.<sup>191</sup>

**6.3.2. Silicon dioxide nanoparticles.** The use of silica as a drug and gene carrier has gradually increased in frequency over the last decade.<sup>192</sup> Mesoporous silica (MS) has tunable size, a large surface area, and porosity, making it suitable for delivery to LCSCs.<sup>193</sup> The ABCG2 protein largely contributes to drug resistance in CSCs.<sup>194</sup> Short hairpin RNA (shRNA) for ABCG2 gene silencing is a promising therapeutic approach to overcome drug resistance.<sup>195</sup> Bioresponsive functionalized mesoporous silica nanoparticles (MSNs) were employed for the co-delivery of DOX and short hairpin ABCG2 (shABCG2) to LCSCs in both *in vitro* and *in vivo* models.<sup>196</sup> This approach effectively targeted LCSCs and prevented the premature release of DOX. The coexistence of DOX and shABCG2 may have synergistic cytotoxic effects on LCSCs, thereby improving their drug resistance. Blocking differentiation and self-renewal is also a method to eliminate LCSCs.<sup>197</sup> Hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ), a target of HCC differentiation therapy, is a transcription factor responsible for maintaining the differentiated state and functional activity of hepatocytes.<sup>198</sup> Cisplatin is a popular chemotherapeutic agent. Cai *et al.* used polyethyleneimine-modified mesoporous silica nanoparticles (PMSN) as delivery vehicles to deliver the gene encoding HNF4 $\alpha$  and cisplatin for differentiation therapy.<sup>199</sup> The results showed that the dual delivery of the HNF4 $\alpha$ -encoding plasmid and the drug cisplatin could target LCSCs, induce apoptosis, and inhibit tumor growth in hepatocellular carcinoma cells. Importantly, HNF4 $\alpha$  down-regulated stemness-related genes and up-regulated hepatocyte-specific genes.

**6.3.3. Arsenic trioxide (ATO) nanoparticles.** Arsenic trioxide (ATO) is a first-line therapeutic agent for treating acute promyelocytic leukaemia (APL) and has been reported to be effective in the treatment of breast cancer, prostate cancer, and HCC.<sup>200,201</sup> ATO can inhibit stemness markers, affect epithelial-mesenchymal transition (EMT), and induce differentiation

of CD133+ LCSCs. However, ATO has the disadvantage of poor bioavailability and significant adverse effects.<sup>202,203</sup> Therefore, there is a need to explore new strategies to enhance the bioavailability of ATO; for example, combining ATO with nanomaterials is a promising option. In one study, Huang *et al.* prepared ATO-based nanoparticles, ZnAs@SiO<sub>2</sub> NPs, using a “one-pot” inverse emulsification method and found that ZnAs@SiO<sub>2</sub> NPs inhibited apoptosis, migration, and invasion of HCC cells and significantly inhibited CD133+ LCSCs.<sup>204</sup> ZnAs@SiO<sub>2</sub> NPs inhibited LCSCs’ stemness and EMT by modulating the SHP-1/JAK2/STAT3 signaling pathway.

**6.3.4. Nanodiamonds (NDs).** Nanodiamonds (NDs) have a truncated semi-octahedral carbon structure with a diameter of approximately 5 nm. They have a narrow size distribution, simple surface functionalisation, excellent mechanical properties, and biocompatibility.<sup>205</sup> These properties allow NDs to demonstrate great potential as a drug delivery platform against drug-resistant tumour cells. The anthracycline epirubicin is a therapeutic agent for HCC, but epirubicin is recognized and excreted by ABC transporter proteins in HCC therapy, which makes it resistant to epirubicin.<sup>206,207</sup> Wang *et al.* developed a nanodiamond–epirubicin drug complex (EPND) to demonstrate an effect on LCSCs.<sup>208</sup> The results showed that EPND targeted tumour-specific cells. When EPND and single-agent epirubicin were compared after 48 hours of treatment of LCSCs, epirubicin treatment resulted in a significant increase in LCSCs. In contrast, EPND treatment resulted in a significant decrease in the percentage of LCSCs.

Inorganic nanoparticles have gained significant attention in diagnostic and imaging applications due to their tunable size and facile surface modification. However, their clinical translation is hindered by inherent limitations, including slow degradation rates and potential toxicity.

## 7. Limitations and challenges

Although nanomedicines have shown potential advantages in targeting liver cancer stem cells (LCSCs) for the treatment of hepatocellular carcinoma (HCC), bringing new hope for overcoming this malignant tumor, numerous limitations and challenges need to be addressed in the process of translating basic research into clinical applications, as revealed in several recent studies.



### 7.1. Biological barriers: tumor microenvironment and LCSC heterogeneity

The complex tumor microenvironment (TME) of HCC is like a solid fortress, seriously hindering the efficacy of nanomedicines. Among them, the excessive accumulation of hyaluronic acid in the extracellular matrix (ECM) significantly alters the physical properties of the TME. Studies have pointed out that high concentrations of hyaluronic acid increase the interstitial fluid pressure and form a dense network structure, which blocks the diffusion path of nanomedicines, greatly reduces their penetration and distribution efficiency in tumors, and severely impairs their targeting effect on LCSCs.<sup>209</sup>

In addition, hypoxic regions are widespread in the TME, which significantly affect the biological behavior of LCSCs. Studies have shown that high expression of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) can activate stemness-related signaling pathways in LCSCs and enhance their self-renewal and drug resistance capabilities. This heightened resistance makes it difficult for conventional nanomedicines to effectively eradicate LCSCs, resulting in a high risk of tumor recurrence and metastasis.<sup>210</sup>

The high heterogeneity of LCSCs themselves is also a major challenge in treatment. Different subpopulations of LCSCs differ in the expression of surface markers (such as CD133, EpCAM, CD44, *etc.*) and have different functional characteristics. As mentioned in some studies, nanomedicines targeting a single marker can only act on some LCSC subpopulations, making it difficult to achieve complete clearance of cancer stem cells, which poses hidden dangers for tumor recurrence.<sup>211</sup>

### 7.2. Nanoparticle-related limitations

The biological distribution and clearance of nanomedicines *in vivo* are difficult to precisely regulate. Most nanoparticles are easily recognized and taken up by the reticuloendothelial system (RES) quickly, especially macrophages in the liver and spleen, resulting in a large number of nanomedicines being cleared before reaching the tumor site. Unmodified poly(lactico-glycolic acid) (PLGA) nanoparticles have a short half-life in mice, and most of them are taken up by liver non-parenchymal cells, with only a small amount accumulating in tumor tissues, which seriously affects the therapeutic effect.<sup>212</sup>

The potential toxicity of nanoparticles cannot be ignored. Some inorganic nanomaterials (such as titanium dioxide, carbon nanotubes, *etc.*) are metabolized slowly in the body, and long-term accumulation may cause cytotoxicity, inflammatory reactions, and even genotoxicity. Summarizing multiple *in vivo* studies, it is pointed out that carbon nanotubes can cause adverse reactions such as pulmonary inflammation and liver and kidney damage. Although they are rarely used in HCC treatment, they sound an alarm for the safety of nanomedicines.<sup>213</sup> Even organic nanocarriers like liposomes and polymeric micelles may have adverse effects, as their components or degradation products can cause immune reactions and disrupt normal cell metabolism.

### 7.3. Bottlenecks in clinical translation and large-scale production

The clinical translation of nanomedicines faces challenges in large-scale production and quality uniformity. Laboratory-prepared nanoparticles often rely on precision instruments and complex processes, such as the controlled synthesis of mesoporous silica or the precise modification of exosomes, which are difficult to scale up in a standardized manner. In addition, different batches of nanomedicines may differ in particle size distribution, surface charge, and drug loading rate, which directly affect the stability of their *in vivo* pharmacokinetics and therapeutic effects.<sup>214,215</sup> At the same time, long-term safety data of nanomedicines are lacking; particularly, the accumulation effect of inorganic nanoparticles in the body may cause chronic organ damage, which needs to be verified through long-term animal experiments and preclinical toxicity evaluation.<sup>216</sup>

## 8. Conclusions

LCSCs have been demystified in the past decade, but this is still only the tip of the iceberg for LCSCs that are implicated in the occurrence, metastasis, drug resistance, and relapse of HCC and can be diagnosed and prognosticated by biomarkers on their surfaces, such as EpCAM and CD44. LCSCs are a class of self-renewing, tumorigenic cell populations, so there is a strong need for targeted therapy. Currently, nanotechnology has an enormous potential for application in the treatment of LCSCs. Nanotechnology offers targeting strategies for LCSCs that can increase cellular uptake of drugs, improve biodistribution, and increase drug stability. Currently, three main classes of nanoparticles have been investigated for targeting LCSCs: polymeric nanoparticles, biological nanoparticles, and inorganic nanoparticles. In particular, as natural nanomaterials, biological nanoparticles show certain advantages. For instance, cell-secreted exosomes contain diverse molecular components that mediate intercellular and cell-tissue communication. Modified exosomes can directly target tumor cells to inhibit tumor growth. However, the application of nanotechnology in LCSCs is still somewhat in its infancy, and more efforts are needed to promote the application of nanotechnology in this area. To achieve this, we need to understand the microenvironmental biology of LCSCs as well as their stemness properties and master their epigenetic characteristics. In terms of drugs, better biocompatible drug delivery systems with high drug delivery capacity should be developed to focus on targeting LCSCs.

This article mainly describes the origin and characteristics of LCSCs, describes the relationship between LCSCs and HCC, and summarizes the nanoparticles used to target LCSCs in recent years.

## Author contributions

Fenglan Huang: writing – review & editing, writing – original draft, visualization, methodology, conceptualization. Li Chen:



writing – review & editing, writing – original draft, visualization. Xin Zhang: writing – review & editing, writing – original draft. Shengqian Tian: writing – review & editing, writing – original draft. Yuxin Han: writing – review & editing, resources, methodology, conceptualization. Minghui Hu: writing – review & editing, supervision, methodology, conceptualization. Lili He: writing – review & editing, supervision, methodology, conceptualization. Rong Luo: writing – review & editing, writing – original draft, visualization, supervision, methodology, conceptualization.

## Conflicts of interest

There are no conflicts to declare.

## Data availability

The authors declare that no data were generated in this review article.

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