BioDrugs (2025) 39:237–260
https://doi.org/10.1007/s40259-024-00702-0

REVIEW ARTICLE

State-of-the-Art Liver Cancer Organoids: Modeling Cancer Stem Cell Heterogeneity for Personalized Treatment

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Accepted: 21 December 2024 / Published online: 18 January 2025

Abstract

Liver cancer poses a global health challenge with limited therapeutic options. Notably, the limited success of current therapies in patients with primary liver cancers (PLCs) may be attributed to the high heterogeneity of both hepatocellular carcinoma (HCCs) and intrahepatic cholangiocarcinoma (iCCAs). This heterogeneity evolves over time as tumor-initiating stem cells, or cancer stem cells (CSCs), undergo (epi)genetic alterations or encounter microenvironmental changes within the tumor microenvironment. These modifications enable CSCs to exhibit plasticity, differentiating into various resistant tumor cell types. Addressing this challenge requires urgent efforts to develop personalized treatments guided by biomarkers, with a specific focus on targeting CSCs. The lack of effective precision treatments for PLCs is partly due to the scarcity of ex vivo preclinical models that accurately capture the complexity of CSC-related tumors and can predict therapeutic responses. Fortunately, recent advancements in the establishment of patient-derived liver cancer cell lines and organoids have opened new avenues for precision medicine research. Notably, patient-derived organoid (PDO) cultures have demonstrated self-assembly and self-renewal capabilities, retaining essential characteristics of their respective in vivo tissues, including both inter- and intratumoral heterogeneities. The emergence of PDOs derived from PLCs serves as patient avatars, enabling preclinical investigations for patient stratification, screening of anticancer drugs, efficacy testing, and thereby advancing the field of precision medicine. This review offers a comprehensive summary of the advancements in constructing PLC-derived PDO models. Emphasis is placed on the role of CSCs, which not only contribute significantly to the establishment of PDO cultures but also faithfully capture tumor heterogeneity and the ensuing development of therapy resistance. The exploration of PDOs' benefits in personalized medicine research is undertaken, including a discussion of their limitations, particularly in terms of culture conditions, reproducibility, and scalability.

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Key Points

Patient-derived organoids serve as patient avatars, offering precise models of tumor heterogeneity and therapy resistance for preclinical testing and personalized treatment strategies.

Investigating cancer stem cell heterogeneity and plasticity is essential for overcoming therapy resistance in liver cancer.

Organoids enable effective drug screening and treatment optimization, improving therapy outcomes.

1 Introduction

The global cancer burden is responsible for nearly 10 million deaths per year and is expected to increase. Among the different organs affected, the liver is the sixth most common site of primary cancer in humans with approximately 905,000 cases in 2020 [1]. Primary liver cancers (PLCs) include a heterogeneous group of tumors with distinct histological features and poor prognosis rates: hepatocellular carcinoma (HCCs) represents 80–85% of all PLCs, followed by intrahepatic cholangiocarcinoma (iCCAs) and a combined hepatocellular-cholangiocarcinoma subtype (CHCs). While hepatitis B virus (HBV) and hepatitis C virus (HCV) infections remain pivotal external risk factors, the significant rise in PLC cases is also attributed to excessive alcohol consumption and the related conditions of metabolic syndrome, obesity, type 2 diabetes, and non-alcoholic fatty liver disease [2, 3]. The majority of these cancers are still diagnosed at advanced stages, where treatments are not very effective and which remains associated with a poor prognosis (5-year survival less than 18%) [4–6]. All targeted therapies have to contend with the emergence of resistance, systematically leading to therapeutic failure [5, 7–10].

The notable heterogeneity observed in PLCs accounts for the resistance of tumors to therapeutic interventions. Moreover, the varied etiology of PLCs is reflected in the molecular heterogeneity of cancers, either within an individual (intratumoral heterogeneity) or within patients (intertumoral heterogeneity) further supporting the need for patient-tailored therapeutics, also called personalized medicine. The current comprehension of PLC heterogeneity primarily revolves around intertumor heterogeneity, emphasizing molecular subclassification through genomic profiling. This approach has successfully identified distinct patient subtypes based on genomic profiles, guiding targeted therapy selection [7–10]. However, relying solely on intertumor heterogeneity for subclassification might not encompass the entire tumor spectrum. Consequently, there is a need to integrate molecular features considering both intertumor and intratumor heterogeneity, along with functional heterogeneity, to enhance patient subclassification and optimize therapy response. Studies have consistently observed intratumor heterogeneity in PLCs through various methods, such as histology, analyses of ploidy patterns, DNA fingerprinting, and whole-genome sequencing (WGS) [11–13]. This intratumor heterogeneity is partly explained by the presence of cancer stem cells (CSCs) sub-populations [14] and their dynamic evolution/plasticity dependent on internal (genetic and epigenetic) or external [tumor micro-environment (TME), drug pressure] signals [15]. This complexity introduces the concept of "functional" intratumor heterogeneity, which characterizes the capacity of cancer cells to adapt to a well-defined microenvironment linked to a specific etiology and develop drug resistance [16].

To date, there are still few predictive models available to understand PLC heterogeneity, to assess the efficacy of planned oncology treatment, and to potentially develop effective new therapies. To achieve this, it is important to develop replicable and accurate models that mimic the structure, TME, and pathological function of PLCs. Specifically, these models must aim to accurately capture and maintain the diversity within a tumor cell community, which is intricately linked to patient outcomes, including responses to treatment. While genetically engineered mouse models (GEMM) and two-dimensional (2D) cell lines have furthered our understanding of liver cancer [17–20], they suffer in recapitulating key features of human liver tissue, in particular its complex three-dimensional (3D) architecture and metabolic functions. In addition, iCCAs have proven difficulty in propagating in vitro [21]. On the other hand, Patient-derived xenografts (PDXs), established for both HCCs and iCCAs, recapitulate the genetic and histological features of the original tumor and show great translational potential to direct treatment in a patient-tailored manner. However, this strategy has several drawbacks: PLCs tend to display relatively low engraftment rates (generally between 5 and 20% engraftment efficiency as reported) [21–25], rendering liver cancer PDX models impractical for functional diagnostics, especially high-throughput drug screening processes. As the use of these animal models is both time-consuming and expensive, alternative methods have been developed, such as the production of ex-vivo three-dimensional (3D) tumor models, which represent relevant physiologically models that are able to closely resemble the in vivo tumor.

In this review, we will give the state of the art concerning the production of PDOs from PLCs, highlighting both potential and limitations in the context of personalized medicine. We provide an overview of advances in PLC-derived PDO models, focusing on CSCs as key players both in the establishment of PDOs but also in recapitulating the tumor heterogeneity and related therapy resistance.

2 Building Tumor Replicas: Liver Organoids Model and Engineered Tumor Organoids

Organoids are self-organized cell aggregates derived from stem cells that are capable of self-renewal, typically organized in three-dimensional (3D) constructs able to replicate the complex structure and in many cases the function of the in vivo tissue, mimicking its in vivo physiology.

In 2009, Sato et al. paved the way for organotypic culture by demonstrating the ability to induce a single mouse

LGR5+ intestinal stem cell to form complex villus-crypt structures on an extracellular matrix (ECM), in the presence of niche-specific growth factors, including epidermal growth factor (EGF), R-spondin-1 (Rspo1), and noggin [26]. Two signaling pathways are essential for the growth of most organoid types: the EGFR pathway, which promotes cancer cell proliferation and requires EGF supplementation in the culture medium [27], and stimulation of the Wnt pathway, via the addition of Rspo-1 and Wnt3a, agonists of the LGR (leucin-rich repeat-containing G-protein-coupled receptor) and Frizzled receptors, and its coreceptor LRP (low-density lipoprotein receptor-related protein). This pathway is involved in the control of numerous processes, such as proliferation, adhesion, and cell differentiation, via the stabilization of a transcriptional cofactor, β-Catenin [28, 29]. This groundbreaking discovery by Sato et al. has since sparked the development of a vast array of human and mouse organoid cultures, revolutionizing our understanding of organ development and function. These versatile 3D ex-vivo models now serve as invaluable tools for investigating a wide range of organs and diseases. On the basis of this work, organoids can be established from two types of stem cells, both with the capacity for self-renewal and self-organization: adult tissue-specific stem cells (ASCs) [30] or pluripotent stem cells (PSCs) [31] which include both induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs). Once formed under proper culture conditions, organoids can be expanded and remain genetically and phenotypically stable in long term culture. They can also be genetically modified and cryopreserved. The advent of a large number of human organoid models has provided a path toward dynamic observation and mechanistic studies of human development [32, 33]. Organoids have also been used to build customized models of specific human diseases and also for clinical applications, such as transplantations. Of note, iPSCs can be used to create 3D liver organoids that mimic the complex structure and functions of the human liver. Takabe et al. demonstrated the successful generation of transplantable hepatic buds with functional characteristics through a combination of iPSC-derived endoderm directed toward hepatic differentiation, mesenchymal stem cells (MSCs), and human umbilical vein endothelial cells (HUVECs) [34, 35]. Hepatic organoids can be derived from both normal and patient-derived iPSCs, enabling the modeling of human liver diseases [36, 37]. iPSC-derived liver organoids can have a potential advantage of their expansion ability, making them valuable tools for toxicology studies, allowing for the rapid screening of a large number of compounds in industrial settings [38]. Recently, organoids genetically manipulated using the gene-editing tool Clustered regularly interspaced palindromic repeats (CRIPSR)/Cas9 [39], provide a better understanding of the pathways involved in the pathogenesis and pathophysiology of PLCs, enabling studies of gene function, disease modeling, and the identification of potential therapeutic targets. Although engineered tumor organoids may serve a purpose in modelling cancer initiation, studying the origin of specific mutations, and identifying potential preventive therapies, it is uncertain whether these organoids fully replicate the original tumor complexity.

3 PDOs: Capturing Cancer Complexity with CSC Insights

Using organoid technology, scientists are actively developing advanced models of cancer tissues known as PDOs or "tumoroids" (meaning "tumor-like organoid"). These models are typically derived from primary or metastatic tumors collected from oncology patients. PDOs establishment is primarily driven by the presence of Tumor initiating cells (TICs), also known as CSCs, within human tumor tissues [40]. CSCs, akin to normal stem cells, possess the dual capabilities of self-renewal and differentiation [41]**.** Unlike normal stem cells, which typically remain quiescent until their regenerative potential is needed, accumulating evidence suggests that CSCs are a pivotal driving force, responsible for tumor initiation, disease progression, cancer recurrence, and resistance to treatment.

3.1 CSCs: Unveiling Heterogeneity and Resistance in PLCs

3.1.1 CSCs: Beyond the Tip of the Iceberg in PLCs

Traditionally, tumor heterogeneity has been attributed to genetic and epigenetic alterations that accumulate during the clonal evolution of cancer cells [42, 43]. However, a newer concept, the "CSCs hypothesis," proposes that a distinct subset of tumor cells, possess stem cell-like characteristics, enabling them to self-renew and divide asymmetrically in order to generate heterogenous cell populations, through multilineage differentiation potential [41]. These CSC traits contribute to tumorigenicity, tumor cell heterogeneity, and the hierarchical organization of cells within tumors [44, 45], (Fig. 1).

Beyond their stem cell-like characteristics, CSCs exhibit various adaptive features that enhance their survival under environmental stress. These include increased drug efflux capacity to actively pump out chemotherapy drugs, aberrant DNA repair mechanisms, activation of various cytoprotective and survival signaling pathways, dysregulation of stemness signaling pathways, increased quiescence, increased immune evasion, deficiency of mitochondrial-mediated apoptosis, and upregulation of anti-apoptotic mechanisms [46]. As a result, these cells, exhibiting inherent

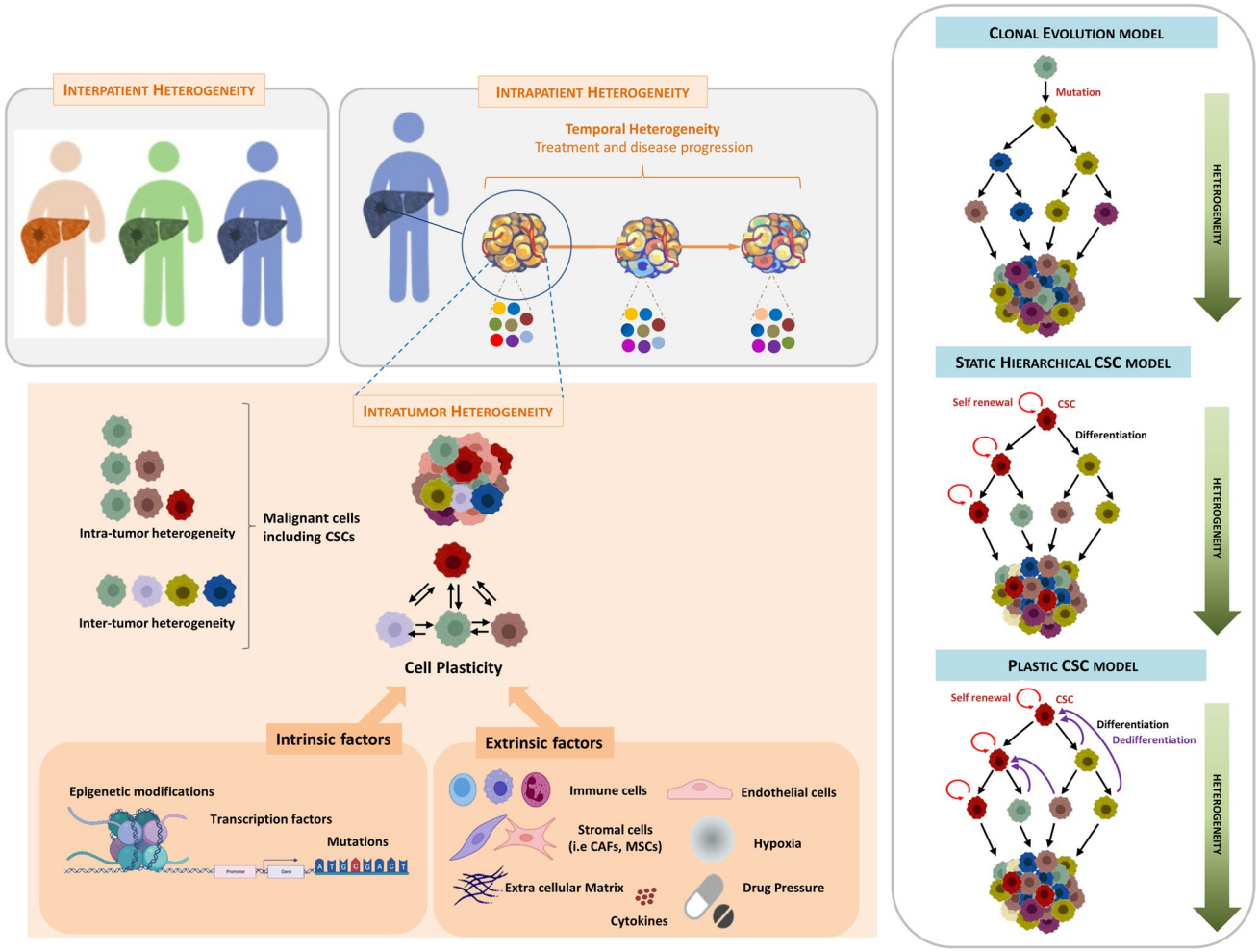

Fig. 1 Tumor heterogeneity in PLCs presents a major challenge to personalized cancer medicine where it drives therapy resistance. Heterogeneity occurs both within individual tumors (intratumoral) and across patients (intertumoral), reflecting the complexity of cancer biology. Moreover, temporal heterogeneity, arising from treatment and disease progression, compounds intratumoral heterogeneity, further complicating therapeutic outcomes. Traditionally, tumor heterogeneity was explained by the clonal evolution model, which attributes it to the accumulation of genetic and epigenetic alterations in cancer cells. However, the "CSC hypothesis" introduces a distinct subset of cells with stem-like properties as key drivers of intratumoral heterogeneity. Initially described as a static subpopulation in the hierarchical CSC model, recent evidence supports the plastic CSC model, emphasizing their dynamic ability to dedifferentiate and switch between distinct cell states, including hybrid epithelial-mesenchymal (E/M) states. This plasticity is tightly regulated by intrinsic factors, such as genetic and epigenetic modifications, and extrinsic factors, including the TME. The TME, composed of stromal cells, such as CAFs, MSCs, endothelial cells, and adipocytes, creates a niche that promotes CSC functionality and therapy resistance. *CSCs* cancer stem cells, *PLCs* primary liver cancers, *TME* tumor microenvironment, *CAFs* cancer-associated fibroblasts, *MSCs* mesenchymal stem cells

resistance to conventional anticancer therapies and hostile microenvironmental conditions, are extremely resilient and are often the driving force behind tumor heterogeneity and treatment failure [44, 45].

The traditional notion of CSCs as a static subpopulation of tumor cells has been challenged by recent research revealing their remarkable plasticity and ability for dedifferentiation and as well as their capability to switch between distinct cell states [47]. These transitions include the epithelial-mesenchymal transition (EMT), promoting dissemination, and its reverse mesenchymal-epithelial transition (MET), facilitating colonization at distant sites [48, 49]. Furthermore, CSCs can shift between differentiated (non-CSCs) and dedifferentiated (CSCs) states, adopting hybrid E/M states, further complicating their behavior [48]. As a result, the dynamic and adaptable nature of CSCs, as proposed by the "plastic CSC model," challenges the traditional static view of CSCs as a distinct tumor subpopulation. Accumulating evidence indicates CSC plasticity is finely regulated by both cell intrinsic (genetic [50] and epigenetic [51]) and extrinsic factors (environmental injury, tumor niche microenvironment) [44, 52]. Thus, the tumor microenvironment (TME),

a complex interplay of cellular and noncellular components, plays a pivotal role in tumor development, progression, and therapy resistance. Stromal cells, including cancer associated fibroblasts (CAFs) [53], MSCs [54], endothelial cells [55], and adipocytes [56], play a critical role in maintaining the TME and promoting CSC functions. This concept of CSC plasticity adds complexity to our understanding of cancer and contributes to intratumoral heterogeneity and therapeutic resistance [45]. However, owing to the lack of a standardized in vivo validation method, the plastic CSC model remains under debate. Deciphering the complex biology and behavior of CSCs is imperative for advancing therapeutic approaches that specifically target these resilient cells, ultimately improving patient outcomes in cancer treatment [57].

3.1.2 CSC Subpopulations: Their Roles in Liver Cancer

Many studies have shown that the phenotypic and functional heterogeneity of tumors, including PLCs, is likely caused by the presence of subpopulations of CSCs within the tumor [14]. Successful identification of CSCs and CSC plasticity comprehension are prerequisite for a better understanding of the molecular mechanisms by which liver cancer evades treatment. Various cell markers have been utilized to identify and isolate CSCs within PLC specimens. These markers include CD133, CD90, CD44, EpCAM, CD13, CD24, OV6, DLK1, α2δ1, ICAM-1, CD47, LGR5, and CK19 [15, 58–61], which are commonly expressed in hepatoblasts or hepatic progenitor cells but not in mature hepatocytes. Additionally, multiple CSC markers have been identified for iCCAs, such as ALDH, CD44, CD44v9, CD90, CD133, CD147, EpCAM, and SOX2 [62, 63]. Intriguingly, most CCA cells coexpress CK19 and albumin, a characteristic of hepatobiliary stem/progenitor cells [62]. Other markers, such as ALDH, side population (SP), and EMT markers, have also been employed to differentiate CSCs from PLCs [64, 65]. So far, no universal markers for liver CSCs have been identified and characterization of CSC subpopulations and their hierarchy organization among tumor on the basis of these markers remains problematic.

Importantly, the presence of high levels of CSC markers is a significant predictor of poor clinical outcomes in patients with iCCAs [63] and HCCs [66]. Yamashita et al. demonstrated that CSCs heterogeneously and largely represented in human iCCAs (> 30%), indicating that these cancers as diseases rich in stem/progenitor cells [67]. The high CSC prevalence in iCCAs stands in contrast to HCCs and other solid tumors [68], where these markers identify only 0.5 to 3% of tumor cells. However, the rate of CSCs in HCCs differs between various clinical stages. Compared with well-differentiated HCCs, poorly differentiated HCCs display higher CSC percentages [66, 69] and are associated with reduced overall and disease-free survival after treatment [70].

Within the tumor, distinct CSC subpopulations coexist, each with its own functional characteristics [14]. However, the composition of these subpopulations varies depending on the cancer subtypes and clinical stages from patients with PLCs [71]. In primary iCCAs cultures and established cell lines CSCs with mesenchymal characteristics (CD90, CD13) overwhelmingly dominate over CSCs with epithelial markers (EpCAM, and LGR5). Moreover, these CSC subpopulations exhibit distinct functional roles depending on the microenvironment (spheroids versus xenografts) [71]. Similarly, in primary HCC tissues and cell lines, there are at least two distinct CSC types that differ in their gene and protein expression patterns and functions. While tumorigenic epithelial EpCAM+ CSCs primarily produce AFP, a well-known HCC marker, metastatic mesenchymal CD90+ CSCs produce a specific basement membrane component, laminin γ2 monomer (LG2m), and exhibit enhanced metastatic potential [59, 72].

The roles of CSC subpopulations in the various responses to treatment remain ambiguous. Some subpopulations exhibit intrinsic resistance by overexpressing efflux pumps, such as ABC transporters [73], or entering a quiescent state, allowing them to evade therapies targeting proliferative cells. Differences in the activation of signaling pathways, such as Notch, Hedgehog, or Wnt/β-catenin [73], further complicate therapeutic strategies as some subpopulations remain unaffected by inhibitors targeting specific pathways. Metabolic diversity also contributes to resistance, with certain subpopulations relying on glycolysis while others depend on oxidative phosphorylation, making metabolic targeting challenging [74]. Finally, CSCs exhibit phenotypic plasticity, favoring dynamic switching between CSC and non-CSC states, which complicates efforts to eradicate them. In addition to intrinsic factors, CSC plasticity is significantly influenced by TME components, including selective stimuli such as hypoxic conditions [75] and chemotherapy.

Favoring the dynamical switch between CSC and non-CSC states, CSC plasticity plays an important role in the evolution of therapeutic resistance, tumor relapse and metastasis. In addition to intrinsic factors, CSC plasticity is influenced by TME components, such as selective stimuli, like chemotherapy. While sorafenib, 5-FU, and epirubicin treatment lead to EpCAM+ cell enrichment in HCC cell lines [76], only 5-FU or epirubicin treatment induces de novo generation of CD90+ and CD105+ mesenchymal liver CSCs [77]. This study demonstrates that liver CSCs and non-CSCs can adopt functional characteristics similar to those of stem cells, induced by intrinsic or acquired cellular plasticity, which contributes to their resistance to treatments and patient relapse.

3.2 Establishing PDOs from PLC Patients

The processes used to generate distinct PDOs differ in varying degrees from organoid generation but generally share several key steps. A commonly used PDO method is a reconstituted model, in which cells dissociated from mechanically and enzymatically dissociated tumor tissues are cultured in a dome or flat gel of 3D scaffolding matrix [e.g., Matrigel or Basement Membrane Extract Type 2 (BME-2)], underneath cell culture medium. In this "submerged Matrigel" procedure, various growth factors and/or pathway inhibitors are supplemented depending on tissue type but often include additives, such as Wnt3a, R-spondin-1, EGF, and bone morphogenetic (BMP) inhibitor noggin, which mimic factors present during normal stem cell homeostasis [26] (Table 1). Thanks to their self-renewal and differentiation properties [41], these culture systems allow CSCs to self-organize into functional units or specific tissue architectures that contain both differentiated cells and stem cells. By this method, PDOs have been successfully developed and amplified from primary tumors and metastatic lesions from various organs including liver, with an establishment rate ranging from less than 20 to more than 90% depending on the type of tumor considered [78].

Thus, organoid technology has been optimized for human PLCs allowing the generation of PDOs from all three common liver tumor subtypes (HCCs, iCCAs and CHCs). An important work performed by Broutier et al. demonstrated that organoid cultures derived from human liver donor/healthy tissues could be expanded long-term in vitro while preserving most of their liver functionality and genetic stability over time [79]. In a second work, the authors have demonstrated the proof-of-concept that liver organoid cultures replicate human PLCs in vitro and successfully established cultures from eight patients, representing three common subtypes of PLC [80]. Thereafter, PLC-derived PDOs were subsequently established from resected early stage disease, advanced cancers, and highly chemorefractory tumors (Table 2).

The most effective method for obtaining tissue to promote the growth of tumor organoids is through surgical resection, as it offers ample starting material for the robust propagation of these organoids. Importantly, the challenge of restricted access to surgically resected PLC specimens can be addressed by utilizing tumor needle biopsies. Studies have demonstrated that tumor needle biopsies serve as a viable source for the generation of liver organoids [81]. While most PLC-derived PDO collections come from fresh tumor tissues, studies demonstrated the feasibility of establishing PDOs from frozen tissues. PDOs could thus be generated from flash-frozen and DMSO frozen samples [82, 83], with only minimal impact on their growing potential. Although this has not been demonstrated for PLCs, Nantasanti et al. have shown that normal organoids can also be generated from frozen liver tissue [84].

Of note, the majority of organoid-based studies on liver cancer focus on PLCs. In contrast, there is limited research on metastatic hepatic carcinoma and other tumors that have metastasized to the liver. An interesting approach was taken by Skardal et al., who introduced colon carcinoma cells into liver organoids, creating 3D liver-tumor organoids for in vitro modeling of liver metastasis [85]. This innovative model demonstrated superior efficacy in representing metastatic tumors compared to traditional 2D cell cultures. While it may not fully replicate the complexity of liver metastatic tumors, this model merits further exploration in research.

3.3 Mirror Images: PDOs Reflecting the Architecture and Expression Profile of the Original Tumor

Several studies have shown that PLC-derived organoids recapitulate the histological architecture and functional characteristics of the corresponding parent tumor. While healthy liver-derived organoids form single-layered epithelial ductal-like cells surrounding a central lumen, PDOs formed compacted structures that resembled the corresponding tumor-of-origin. Notably, PLC-derived organoids also retain the specific histological features between patients as well as between tumor subtypes. HCC-derived PDOs, such as their parental tissue, exhibit pseudoglandular rosettes, a hallmark of HCCs while CCA-derived PDOs exhibit extensive glandular domains, similar to the patient's tumor [80, 81, 86].

Remarkably, PLC-derived PDOs are also characterized by the fact that they retain the expression of markers (such as epithelial and stem cell markers) observed on the tissues of the original patient, maintaining inter-patient and/or tumor subtype differences with specific expression profiles between HCCs and iCCAs [80, 81, 86–88]. Thus, well-established markers include preferentially HepPar1, Glypican-3 (GPC3), hepatocyte nuclear factor 4 (HNF4) for HCCs, and SOX2, EpCAM for iCCAs. Cytokeratins 7 and 19 (CK7 and CK19) serve also as valuable histochemical markers for distinguishing between HCCs and iCCAs, since both markers are higher expressed in iCCAs [89]. Moreover, others stem cell markers such as LGR5 and SOX9 are used to characterize PLC-derived PDOs [88]. On the other hand, the most significant HCC markers are albumin (ALB) and α-fetoprotein (AFP). This latter one represents a marker of liver function, such as synthesis and secretion, typical of differentiated hepatocytes [90]. PAS and mucicarmine staining showed that mucus including mucin was present in the lumen of primary iCCA tissues and matched PDOs demonstrating that PLC-derived PDOs mimic the primary tissues in both architecture and function [86]. Likewise, AFP, spalt-like transcription factor 4 (SALL4), EpCAM are "stemness"-related markers for CHCs [91].

Table 1 Composition and functions of culture medium components used in the generation of primary liver cancer-derived organoids as reported in published articles

Organoid isolation mediums for primary liver cancer-derived organoids					
References		[80]		[81]	[88]
		"Classical IM"	"Tumouroid IM"		
Organoid Splitting Media	Advanced DMEM/F12/Hepes/Glutamax/B27 supplement 1X/N2 supplement 1X	+	+	+	+
Y-27632	A Rho kinase inhibitor that effectively reduces the anoikis of dissociated stem cells	10 μM	10 μM	–	10 μM
A83-01	Potent inhibitor of TGF-βR ALK5, ALK4, and ALK7 that suppresses the proliferation of organoids	5 μM	5 μM	5 μM	0.5 μM
Noggin	An inhibitor of bone morphogenetic proteins that modulates cellular differentiation, proliferation, and apoptosis	25 ng/mL	–	–	100 ng/mL
EGF	Key factor in epithelial malignancies, and its activity enhances tumor growth, invasion, and metastasis	50 ng/mL	50 ng/mL	50 ng/mL	50 ng/mL
FGF-10	FGF10/FGF receptor 2IIIb axis is important for the organ development, including the stomach, liver, breast, and prostate	100 ng/mL	100 ng/mL	100 ng/mL	100 ng/mL
HGF	HGF/Met signaling promoted oncogenesis, tumor angiogenesis, tumor invasion of multiple tumor types; HGF profoundly enhances organoid growth	25 ng/mL	25 ng/mL	25 ng/mL	30 ng/mL
Gastrin I	Gastrin stimulates tumor growth through promoting the proliferation and suppressing the apoptosis of cancer cells	10 nM	10 nM	10 nM	10 nM
N-acetylcysteine	Antioxidant directly scavenging ROS and partially via ERK1/2 activation. It is also a source for cysteine in the generation of the antioxidant glutathione	1.25 mM	1.25 mM	1.25 mM	1.25 mM
Nicotinamide	Vitamin PP is a nutrient that is required for long-term culture of organoids	10 mM	10 mM	10 mM	12.5 mM
Forskolin (FSK)	A CFTR activator used to induce organoid swelling due to chloride and fluid flux into the lumen	10 μM	10 μM	10 μM	12.5 μM
Wnt 3a conditioned medium	A master regulator in regulation of cell development, proliferation, differentiation, adhesion, and polarity; The aberrant activation of Wnt signaling promotes carcinogenesis and progression of cancers	30%	–	30%	30%
R-Spondin 1 conditioned medium	The ligand of LGR5 and a niche factor that is required for the self-renewal of stem cells and activates Wnt signaling; R-spondin-1 facilitates the growth and metastasis of cancer cells	10%	–	10%	20%
Dexamethasone	It acts as a glucocorticoid that helps in modulating cellular stress responses, promoting cellular differentiation, and reducing inflammatory signaling	–	3 nM	–	–

EGF epidermal growth factor, *FGF* fibroblast growth factor, *HGF* hepatocyte growth factor, *Wnt3a* wingless-type MMTV integration site family, member 3A

Table 2 Published articles on establishment of patient-derived organoids for primary liver cancer

Tissue collection	PLC subtype	Number of patients—biopsies	Number of PDO cell lines	Success rate	Treatment	References
Surgical specimen	HCCs	11 – N/A	3	~ 27%	29 anticancer compounds	[80]
	iCCAs	6 – N/A	3	50%		
	CHCs	2 – N/A	2	100%		
Needle biopsies	HCCs	26 – 36	10	26%	Sorafenib	[81]
	iCCAs	4 – 6	2	~ 33%		
Surgical specimen	HCCs	2 – N/A	10	N/A	129 anticancer compounds	[88]
	iCCAs	3 – N/A	17	N/A		
Surgical specimen	HCCs	26 – N/A	17	~ 47%	Cabazitaxel, oxaliplatin and sorafenib	[92]
	CCAs	2 – N/A	1	50%		
Surgical specimen	HCCs	20 – N/A	40	N/A	Omacetacxine	[164]
Surgical specimen	iCCAs	N/A	N/A	N/A	55 anticancer compounds	[165]
Surgical specimen	HCCs	14 – 14	4	~ 28%	Tumor infiltrating lymphocytes and peripheral blood lymphocytes	[132]
PDX lines and surgical specimen	iCCAs	N/A	>3	N/A	Differentiation medium and macrophage conditioned medium	[135]
Surgical specimen	iCCAs	6 – N/A	3	50%	339 anticancer compounds	[86]
Surgical specimen	HCCs	153	52	29%	Sorafenib, phenformin	[126]
Surgical specimen	CCAs	29 – N/A	20	~ 69%	Gemcitabine, sorafenib, cisplatin, and doxorubicin	[166]
Surgical specimen	HCCs	8 – N/A	4	50%	Sorafenib, GANT61	[87]
Surgical specimen	HCCs	N/A	N/A	N/A	Knockdown of *PRMT6*, 5-FU, cisplatin, and sorafenib	[110]
Surgical specimen	iCCAs	57 – N/A	44	~ 75%	Gemcitabine, cisplatin, 5-FU, oxaliplatin and others	[93]
Surgical specimen	HCCs	N/A	N/A	N/A	Veterporfin (YAP inhibitor)	[167]
Surgical specimen	HCCs	N/A	N/A	N/A	SHP099, sorafenib	[168]
Surgical specimen	HCCs	N/A	27	60%	CD8 cells	[169]
PDX lines	HCCs	N/A	14	N/A	268 anticancer compounds	[161]
PDX lines	CCAs	N/A	19	N/A	Pemigatinib, niraparib	[25]
Surgical specimen	HCCs	N/A	4	N/A	Ifenprodil, sorafenib	[127]
Needle biopsy	HCCs	N/A	3	NA	CAR-T cells, CD8+ T cells	[170]
Surgical specimen	HCCs	N/A	N/A	N/A	EpCAM-apt-Dox	[124]
Surgical specimen	HCCs	13 – N/A	N/A	N/A	Knockdown of *OPA1* and *MFN1*	[111]
Surgical specimen	iCCAs	1 – N/A	N/A	N/A		
Surgical specimen	CCAs	N/A	4	N/A	CAFs cocultures, sorafenib, regorafenib, and 5-FU	[145]
Surgical specimen	HCCs	N/A	N/A	N/A	CAFS conditioned medium, sorafenib	[146]
PDX lines	HCCs	N/A	N/A	N/A	HCC-endothelial co-cultures	[171]
Needle biopsy	HCCs	N/A	N/A	N/A	CD8+ TILs	[172]

CAFs cancer-associated fibroblasts, *CAR-T* chimeric antigen receptor -T cells, *CCAs* cholangiocarcinoma, *CHCs* combined HCC-CCA, *EpCAM-apt-Dox* epithelial cell adhesion molecule aptamer with doxorubicin, *HCCs* hepatocellular carcinoma, *MFN1* mitochondrial fusion protein mitofusin-1, *OPA1* optic atrophy 1, *PDX* patient-derived xenografts, *PRMT6* protein arginine methyltransferase 6, *TILs* tumor-infiltrating lymphocytes, *5-FU* 5-fluoruracil, *N/A* not available

3.4 Preserving the Blueprint: PDOs Retaining Genetic Landscape, Even After Long-Term in Vitro Expansion

Comprehensive sequencing analyses have uncovered the substantial genetic diversity of PLCs, with high somatic alteration rate (mutations, fusions, or amplifications). Some of these alterations are potential therapeutic targets. To effectively translate these genomic findings into novel therapeutic approaches, in-depth functional characterizations are required, employing experimental models that

faithfully recapitulate the cancerous characteristics of primary PLCs, in particular the mutational landscape.

Several groups have succeeded in establishing PLC-derived PDOs that phenotypically and genetically mimic the tumor from which they are derived. Furthermore, they have demonstrated that PDOs can be grown stably for over a year without significative changes in the histological architecture, expression profile but also in the genomic landscape. Indeed, when comparing the global variant profile, several studies have demonstrated a good concordance rate of whole exonic variants (~ 90%) between the primary tumor tissues, xenograft tumor tissue, and PDOs derived [80, 81, 86]. Importantly, mutations and amplifications affecting bona fide cancer genes found in the original cancer tissues were preserved in matched PDOs [92, 93]. Consistent with prior studies [78, 94], each PLCs-derived PDO retained a remarkable intratumoral mutational heterogeneity and tumor subtype-specific mutations present in the primary tissue, even after long-term expansion [80, 81, 86, 92].

This ability of PDOs to be grown in culture over the long term without significant modifications distinguishes them from existing 2D cell lines, which often lose their patient-specific characteristics and genetic landscape, as evidenced by the frequent accumulation of *TP53* mutations in these cell lines [95]. While the exact reasons for these discrepancies remain unclear, it is plausible that ECM interactions play a significant role in maintaining heterogeneous and unselected populations within the PDO culture, thus preventing anoikis [96].

3.5 CSCs' Pivotal Role: PDOs Recapitulate PLC Tumorigenesis

Importantly, upon transplantation into mice, PLC-derived PDOs show metastatic potential, even after long-term expansion in culture [80, 81]. These results show that CSCs, with self-renew and lineage differentiation capacities are still maintained in PDOs cultured during short or prolonged culture period (> 4 months). This result is supported by a recent work revealing that during serial passages, the percentage of CSCs (such as cluster of differentiation-133+ and Wnt+ cells) and their tumor-initiating capacity were constant in colorectal-derived PDOs, while they gradually increased in sphere culture (spheroid) models. Thus, while the sphere formation assay enriches for CSCs and chemoresistant cells, PDOs culture may be useful in long-term maintaining tumor heterogeneity and the levels of chemoresistant cells [97].

The fundamental question by which CSCs function in PLC progression is unclear. The ability of CSCs to initiate and reconstitute tumor lesions together with features such as differentiation state and chemo/radiotherapy resistance has been extensively studied from 2D tumor tissue or cell line culture models but still remains very little studied in 3D tumor culture models, such as PDOs. Mimicking several features of the original tumor, PDOs represent essential tools to better understand CSC functions. These aspects will be discussed later.

4 Liver Organoids: Unlocking Secrets of Liver Cancer

While engineered organoids may serve a purpose in modelling cancer initiation, these models have limitations, particularly in replicating the intricate tumor complexity observed in patients. Thus, collections of PLC-derived PDOs from a variety of histological cancer subtypes and patient clinical stages are indispensable for studying PLC progression mechanisms, including CSC research, discovering biomarkers and transcriptomic profils, and improving therapy response prediction in patients (Fig. 2).

4.1 Deciphering the Journey: Studying Liver Cancer Initiation and Progression

While existing cancer cell lines and PDOs from PLCs mirror the mutations and gene expression patterns of real tumors, they cannot tell us how these cancers start. Organoids derived from normal liver tissues can be used to model liver tumor initiation by investigating the effects of sequentially introduced driver mutations in an isogenic genetic background. In particular, researchers have successfully applied CRISPR/Cas9 gene editing technology to liver organoids to model the initial alterations in human PLCs. Artegiani et al. discovered that human cholangiocyte organoids with four common cholangiocarcinoma mutations (*TP53*, *PTEN*, *SMAD4*, and *NF1*) became malignant after loss-of-function of the tumor suppressor *BAP1* by CRISPR/Cas9 [98]. A similar strategy has also been employed with murine CCA-derived organoids to produce tractable CCA PDOs that recapitulate the multiple steps involved in liver tumorigenesis [99]. Sun et al. created also organoids with liver architecture and function by directly reprogramming human hepatocytes (hiHeps) and inactivation of p53 and RB. Thus, genetically engineered HiHep organoids models demonstrated that HCCs could be formed via c-MYC overexpression, while iCCAs could be induced by oncogenic RAS-driven lineage conversion [100]. Thus, combining miniature "organ replicas" (organoids) with precise gene editing (CRISPR/Cas9) offers a powerful tool to study how genes contribute to cancer in a human-like setting.

In the case of chronic HBV infection, the primary cause of liver cirrhosis and HCCs worldwide, our understanding of the mechanisms behind HBV-induced HCC mainly comes from epidemiological studies [101, 102], genomic analyses [103, 104], and in vitro research [105, 106]. However,

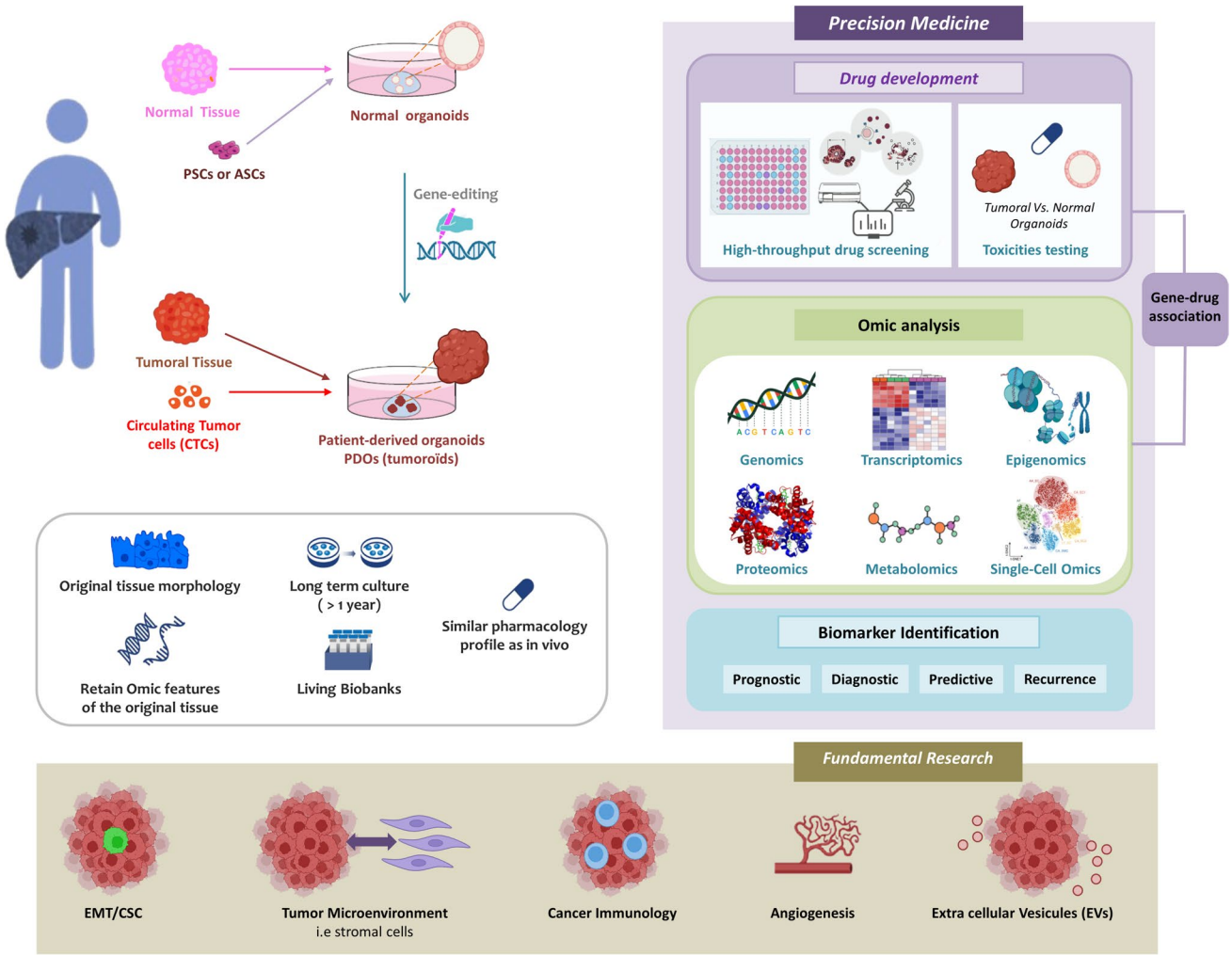

Fig. 2 PDOs, driven by CSCs, are considered the most predictive models in basic and translational cancer research. On the basis of the capacity of stem cells for self-renewal and self-organization, liver organoids can be derived from normal liver tissue, ASCs, or PSCs. Employing CRISPR/Cas9 gene editing technology enables the engineering of tumor organoids, providing a valuable tool for modeling cancer initiation, exploring the origins of specific mutations, and identifying potential therapies. Furthermore, utilizing organoid technology, PDOs have been established from tumor biopsies or CTCs (not yet with PLC-derived CTCs). These PDOs maintain crucial genetic and phenotypic characteristics of their respective in vivo tissues, even after prolonged in vitro expansion. The ability to freeze and recover PDOs facilitates the creation of living PDO biobanks. PLC-derived PDOs act as patient avatars, facilitating preclinical investigations for biomarker identification, anticancer drug screening, efficacy testing, and advancements in precision medicine. Additionally, PDOs contribute to fundamental research, particularly in understanding the behavior of CSCs, CSC plasticity, and their interactions with the tumor microenvironment (stromal cells, immune cells, endothelial cells, extracellular vesicles, etc.) *ASCs* adult tissue-specific stem cells, *CSCs* cancer stem cells, *CTCs* circulating tumoral cells, *PDOs* patient-derived organoids, *PLCs* primary liver cancers, *PSCs* pluripotent stem cells

the complexities of HBV-mediated tumorigenesis remain largely unclear owing to a lack of suitable model systems. De Crignis et al. propose human liver organoids as valuable models for studying HBV infection and tumor development [107]. For that, study developmental processes with organoids can also give critical information concerning tumor development. Transcriptomic and metabolomic analysis of organoid development indicated that the phosphatidylethanolamine biosynthesis pathway plays a vital role in both early liver development and HCC progression [108]. Other studies utilizing liver organoids establish a mechanistic connection between tumorigenicity and changes in glucose metabolism [109, 110] and alterations in mitochondrial structure and function [100]. Notably, Sun et al. demonstrated that excessive coupling of mitochondrial-associated endoplasmic reticulum (ER) may serve as the key mechanism driving c-MYC dependent hepatocarcinogenesis, as evidenced in a forced c-MYC transfection model in liver organoids (hiHeps), ultimately leading to the formation of HCCs [100]. These conclusions are supported by a study of Li et al., who

demonstrated that excessive activation of mitochondrial fusion in CCA- and HCC- derived PDOs altered cellular metabolism and fueled tumor cell growth [111].

4.2 Finding the Needle in the Haystack: Identifying Biomarkers

The poor prognosis of PLCs results from delayed detection. Late diagnosis hinders timely treatment and contributes to lower survival rates. Consequently, there is a critical need to innovate and develop novel diagnostic methods for PLCs. By integrating banked liver PDOs into genetic and epigenetic screens, the use of molecular profiling approaches, including transcriptomics, proteomics and metabolomics, enables a better understanding of the genetic and epigenetic landscape of different cancer subtypes and patient clinical stages, improving the robustness and generalizability of results. In this way, PDO cultures could become a valuable resource for the discovery of biomarkers with an essential function in aiding diagnosis and guiding precision medicine. Indeed, the identification of reliable biomarkers capable of establishing more effective patient stratification and accurately predicting drug responsiveness is the key to effective cancer treatment. Using omics approaches, several studies have revealed the promising application of PLC-derived PDOs as a biomarker discovery tool. Broutier et al. identified novel genes associated to poor prognosis for PLCs [80], demonstrating that culturing PLCs as PDOs models retains critical tumor cell characteristics, enabling the identification of novel genes that hold promise as prognostic biomarkers for PLCs. However, further research is required to validate their usefulness as predictive biomarkers and/or to establish their direct involvement in the progression of the disease.

4.3 Testing Grounds: PLC-Derived PDOs as Promising Drug Screening Platforms

Although being an indispensable tool for carrying out preclinical studies, the use of PDX in vivo systems for large-scale screening during early drug discovery is hampered by ethical, economic and throughput constraints that limit the number of drug tested. Moreover, due to their low engraftment rates, PLCs make liver cancer PDX models unsuitable for functional diagnostics [21–25]. Thus, recent findings indicate that PDOs hold promise for high-throughput drug screening and target discovery in a three-dimensional context. A number of studies using PDOs derived from different cancer types have demonstrated good reliability between the results of PDO-based in vitro drug screening and clinical response. In a clinical study, the responses of metastatic colorectal cancer-derived PDOs to irinotecan correlated with patients' responses to the drug, suggesting that PDOs could help avoid giving irinotecan to patients who would not benefit [112]. In a systematic approach, de Witte et al. demonstrated that ovarian cancer-derived PDO drug response to carboplatin and paclitaxel combination treatment correlated with several clinical response measure [113]. By comparing clinical responses observed in patients with ex vivo-response data gathered in patients-derived PDO and PDX models, Xiaoxue et al. reports that PDOs also faithfully recapitulated treatment responses of biliary tract cancers (BTCs). The drug screening results in PDOs are further validated in PDO-based xenografts and confirmed in 92.3% (12/13) of BTC patients with actual clinical response. In primary liver cancers, Broutier et al. pioneered a proof-of-concept drug sensitivity test using PDOs and PDO-derived xenografts, demonstrating a correlation between some drug sensitivities and mutational profiles. Thus work showed that PLC-derived PDOs facilitate both the prediction of drug sensitivity/resistance in a patient-specific manner but also enabled target identification, revealing the efficacy of ERK inhibitors in cells resistant to BRAF and MEK inhibitors [80]. Nuciforo et al. tested different sorafenib concentrations on tissue-derived PDOs from PLC patients with various clinical stages and etiologies. The study revealed a remarkable dependence of PDO growth on sorafenib dosage, highlighting significant inter-patient variability in treatment response. This result indicates that PDOs derived from PLCs biopsies could be used to test the specificity and sensitivity of drugs to the tumor [81]. Remarkably, the pharmacotyping of PDO culture remained consistent across numerous passages, with occasional minor variations [80, 114]. This could be explained in part by the fact that CSCs are consistently maintained in PDOs even after long-term expansion in culture [80, 81]. This strongly differentiates them from spheroid models in which CSC enrichment and an evolution of chemoresistance have been observed during serial passages [97]. These elements show a perspective for research on drug resistance and personalized medicine. In 2019, Li et al. demonstrated the promise of PDOs for drug screening in the treatment of iCCAs. They established several PDOs from distinct regions of each primary surgical specimen and tested 129 FDA-approved cancer drugs on them. While the majority of drugs proved either ineffective or effective only in specific PDOs, a subset of drugs exhibited pan-efficacy, displaying at least moderate activity in most of the PDOs. Among them, four drugs identified in the study (idarubicin, panobinostat, topotecan, and bortezomib), and are undergoing clinical trials for both HCCs and iCCAs. Moreover, this study revealed substantial intrapatient heterogeneity of responses to different drugs [88].

Precision therapy faces limitations owing to the complex and dynamic nature of cancer. Mechanisms of resistance to conventional and targeted therapies are dynamic and sequential and evolve over time. They involve reversible phenotypic changes, such as transient senescence [115], metabolic

reprogramming [116], epigenetic changes [117], EMT [118], and/or irreversible mutational changes [119]. Studying these processes directly in patients require a multiplication of samples during therapeutic management, which is often unthinkable. Enter PDO models, which offer a valuable tool to address these challenges. PDOs make it possible to follow the sequence of resistance acquisition in a controlled setting and identify the mechanisms involved in a reproducible and more relevant way than 3D culture in spheroids [97].

High-throughput drug screening using PLC-PDOs holds immense potential, but a comprehensive analysis integrating drug response profiles and genomic data is still missing. In this context, transcriptomic signatures, based on the analysis of expression levels of transcripts for selected groups of genes, offer a powerful tool. Not only can they illuminate tumor behavior [120–122], but they also possess the exciting potential to predict drug efficacy, even for therapies without specific known targets [123]. Thus, PDOs hold significant potential for establishing the pharmacogenomic landscape in PLCs, a goal that hinges on a systematic analysis of gene–drug associations within PDO biobanks.

4.4 Shining a Light on Cancer Stem Cells: Applications of PLC-Derived PDOs in CSC Research

Current PDOs are not only useful in uncovering novel treatments but can also be used to carry out fundamental research, particularly the behavior of CSCs, CSC plasticity, and their relationship with the TME. Thanks to their capacity for self-renewal and differentiation, CSCs possess unique properties that enable them to contribute to tumor formation, intratumoral heterogeneity and the hierarchical organization of cancer cells [14]. The analysis of stem cells in human cancers is challenging because it is difficult to identify and track tumor cells in their natural environment. Traditional cancer models, such as cell lines, do not replicate this complexity, which limits research. Thus, PDOs that could be maintained in culture for extended periods are good models to better understand stem cell hierarchies, allowing researchers to track the behavior of CSCs over time and study their response to different treatments. Single-cell transcriptome analysis revealed intratumoral CSC heterogeneity within HCC-derived PDOs. Notably, within CSC markers, such as CD133, EpCAM, and CD44, it was discerned that the CD44+ CSC subpopulation contributes to drug resistance by intensifying the Jak-STAT signaling pathway. This activation is instigated by the upregulation of nuclear paraspeckle assembly transcript 1 (*NEAT1*) under hypoxic conditions [75].

To adapt to their environment, CSCs display both remarkable plasticity, moving from a "stem" to a differentiated state, and remarkable resilience, employing a variety of adaptive features, as we saw earlier. These CSC features contribute to their role as a major driver of treatment failure [44–46]. Knowledge of cancer pathophysiology can be enhanced by specific studies on certain important aspects of the TME, which functions as a CSC niche, protecting and maintaining CSC characteristics. PDOs could be a suitable model to identify the mechanisms that mediate stemness-related drug resistance induced by the TME and evaluate strategies aiming to target CSCs. Several approaches have been explored to kill CSCs to deplete the pool of CSCs, disrupt the hierarchical organization of tumors [50]. This strategy will allow reducing tumor cell heterogeneity, and making tumors more susceptible to conventional anti-cancer treatments (Fig. 3). In this context, the identification and targeting of surface markers specific to CSCs hold significant potential for selectively eliminating CSC populations, thereby interrupting tumor growth and preventing disease relapse. EpCAM expression in HCC-derived PDOs correlated with the expression of multiple CSC markers (CD133, CD24, ALDH1A2, CD90) and proteins related to stemness (Notch pathway), angiogenesis (PDGF, VEGFB) and EMT [124]. On the basis of these results, Zhou et al. used aptamer-based drug delivery agents (CD133-apt-Dox and EpCAM-apt-Dox) to selectively and effectively kill liver cancer stem-like cells. Both combinations of drugs and antibodies significantly inhibited the growth of HCC-derived PDOs but exerted minimal cytotoxicity to normal liver organoids, demonstrating that aptamer-based drug delivery agents could be a highly promising approach for liver cancer therapy [124, 125].

Among the targeted therapies against CSCs, targeting epigenetic modifications, including histone methylation, histone acetylation, and DNA methylation, presents promising strategies to subvert tumor metastasis and improve the efficacy of cancer treatments in CSCs. The emergence of CSCs has been linked to abnormal epigenetic alterations in normal cells, leading to sustained primed epigenetic modifications that perpetuate aberrant differentiation and tumorigenesis, even after the oncogene's cessation [15]. In this context, Chang et al. showed that silencing of the protein methyltransferase 6 (*PRMT6*) promoted cancer stemness and therapy resistance of patient-derived organoids through enhanced expression of CSC markers (CD133, SOX2, NANOG), enhanced cell-like properties (migration, invasion, and oncosphere formation), and increased resistance to cisplatin, 5-FU, and sorafenib. They propose a critical repressive role for PRMT6 in HCC cell maintenance via regulation of RAS binding and MEK/ERK signaling through methylation of CRAF on arginine 100. These results provide a mechanistic link among tumorigenicity, therapy resistance, and glucose metabolism [109].

If targeting CSCs directly is a promising strategy for developing more effective cancer therapies, evidence

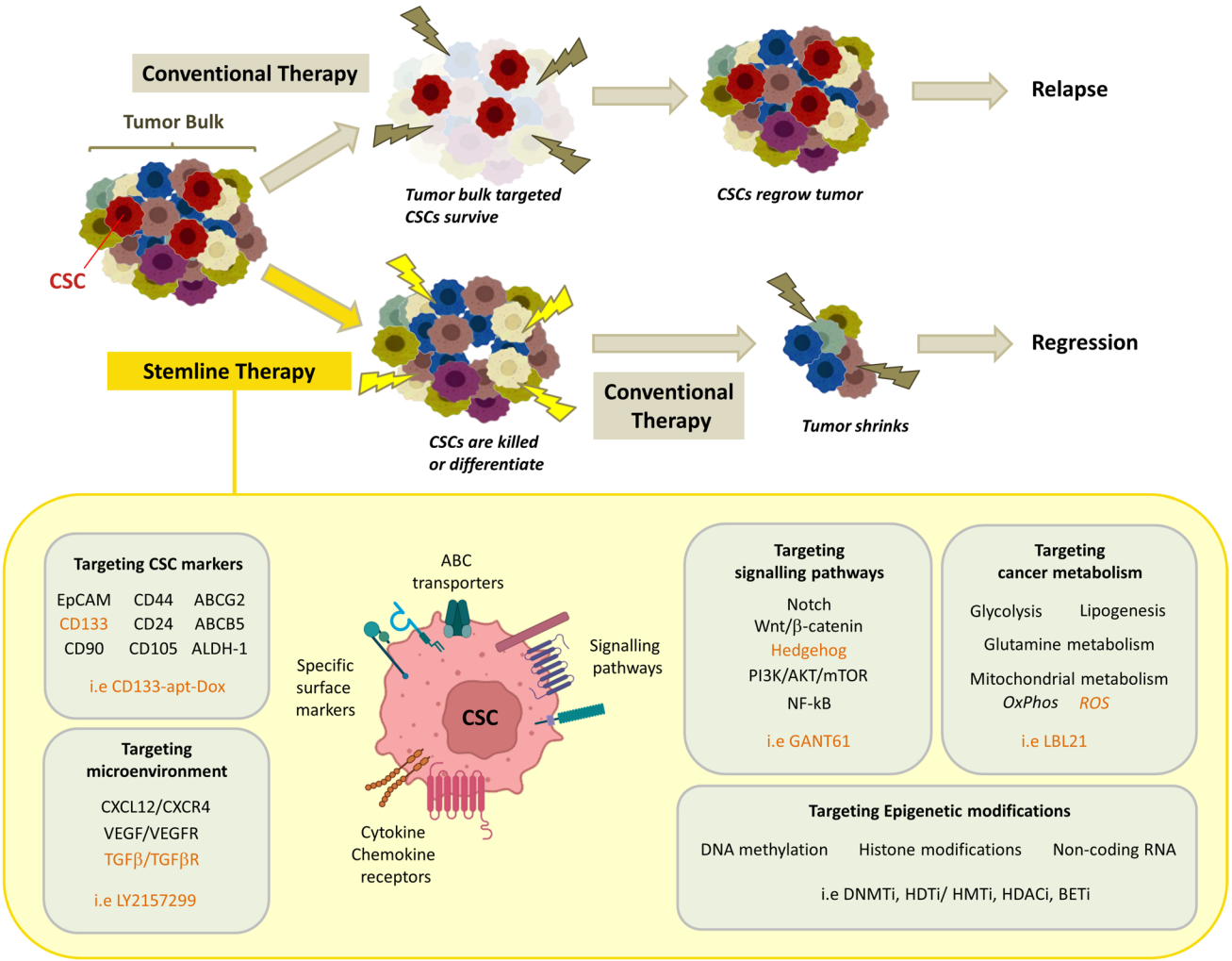

Fig. 3 Schematic diagram summarizing the therapeutic strategies targeting CSCs. Unlike bulk tumor cells, CSCs are inherently resistant to most conventional chemotherapeutic agents, allowing the surviving CSC subpopulation to regenerate tumors in patients, leading to tumor relapse. Several strategies have been developed to eliminate CSCs, aiming to deplete the CSC pool and disrupt the hierarchical organization of tumors. These approaches notably include targeting surface markers specific to CSCs, modifying epigenetic alterations, inhibiting key signaling pathways, disrupting cancer metabolism, and altering the tumor microenvironment. Targeting CSCs reduces tumor cell heterogeneity and enhances the tumor's susceptibility to conventional anticancer treatments. In orange, examples of inhibitors or drugs used to target CSCs. *OXPhos* oxidative phosphorylation, *PRMT6* protein arginine methyltransferase, *ROS* reactive oxygen species, *CD133-apt-Dox* CD133-specific aptamer conjugated with doxorubicin

demonstrated that targeting both CSCs and the TME may represent the best option in the anticancer approach. In regards to targeting CSC regulating pathways, a combination of agents which can affect multiple cellular signaling pathways is likely to be the most robust targeting strategy. Since CSCs regulate myriad cellular functions, conventional therapies could also be combined with novel treatment options to overcome resistance mechanisms induced by the conventional therapies such as sorafenib, the first-line drug for HCC. PDOs can be used to study how CSCs become resistant to therapy and how this information can be used to develop new strategies to counteract resistance. Xian et al. has been demonstrated that acquired sorafenib resistance in PDOs promotes HCC aggressiveness via facilitating stemness, dedifferentiation and EMT. However, specific targeting the mammalian target of rapamycin (mTOR) signaling pathway has been shown to be effective in treating acquired sorafenib-resistant HCC-derived PDOs, possibly via inducing phosphorylated S6 kinase [126]. Using a combinatorial CRISPR-Cas9 screen to identify druggable targets that synergize with sorafenib, Xu et al. found that NMDAR1, a glutamate receptor, could be a potential target for HCC therapy. Combination of ifenprodil, a clinically approved NMDAR antagonist, and sorafenib significantly reduced tumor growth on HCC-derived PDOs. Authors attributed these magnified

effects to the upregulation of unfolded protein response, which triggers the arrest of the cell cycle, and the downregulation of genes linked to Wnt-signaling and stemness. They suggested that ifenprodil could be repurposed as an adjunct to sorafenib for HCC treatment, as it has a known safety profile and could improve the clinical outcome and prevent tumor recurrence [127]. Another study on HCC-derived PDOs demonstrated that the induced sensitivity to sorafenib by GANT61, an Hedgehog signaling inhibitor, was correlated with a decrease in stemness features (SOX2, NANOG, OCT4) [87].

Ongoing studies are also investigating the potential of regulating reactive oxygen species (ROS) levels in the TME of CSCs as a viable approach for cancer treatment since maintaining low ROS levels in CSCs preserves stemness and associated therapy resistance [128]. In this context, Wang et al. have reported that the novel ROS-modulating agent LBL21 has promising anticancer activity by effectively eliminating stem-like cancer cells [129]. Despite these advancements, the precise mechanism of regulating ROS levels in CSCs remains unclear. By silencing kinesin family member 15 (*KIF15*) in HCC-derived PDOs, Li et al. revealed that KIF15 markedly decreased intracellular ROS levels, by inhibiting proteasomal degradation of phosphoglycerate dehydrogenase (PHGDH). This process, in turn, promoted the CSC phenotype characterized by the expression of CD133, CD44, CD24, CD90, and EpCAM, along with enhanced malignancy features, such as tumor initiation, self-renewal, metastatic potential, and resistance to therapy [130].

Investigating the intricate connection between CSCs and the TME in PDO model could lead to identify new CSC molecular markers, to decode the mechanisms that drive CSC plasticity and develop innovative strategies to disrupt CSC niches. Addressing these challenges using suitable preclinical models, such as PLC-derived PDOs, is decisive to advancing our knowledge of CSCs and their interactions with the TME, thus paving the way for more effective CSC-targeted therapeutic approaches.

5 Challenges and Hurdles: Bridging Gaps in Organoid Technology

While PDOs have emerged as promising tools for PLC studies, their full potential remains constrained by limitations in culture conditions, reproducibility, and scalability. Effectively incorporating organoids into liver cancer treatment approaches for clinical use requires thorough preclinical and clinical validation to ensure both their effectiveness and safety.

5.1 Seeding the Future: Enhancing Success in Establishing PLC-Derived PDOs

PLC-derived PDOs could be derived from patients with all major underlying liver diseases and different clinical stages of the tumors, demonstrating the potential of the organoid technique for building up larger biobanks representing the entire clinical spectrum of liver cancer. However, liver PDO culture faces challenges in achieving high success rates compared with other tumor organoids such as pancreatic PDOs (75–83%) and colorectal PDOs (90%) [78]. The success rate, notified, in few works, varies considerably between hepatocyte and cholangiocyte origin. Indeed, HCC PDOs generation exhibit a particularly low rate of around 26% from needle biopsies [81, 131] or 29 to 50% from surgical resection pieces of approximately 1–4 cm^3 [80, 87, 126], while CCA organoids have a success rate of 50% [86].

While the success rate of culturing PLC-derived PDOs was first not significantly associated with a range of clinically relevant patient characteristics [81], more recent work has shown that larger tumor size, microvascular invasion, macrovascular invasion, advanced TNM stage, and advanced Barcelona Clinic Liver Cancer (BCLC) stage were associated with successful development of HCC-derived PDOs [126]. There are many reasons for this low success rate of PLC-PDOs establishment (Fig. 4):

(i) HCC organoids can be generated only from a restricted subset of HCCs. Thus, it has been demonstrated that the rate of organoid establishment from PLC tissue is strongly correlated with the histopathological grading of the HCCs. Indeed, only moderately to poorly differentiated tumors (Edmondson grades III and IV) with a KI-67 index > 5% were able to generate HCC-derived PDOs. It is conceivable that the generation of HCC PDOs requires a cell proliferation rate threshold that is not reached in highly differentiated, slow-growing Edmondson grade I and II tumors [80, 81, 131]. More importantly, multiple prior studies support the notion that the presence of CSCs, based on the expression of CD133, CD90, CD44, and EpCAM, is correlated with poor differentiation and aggressive phenotype of patients with HCCs and lower overall survival compared to well-differentiated HCCs [66, 69, 70, 126, 132]. Thus, HCC tissues with stronger stem features and proliferative capacity are more likely to succeed in establishing PDOs. This conclusion is supported by a recent study from Ren et al., which analyzed the molecular characteristics of 72 BTC tissues (including 57 iCCAs) that yielded successful or failed PDOs cultures. This work showed that tumor tissues with enhanced stemness- and proliferation-related gene

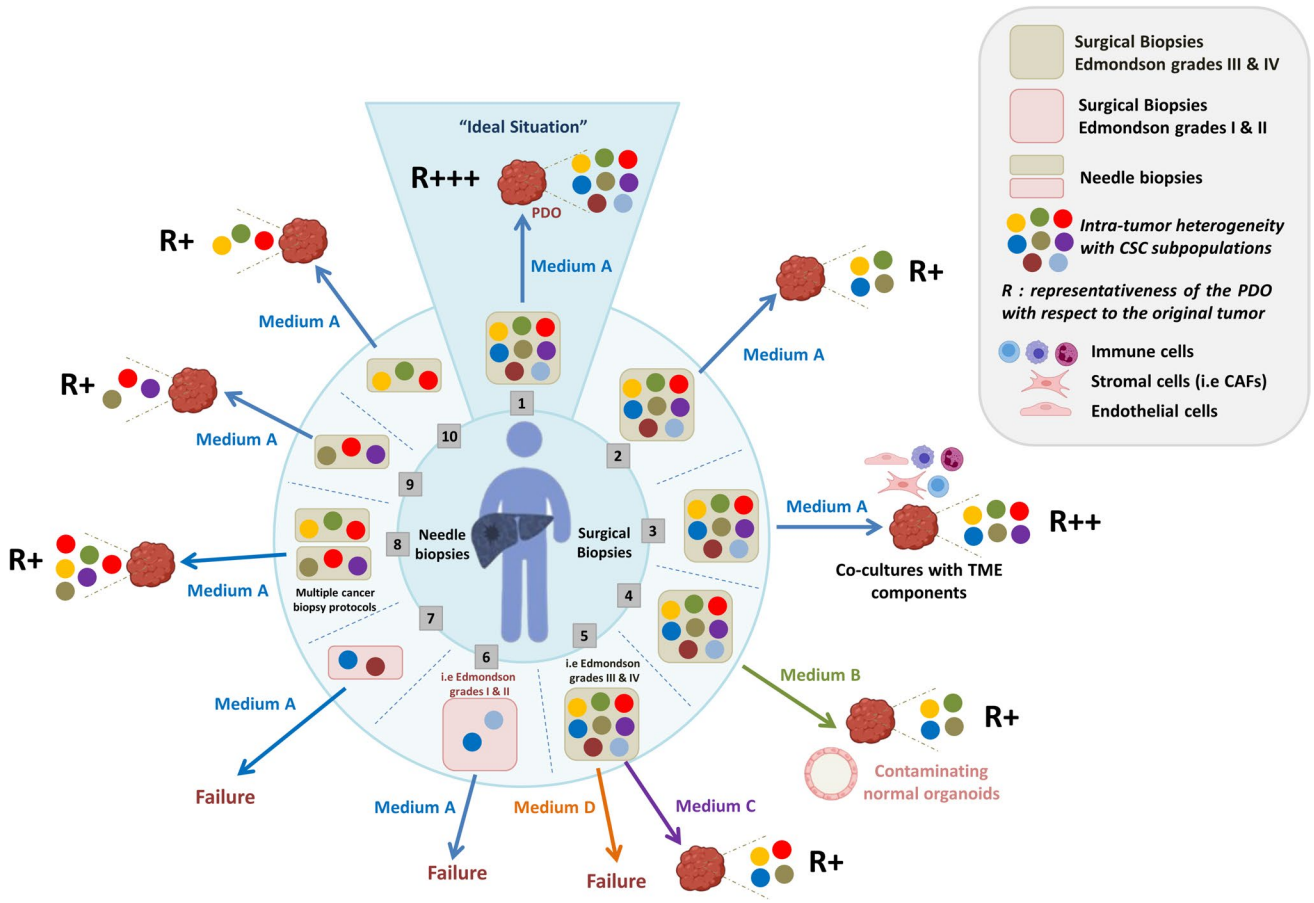

Fig. 4 PLC-derived PDO culture faces challenges, both in achieving high success rates and in faithfully representing the CSC related heterogeneity of original tissue, thereby biasing the response of tested anticancer therapeutics. Case 1 represents the ideal situation where the PDO accurately reflects the CSC composition of the original tumor. Several factors influence this outcome: **A** stemness rate in the biopsy. The establishment of HCC PDOs is closely linked to the histopathological grade, stemness features, and proliferation capacity of the original tumor. Poorly differentiated tumors with stronger stem features are more likely to form PDOs (e.g., Edmondson grades III and IV, case 2) compared with highly differentiated Edmondson grade I and II tumors in HCCs (case 6). **B** Insufficient amounts of tissue with CSC features. The availability of fresh tumor tissue containing viable CSCs is essential for successful PDO production. In addition to resected tumors (cases 1–6), needle-biopsies (cases 7–10) offer a less invasive alternative but present drawbacks, such as limited material and CSC subpopulations. Consequently, needle biopsies may fail to generate PDOs (case 7) or inadequately capture the full spectrum of tumor heterogeneity (cases 9–10), contributing to variability in PDO accuracy, transcriptomic fidelity, and drug response prediction. Using multiple biopsy protocols (case 8) can help ensure that functional intratumor heterogeneity is well represented in PDO-driven platforms. **C** Contamination by epithelial organoids. Healthy epithelial organoids often outgrow tumor-derived PDOs due to the slower proliferation of cancer cells (case 4). Modified protocols, such as extending tissue digestion and using specifically designed specific isolation media, help to suppress normal organoids. However, even these improved methods do not guarantee successful PDO generation from all tumor samples (case 5). **D** Current uncontrolled and non-standardized protocols, including the culture medium (i.e., medium A, B, C, or D). Variability in culture media and protocols exerts selective pressures on PDOs, which can bias the representation of tumor subclones and hinder genetic fidelity to the original tumor (cases 2, 4, 5). **E** The absence of tumor micro-environment components. Cellular co-cultures of PDOs enriched with a specific cell (mainly stromal, endothelial and immune cells) overcome partly this limitation (case 3). *CSCs* cancer stem cells, *HCCs* hepatocellular carcinoma, *PDOs* patient-derived organoids, *PLCs* primary liver cancers

can more easily form PDOs [93]. A similar finding was observed by Xian et al. demonstrating that the higher success rate of PDO generation is associated with the presence of more aggressive PLC tumor cell subpopulations in these samples, with elevated expression of genes associated with stemness and proliferation, such as *POSTN*, *SLC1A7*, *MMP12*, *TREM1*, and *CLEC5A* [126].

(ii) Another major obstacle to establishing successful PDO cultures is the limited availability of fresh tumor tissue containing viable tumor cells presenting stemness features. While traditionally derived from resected tumors, PLC-derived PDOs can also be generated from minimally invasive fine-needle biopsies [81], expanding access to a broader patient population. However, this biopsy approach presents limita-

tions. The amount of material obtained is often insufficient, and more specifically the number and types of CSC subpopulations captured can be limited, both of which affect the success rate of generating PDOs. Additionally, owing to intratumor heterogeneity, biopsies from different tumor regions often contain distinct cellular populations, leading to a variety of PDOs. This inherent intratumoral heterogeneity contributes to the observed variability in drug responses across these models [88]. This variability may explain why some studies report transcriptomic differences between primary tumors and corresponding PDOs. These differences can impair the ability of organoids to accurately reflect the parental tumor and limit their usefulness in guiding clinical treatment [86]. Thus, the derivation of PDOs from multiple tumor locations of individual patients may allow better estimation of intratumoral heterogeneity and risk of resistance, thus contributing to improved treatment allocation. In all cases, increasing the tumor tissue available for PDOs production would lead to a greater success rate and better ameliorate the similarity in term of heterogeneity between PLC-tissues and their paired PDOs.

(iii) A crucial factor for failure of PDO establishment is via contamination by normal epithelial organoids [133]. Owing to the gradual telomere shortening [134], PDOs grow at a slower rate than normal epithelial cells and are often out competed by them [86]. This is a problem which plagues all organoid cultures despite efforts to refine the tissue extraction process to minimize contaminating cells. In their study on PLC-derived PDO production, Broutier et al. [80] observed that healthy contaminating organoids can rapidly outcompete tumor-derived organoids when cultured in a "Classical human liver organoid isolation medium" (Classical IM), whose composition is relatively similar to that employed by other research groups [81, 88] (Table 1). To address this, they proposed manually separating healthy liver-derived organoids from tumor-derived organoid cultures based on visual inspection. Initially, healthy liver-derived organoids were distinguished by their typical morphology, a single layer of ductal-like epithelial cells surrounding a central lumen, while tumor-derived organoids generally formed compact structures. However, this strategy has limitations, as tumor tissues can also exhibit a monolayered cystic morphology depending on their differentiation state (e.g., poorly or moderately differentiated tumors), as demonstrated in studies by Saito et al. [135] and Broutier et al. [80]. To prevent the growth of these healthy contaminating organoids, the researchers modified their derivation protocol in several ways. They extended the tissue digestion time from under an hour to more than 2 h or even overnight. They further adjusted the initial culture conditions by removing R-spondin-1, Noggin, and Wnt3a, and incorporating dexamethasone as a supplement. This "tumoroid-specific isolation medium" selectively generates tumoroids; however, it did not successfully result in tumoroid generation from all tumor specimens.

(iv) Current uncontrolled and nonstandardized protocols for organoid culture, including in particular the culture medium used, largely affect the rate of PDO generation and genetic disparity to the original tumor. Actually, there are no defined culture conditions to grow PDOs specific to different type of tumors. Studies have shown that the composition of the medium used to culture cancer organoids can exert selective pressure on them, which can influence the genetic landscape of PDOs by favoring the growth of certain tumor subclones over others [136]. This can lead to either the failure to establish PDOs [80] or the establishment of PDOs that do not accurately represent the original tumor.

5.2 Recreating the Ecosystem: Optimizing TME in PDOs

Cancer is now considered a TME disease, although it was originally thought to be a cell and gene expression disorder. The intricate and varied composition of the TME, encompassing a variety of cellular (epithelial cells, fibroblasts, stem cells, endothelial, and immune cells) and non-cellular (ECM, cytokines, chemokines, and growth factors) components, holds pivotal roles in tumor development and progression and therapy resistance. This resistance against chemotherapy, radiotherapy, and immunotherapy is highly influenced by the TME, which provides a survival niche for CSCs to maintain the immature CSC phenotype and influence CSC plasticity [44, 45, 52]. Therefore, understanding the mechanisms underlying the interaction between the TME and CSCs is essential to better study phenotypic heterogeneity, cell plasticity and develop strategies to overcome therapeutic resistance in cancer [137].

Owing to the limitations of 2D models, 3D organoid cultures are gaining prominence as tools for modeling the TME and evaluating cancer therapeutics. However, reconstructive methods for establishing PDOs in submerged Matrigel have only focused on expanding the epithelial counterpart of the tumors and generally fail to preserve the non-cancerous cells present in the TME, which are rapidly lost in the PDO cultures. The dense stroma, observed in PLCs, includes fibroblasts, immune cells and ECM, creating a complex ECM,

each component of which has a distinct role in promoting cancer development, invasion and metastasis, as well as chemoresistance [138]. Given that CSCs rely heavily on the TME to maintain their stemness [139], the absence of fibroblasts, vascular structures, or immune cells components in a single 3D PDOs system represents a major drawback for recapitulating the heterogeneity of CSCs in the tumor of origin, thereby biasing the response of tested anticancer therapeutics [140, 141]**.** To overcome this limitation, researchers have optimized PDO culture technology, developing cellular cocultures of PDOs enriched with a specific cell (mainly stromal cells, ECs, immune cells and pathogens), associated with sophisticated approaches, such as microfluidic devices and bio-printer cultures.

CAFs provides a survival niche for CSCs [53]. CAFs have been shown to maintain cancer stemness by directly interacting with cancer cells or secreting growth factors, such as IL-6, chemokine (C-C motif) ligand 2 (CCL2), and hepatocyte growth factor (HGF), to upregulate Notch and Wnt stemness signaling [142–145]. Building on this, Liu et al. successfully established 3D coculture models of PLC-derived PDOs and CAFs [146]. This model, applicable to both mice and humans, delves deeper into the intricacies of interactions between liver cancer cells, particularly CSCs, and CAFs. The study revealed that CAFs not only boosted the growth of PDOs but also enhanced their stemness features, as evidenced by increased expression of markers, such as Lrig1, Muc5ac, CD133, TERT, and NANOG, as well as an increase in tumorigenic potential. Furthermore, the coculture model demonstrated how PDOs acquired resistance to various drugs, such as sorafenib, regorafenib, and 5-FU through both direct cell-cell contact and paracrine signaling mechanisms. In that regard, Loh et al. enhanced hepatocyte resistance to sorafenib by culturing HCC-derived PDO in conditioned medium to mimic the TME. This approach revealed a critical role for Follistatin-like 1 (FSTL1), a pro-inflammatory factor, which was predominantly secreted from the CAFs, to enhance stemness in HCC via deregulated AKT/mTOR/4EBP1 signaling pathways [147].

The combination of an anti-VEGF (bevacizumab) and an anti-PDL1 (atezolizumab) has become the standard of care for the treatment of unresectable HCCs highlighting the importance of the vascular sector and immune system in HCC [148]. Researchers are actively developing techniques to enhance vascularization in organoids, including: endothelial cell cocultures, microfluidic devices, tumor-on-a-chip, 3D bioprinting with pre-formed vascular networks [34, 149, 150]. New research indicates that ECs, beyond their known structural role in forming blood vessels, may be a source of angiocrine factors that could influence the behavior of cancer cells and other stromal cells within the TME, impacting tumor progression and therapy resistance [55, 151–153]. Similarly, the lack of an immune system in current PDO models hinders the study of immunotherapy. In 2022, Zhou et al. demonstrated the feasibility of creating co-culture PDOs from iCCAs with allogenic PBMCs. By optimizing culture conditions they successfully maintained the 3D structure of organoids while enabling immune cell interaction with tumor cells. The coculture system demonstrated an effective antitumor organoid immune response with cytotoxic effects mediated by direct cell–cell contact and the release of soluble factors. This innovative approach eliminates the need for patient-specific blood (autologous), simplifying the process and potentially broadening its applicability [154].

Thus, PLC-derived PDOs represent patient-specific platforms for preclinical testing of angiogenic-, immune- and chemotherapies, leading to individualized treatment decisions.

6 From Bench to Bedside: Bridging the Gap Between PDO-Based Drug Response Assessment and Clinical Application

Beyond current challenges related to the representativeness of the TME in PDO models, the undeniable potential of PDOs for personalized medicine faces a critical challenge: time. Delays in cancer treatment worsen prognosis and often require harsher treatments with more severe side effects. A 4-week delay in treatment can significantly increase mortality across various cancers [155]. Thus, the window of opportunity is narrow, and PDO models must deliver results quickly to inform treatment decisions within clinical timelines. A major obstacle is the expansion bottleneck. Solid tumors, particularly those derived from core needle biopsies, often require 4-12 weeks [81] for sufficient PDO growth to enable drug testing, exceeding acceptable timeframes. Automated organoid platforms offer solutions by enabling rapid production of standardized PDO batches, improving consistency across labs, reducing human error, and producing more reliable, reproducible results. For example, Linsen et al. recently introduced an automated system for sample storage and retrieval [156], significantly reducing handling time. Furthermore, advancements in miniaturization, microfluidics, and robotics, such as liquid-handling robots [157], are under development to allow testing of more molecules with fewer PDOs. Combi-seq is a scalable microfluidic process capable of screening hundreds of drug combinations within picoliter-sized droplets [158], using transcriptome changes as measurable outputs for drug effects. Automated microfluidic platforms also enable sequential delivery of chemotherapeutics in PDOs, replicating real-world treatment combinations in a patient-specific manner [159]. Combined with artificial intelligence, these methods provide rapid, cost-effective assessments of treatment responses, reducing

response times [160]. For example, the Quadratic Phenotypic Optimization Platform (QPOP) efficiently identifies optimal drug compositions and doses from a pool of candidates without requiring mechanistic insights [161]. QPOP identified dinaciclib and ixazomib as an effective combination against HCCs. Validation in HCC-derived PDX organoids confirmed that this combination induces stronger pro-apoptotic and antiproliferative effects compared with sorafenib, likely via the JNK signaling pathway [162]. High-throughput optical imaging of drug response in patient-derived organoids could enable patient-specific drug testing and help clinicians select the optimal drug regimen for each cancer patient. Identifying specific transcriptomic signatures in PLC-derived PDOs will streamline the drug selection process, leading to more efficient identification of effective treatments tailored to individual patients.

Once promising drug combinations have been identified using patient-derived organoids, it becomes crucial to evaluate their effectiveness and clinical applicability. In vitro studies should transition into in vivo experiments using animal models to assess the therapeutic effects observed in organoids under more complex biological conditions. Furthermore, clinical trials play an essential role in validating the findings from preclinical studies. Incorporating information gleaned from organoid testing into trial design can enhance the selection of eligible participants and inform treatment regimens. This translational approach ensures that the insights gained from organoid research not only remain on paper but also find their way into practical and effective cancer treatment methodologies. By adopting a patient-centric strategy, where treatments are based on individual tumor profiles, the field of liver cancer therapy stands to benefit immensely, ushering in a new era of personalized medicine.

7 A Look Ahead: Future Perspectives

Preclinical research using human cancer cell lines and mouse models has led to beneficial discoveries for patient care, but the high failure rate of clinical trials shows the need to improve preclinical investigations. Of note, a large analysis of clinical trial data (406,038 entries for 21,143 compounds) revealed a concerningly low success rate for cancer treatments (3.4%), compared with an overall success rate of 13.8% for all other diseases combined [163]. Systemic therapies for cancers are inefficient because of the disease's considerable heterogeneity and its tendency to develop drug resistance, which leads to therapeutic failure. Accumulating evidence has shown that CSC subpopulations have distinct functional roles in tumor development but remain difficult to identify with current CSC markers. Moreover, their maintenance/regulation in the TME remains unclear. This evidence further support the need for patient-tailored therapeutics. Understanding how the TME affects the properties of liver CSCs could open up new possibilities for innovative therapeutic strategies. Furthermore, the plasticity of CSCs, allowing them to switch between CSC and non-CSC states, emphasizes the need for treatments that can adapt to this dynamic nature, targeting both CSCs and transitioning tumor cells.

Better preclinical models are essential for assessing therapy efficacy and identifying the patients who would benefit the most. PLC-derived organoids (PDOs) from PLC tissue preserve the histological structure, genetic profile, and mutational landscape of their original tumors. Displaying genetic and phenotypic stability, even after long-term in vitro expansion, PDOs can be frozen and recovered, enabling the creation of living PDO biobanks. Thus, PDOs, covering the three common subtypes of liver tumors (HCC, iCCA, and CHC), have been successfully produced from tumor tissues, representing early-stage disease, advanced cancers and highly chemoresistant cancers. These PDO models, which enable tumors to be modelled more accurately, are more appropriate for in vitro tumor biology and research related to treatment. Since PDOs provide biological links with patient data, PDOs have become avatars for precision cancer therapy. This cancer model is valuable for finding genes with prognostic significance and potential new therapeutic targets for PLCs. The PDO model also enables drug sensitivity testing, allowing the link between treatment efficacy and the individual tumor's genetic profile. By identifying specific transcriptomic signatures and adjusting dosage regimens in vitro, the PDO model makes it possible to optimize therapeutic results through personalized treatment strategies.

Challenges remain in liver PDO culture, including achieving high success rates compared with other tumor organoids. Successful organoid generation is related to the level of stemness rate in PLC tissues, highlighting the impact of the number and/or specific types of CSC subpopulations in the tumor samples required. The low success rate in generating PDOs may also depend on criteria specific to the nature of PLCs, which need better understanding.

To address the lack of stromal components in current PDOs, advanced PDO models have been developed, including the TME to provide a niche for CSCs, affecting CSC plasticity and therapy resistance. Coculturing PDOs with specific stromal cell types and using bioprinter approaches, microfluidic cultures and air-liquid interface (ALI), are promising methods for better reproducing physiological components of bulk tumors. Advanced PDO models will be more adept at replicating the traits of the initial tumor stem cells. This proficiency is fundamental for identifying and characterizing subpopulations of CSCs, comprehending stem cell hierarchies, and investigating the mechanisms governing cell plasticity that drives therapeutic resistance. Consequently, they will contribute to a better understanding

of stemness-related drug resistance and develop start-up strategies to target CSCs and overcome therapeutic resistance in PLCs. The development of high-quality TME PDO models is therefore essential for converting in vitro drug screening results or immunotherapy responses into reliable clinical predictions.

Thus, the ability of PDOs to bridge the gap between tumor biology and PLC patient data unlocks a new era of personalized cancer therapy, where treatment is guided by individual patient avatars.

Declarations

Funding Open access funding provided by Université Paris-Saclay. This work is part of the PREMELICAN (Precision Medicine in Liver Cancer) project, supported by funding from AP-HP Carnot Institute.

Conflict of interest Julien Giron-Michel, Maël Padelli, Estelle Oberlin, Hind Guenou, and Jean-Charles Duclos-Vallée have no conflicts of interest that are directly relevant to the content of this article.

Ethics approval No ethics approval was required.

Consent to participate Not applicable.

Consent for publication Not applicable.

Availability of data and materials All data represented are available in the public domain.

Code availability Not applicable.

Author contributions J.G.M. wrote the manuscript and designed the figures; J.G.M., M.P., E.O., H.G., and J.C.D.V. edited the manuscript. All authors read and approved the final manuscript.

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