Organoids for the Study of Liver Cancer

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Abstract

Liver cancer is the second most lethal malignancy worldwide. Cell lines and murine models are the most common tools for modeling human liver carcinogenesis. Most recently, organoids with a three-dimensional structure derived from primary tissues or cells have been applied to liver cancer research. Organoids can be generated from induced pluripotent stem cells, embryonic or adult, healthy or diseased tissues. In particular, liver organoids have been widely employed in mechanistic studies aimed at delineating the molecular pathways responsible for hepatocarcinogenesis. The introduction of clustered regularly interspaced palindromic repeats (CRISPR)-associated protein 9 (Cas9) and microengineered miniorganoid technologies into liver organoids for cancer study has significantly accelerated these investigations. Translational advances have been made by utilizing liver tumor organoids for anticancer drug screening, biobanking, omics profiling, and biomarker discovery. This review summarizes the latest advances and the remaining challenges in the use of organoid models for the study of liver cancer.

Liver cancer is the sixth most common cancer and the second most lethal malignancy worldwide.[1] Primary liver cancer (PLC) includes a heterogeneous group of tumors with distinct histological features and clinical outcomes, with hepatocellular carcinoma (HCC) representing 80% of all PLCs, and intrahepatic cholangiocarcinoma (iCCA) covering most of the remaining cases.[2] A combined hepatocellular–cholangiocarcinoma (HCC/CC) subtype has also been identified among PLCs.[3] PLCs are characterized by a complex and diverse genetic landscape of alterations, including high degree of aneuploidy, DNA

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copy number variations, somatic mutations, and epigenetic changes, all contributing to hepatocarcinogenesis at various degrees.[4] This molecular heterogeneity implies the need for patient-tailored treatments, an approach known as precision medicine.[5] Frequent genetic alterations in PLCs include, among many, *TP53*, cell cycle-related genes, such as *CCND1* and *CDKN2A*, and chromatin-remodeling genes *ARID1A* and *ARID2*.[4,6] HCC is further characterized by mutations leading to the activation of WNT/β-Catenin pathway and *TERT*, whereas mutations of *IDH1/2* and *KRAS* genes, as well as *FGFR2* fusions frequently occur in iCCA.[7–9]

Both in vitro and in vivo models have been employed to study liver cancer pathogenesis. In vitro techniques include cell lines and organoids, whereas in vivo techniques include genetic modified animals and patient-derived xenograft (PDX) models. Among them, the organoid system has increasingly been used to investigate the molecular mechanisms associated with liver carcinogenesis and examine the therapeutic efficacy of anti-tumor therapies.[10,11]

Liver Organoids as a Model for Liver Disease Investigation

An organoid is defined as a three-dimensional (3D) structure derived from primary tissues or cells. It consists of organ-specific cell types, thus mimicking in vivo tissue structures.[12–14] Organoids can be propagated for a relatively long time while maintaining their cellular complexity and 3D structure. They can be amplified from a small tissue sample and adapted to high-throughput research. In the last few years, a wide variety of human and murine organoids have been established from different organs to study numerous physiologic and pathologic conditions.[14]

Embryonic stem cells (ESC), induced pluripotent stem cells (iPSC), and primary tissues are the main sources for the generation of tissue-specific organoids.[15] The culturing and expansion of normal liver organoids was first achieved by Huch et al with mouse Lgr5+ liver stem cells.[16] Subsequently, Takebe et al generated liver organoids from human iPSCs in combination with endothelial and mesenchymal cells in a Matrigel matrix.[17] Two years later, the Clevers' group reported a novel in vitro system to generate long-term organoid cultures derived from human bipotent progenitor liver cells.[18] The authors optimized the culturing system and provided protocol for culturing and establishing self-renewing human and mouse adult liver organoids.[18] Liver cells can grow in a gel-based extracellular matrix (ECM) when supplemented with essential growth factors, including hepatocyte growth factor (HGF), epidermal growth factor (EGF), fibroblast growth factor 10 (FGF10), and R-spondin-1 (Rspo1).[19] However, these liver organoids are primarily generated from biliary epithelial cells (BEC). Aiming at establishing a long-term expansion system for hepatocyte originated liver organoids, Hu et al established a protocol for generating hepatic organoids from Axin2+ hepatocytes with a modified culture medium. In brief, Wnt agonists such as Rspo1 and CHIR99021; EGF, FGF7, FGF10, and transforming growth factor-α (TGF-α); ROCK inhibitor Y27632; HGF and TGF-β inhibitor A83–01 were supplemented to the medium. The study by Hu et al emphasized the critical role of Wnt/Rspo1 and HGF signaling pathways in regulating primary hepatocyte expansion in both mouse and human organoid models.[20] In parallel, Peng et al revealed that tumor necrosis factor-α (TNFα) enabled the in vitro expansion of primary mouse hepatocytes in 3D culture in the

absence of Rspo-1. Single-cell RNA sequencing (RNAseq) of these 3D culture hepatocytes demonstrated the expression of numerous hepatocellular markers but not of other cell types.[21] Since then, several protocols for normal liver organoids have been established by modifying the culturing system or the stem cells oforigin.[22–24] For instance, Sun et al transfected fibroblasts with FOXA3, HNF1A, and HNF4A and expanded them by SV40 large T antigen (SV40LT) to generate human-induced hepatocytes (hiHeps). These cells were able to self-organize into liver organoids with hepatic architecture and functions in a hepatocyte-maintaining medium.[25]

Thus, in summary, liver organoids were developed using several approaches and cells of origin (►Fig. 1) and are widely applied for functional studies.[26]

Generation of Liver Tumor Organoids for the Study of Human Liver Cancer

Early studies on liver organoids have been focusing on testing and validating the accuracy and genetic features of the system. Recently, human and mouse liver tumor organoids (also termed as "liver tumoroids") have been established through various methods (►Fig. 1 and ►Table 1).

Patient-Derived Liver Cancer Organoids

In 2017, Broutier et al reported the development of long-term organoid cultures derived from patients with PLC.[27] Specifically, patient-derived organoid (PDO) cultures were established from eight individuals with PLC, belonging to three tumor histotypes (HCC, iCCA, and HCC/iCCA). Using histological, immunohistochemical, and genomic analyses, the authors showed that PDOs retained the organ site-specific histologic and genetic traits. In addition, PDOs exhibited a similar biological behavior when transplanted into immunocompromised mice.[27] In a similar study, Nuciforo et al reported long-term organoid cultures from needle biopsies of HCC patients with various etiologies and tumor stages. Similar to that described by Broutier et al, HCC organoids retained the morphology and the expression pattern of HCC tumor markers and preserved the genetic heterogeneity of the originating tumors. Interestingly, the success rate for the generation of HCC organoids was 26% based on the number of cultured tumor biopsies (10 of 38 HCC biopsies),[28] a percentage that is significantly lower when compared with the reported success rates for pancreatic cancer (75–83%)[29] and colorectal cancer (90%).[30] The lower success rate using HCC specimens may be due to the fact that hepatocytes, the cell of origin of HCCs, lack the features of epithelial stem cells needed for their propagation in the organoid culture system.[31] Of note, the study by Nuciforo et al adopted the system developed by Broutier et al.[27] Although the authors attempted to optimize the system to improve the success rate by removing compounds with potential negative effects on HCC proliferation such as Forskolin, N-acetyl-L-cysteine, nicotinamide, and HGF from the medium, and adding FGF19, a factor with potential growth-promoting effects for HCCs, these efforts had limited effects on the establishment of additional HCC organoid lines.[28] These protocols have been subsequently applied in several studies.[32–36]

In addition to the traditional Matrigel organoid system, Fong et al attempted to generate HCC patient-derived xenograft originated organoids (HCC-3DPDX) by applying

a 3D sponge system. The system consisted of a cellulose-based hydrogel with interconnected macropores fabricated by leveraging the ability of hydroxypropyl cellulose (HPC) derivatives to undergo thermal-induced phase separation and photo-crosslinking. Methacrylate (MA) was used in the photo-crosslinkable group and dichloromethane was used for the reaction to synthesize MA-HPC in the system. In total, 14 HCC-3DPDX lines were generated from the authors' previously built HCC-PDX biobank. Organoids rapidly formed by Day 2 and remained viable for 1 week, and a few lines maintained viability for almost 3 weeks in culture.[37] HCC-3DPDX lines feasibly maintain various cellular compartments of HCC samples and retain genomic profiles and intratumoral heterogeneity of their in vivo counterpart. Therefore, they might represent a promising tool to determine individualized treatments. However, technical complexities of this bioengineering approach may restrict its widespread application.

Murine Liver Tumor-Derived Organoids

Cao et al reported the establishment of organoids from mouse primary liver tumors induced by the hepatocarcinogen diethylnitrosamine (DEN).[38] The author succeeded in culturing 91 lines from 129 liver tissue/tumors. These organoids could be grown in long-term cultures in vitro and ~20% formed tumors in immunodeficient mice upon serial transplantation, confirming their tumorigenic and self-renewal properties. Interestingly, single cells from the tumor organoids had high efficiency of organoid initiation, and a single organoid derived from a cancer cell was able to initiate a tumor in mice, indicating the enrichment of tumor-initiating cells in the tumor organoids. Furthermore, these organoids recapitulated, to some extent, the heterogeneity of liver cancer in patients, with respect to phenotype, cancer cell composition, and treatment response.[38] Recently, Saborowski et al were able to establish a iCCA organoid from the mouse iCCA model consisting of $Kras^{G12D}$ mutation and loss of $Tp53$.[39] The organoid was cultured using the protocol from Broutier et al and used to probe candidate cancer in genes in vivo by applying RNA interference or CRISPR/Cas9 technology in liver organoids. In a proof-of-principle study, they showed that loss of function mutation of phosphatase and tensin homolog (Pten) either by shRNA against $Pten$ or CRISPR/Cas9 mediated knockout of $Pten$ was able to promote iCCA organoid growth.[40]

Primary Human Hepatocytes (PHH)-Modified Liver Tumor Organoids

As described previously, Sun et al generated hiHeps via reprogramming fibroblasts.[25] These hiHeps were subsequently used to investigate the genes that may promote liver tumor imitation. Specifically, the authors found that HCC could be formed via c-MYC overexpression, while iCCA could be induced by oncogenic RAS-driven lineage conversion.[25] Studies on liver tumor initiation using hiHeps organoids are further discussed in the section "Study of liver tumor initiation and progression mechanisms."

Cancer Cells Self-Assembled Liver Tumor Organoids

Recently, Wang et al sought to demonstrate that liver nonparenchymal cells, including endothelial cells and fibroblasts, support HCC tumor organoid formation in vitro. For this purpose, HCC and nonparenchymal cells were coseeded in a thick layer of Matrigel. Strikingly, HCC and nonparenchymal cells self-organized into 3D cell clusters, exhibiting a remarkable intrinsic organizing capacity. At the molecular level, HCC cells cultured together

with nonparenchymal cells were characterized by higher expression of neoangiogenesis inducers (vascular endothelial growth factor 2 [VEGFR2], VEGF, hypoxia inducible factor-α), tumor-related inflammatory cytokines (CXCR4, CXCL12, TNF-α), and epithelial-mesenchymal transition markers (TGF-β, Vimentin, MMP9) when compared with organoids containing only HCC cells,[41] thus more closely recapitulating the alterations of the human disease. Although further validation is required, the described findings underline the usefulness of this system to study the interactions between cancer cells and nonparenchymal cells in hepatocarcinogenesis as well as for preclinical testing of innovative therapies.

Application of Liver Tumor Organoids for the Study of Liver Cancer

Liver tumor organoids have been used to investigate the mechanisms responsible for tumor initiation and progression. Extensive studies have also utilized liver tumor organoids to improve therapy response prediction in patients. Other applications of organoids for liver tumor include, but are not limited to, omics profiling, biomarker discovery, and biobanking (►Fig. 1 and ►Table 1).

Study of Liver Tumor Initiation and Progression Mechanisms

Cancer cell lines and organoids derived from PLCs roughly recapitulate the mutations and expression profiles of liver tumors but cannot be used to study cancer initiation. In contrast, organoids derived from normal liver tissues can be used to model liver cancer pathogenesis by sequentially introducing cancer driving mutations.[42] The advantage of using such models is that they enable the investigation of the effects of driver mutations in an isogenic genetic background. For instance, Sun et al transfected hiHeps originated liver organoids with c-MYC oncogene. Of note, forced transfection of c-MYC led to HCC formation in the system. Mechanistically, alterations of mitochondrial-associated endoplasmic reticulum (ER) membranes were observed, suggesting that excessive mitochondrial-ER coupling may be the key mechanism underlying c-MYC dependent hepatocarcinogenesis. In addition, a mutant form of RAS (RAS^{G12V}) was able to transform hiHeps into iCCA organoids. Subsequently, these iCCA organoids were able to form tumors in mice after orthotopic transplantation. Further analysis suggested that iCCAs developed via hepatocyte-based lineage conversion. At the molecular level, the NOTCH and JAK/STAT cascades were found to be responsible for the observed hepatocyte-iCCA conversion.[25] The results provide evidence that at least some human iCCAs may derive from mature hepatocytes. BRCA1 associated protein-1 (BAP1) is a deubiquitinating enzyme that is implicated in iCCA development.[43] In a recent study, using CRISPR-Cas9 based gene-editing, Artegiani et al deleted BAP1 in normal human cholangiocyte organoids.[44] It was found that deletion of *BAP1* alone led to loss of several epithelial characteristics of the organoids. When loss of *BAP1* was combined with other common iCCA mutations, including *TP53*, *PTEN*, *SMAD4*, and *NF1*, it resulted in iCCA development when the organoids were transplanted into mice.[44] Excitingly, Artegiani et al later reported a CRISPR-Cas9-mediated homology-independent organoid transgenesis (CRISPR-HOT) methodology for fast and efficient generation of knock-in human organoids.[45] Combining tubulin tagging with *TP53* knockout revealed that TP53 is involved in controlling hepatocyte ploidy and mitotic spindle fidelity, providing evidence that *TP53* mutation-induced aneuploidy may promote liver cancer.[45]

Thus, organoid technology in combination with CRISPR/Cas9 strategy is a powerful tool to investigate cancer gene function in a human context.[46]

PDO liver tumoroids are also widely used to characterize the molecular mechanisms leading to tumor progression. For instance, Chan et al demonstrated that silencing of protein methyltransferase 6 (PRMT6) induced cancer stemness in HCC-derived PDOs. Further analysis suggested that PRMT6 functions via CRAF-ERK signaling.[32] In a follow-up study, it was revealed that PRMT6 regulates ERK-dependent PKM2 expression and Warburg effect in HCC PDOs. The results provide a mechanistic link among tumorigenicity, sorafenib resistance, and glucose metabolism.[33] Similarly, using PDO iCCA organoids, Li et al reported that silencing of mitochondrial fusion regulatory genes, including optic atrophy 1 (OPA1) and mitofusin 1 (MFN1), inhibited the fusion process in iCCA tumor organoids. This led to the inhibition of cell growth in vitro and tumor formation in vivo after tumor cell engraftment in mice. Mechanistically, it was discovered that the increased mitochondrial fusion in liver cancer cells altered metabolism and fueled tumor cell growth.[34] Furthermore, Li et al silenced kinesin family member 15 (KIF15) in HCC organoids and revealed a significant reduction in sphere formation as well as the decreased expression of stemness-related genes. The results indicated that KIF15 might promote the CSC phenotype and malignancy in HCC.[47]

Hepatic and Biliary Lineage Commitment Studies

Both hepatocytes and cholangiocytes exhibit elevated plasticity, and cell fate conversion from hepatocytes to cholangiocytes has been reported under pathological conditions.[48] To investigate whether iCCA cells can be converted to functional hepatocytes, Saito et al established organoids derived from human iCCAs and cultured them under conditions suitable for hepatocyte differentiation. Noticeably, the organoids acquired functions of mature hepatocytes, such as albumin secretion, bile acid production, and increased CYP3A4 activity. Moreover, the malignant potential of iCCA cells was markedly reduced after hepatocytic differentiation. Removal of Rspo1, the ligand of LGR5, induced differentiation of iCCA cells into functional hepatocytes, suggesting that inhibition of the Wnt signaling pathway may induce hepatocyte differentiation.[18,49] Consistently, Rimland et al generated intrahepatic bile duct (IHBD) organoids using culture conditions promoting Wnt signaling. The authors found that IHBD organoids expressed the stem cell/progenitor markers LGR5 and PROM1 as well as the ductal markers KRT19 and KRT7. Interestingly, IHBDs were found to express low levels of hepatocyte markers under differentiation conditions, suggesting the critical role of Wnt signaling in promoting hepatocyte differentiation.[50]

Ochiai et al isolated mouse liver cells and cultured them with a modified protocol to generate cholangiocyte lineage committed organoids. The combination of *Kras* overexpression with suppression of one of the tumor suppressor genes commonly inactivated in iCCA (*Cdkn2a*, *Pten*, *Apc*, and *Tp53*) resulted in iCCA formation in these organoids, supporting the oncogenic potential of these genetic events for iCCA development. The authors also demonstrated that mutant Pik3ca and FGFR2-AHCYL1 fusion possess limited oncogenic potential in these liver-derived organoids, probably requiring additional mutations or a proper hepatic niche to induce iCCA formation.[51] Li et al generated a murine HCC

model consisting of codeletion of *Pten* and *Tp53* together with knocking-in prominin-1 (*Prom1*). HCC tumor organoids were subsequently derived from these mouse HCCs. Subsequently, the authors investigated whether these HCC organoids may acquire iCCA-like traits following their expansion and orthotopic transplantation in mice. Invasive and metastatic tumors developed following this procedure and exhibited iCCA-related pathologic and molecular features, which were absent in the primary HCC tumors. This model partly recapitulates the molecular features of human HCC/iCCA mixed tumors and suggest that this peculiar tumor subtype is the consequence of the evolution of HCC into another cancer entity with iCCA features.[52]

Drug Screening and Precision Medicine

The available options for PLC management have been elegantly reviewed elsewhere.[53,54] As for molecular therapies, sorafenib[55] and lenvatinib[56] multikinase inhibitors are first-line therapies, while regorafenib, cabozantinib, ramucirumab, and nivolumab are second-line therapies for HCC.[57] Excitingly, a recent phase III clinical trial (IMbrave150) showed that atezolizumab (an anti-PDL1 antibody) combined with bevacizumab (an anti-VEGF-A antibody) resulted in better overall and progression-free survival outcomes than sorafenib in unresectable HCC,[58] holding the promises for immunotherapy as a crucial therapy for HCC treatment. Pemigatinib,[59] a FGFR1–3 inhibitor, has been recently approved as the first ever target therapy for iCCA patients with *FGFR2* fusion mutations.

The tumor organoid culture preserves intratumor heterogeneity[60] and the tumor microenvironment.[61] Consequently, organoids may be better suited for drug screening than cancer cell lines; in addition, organoids are significantly cheaper and more efficient than murine tumor models. Organoids have also been applied as the platform to evaluate drug efficacy, opening the doors for precision oncology. Since the liver is the major organ responsible for drug metabolism, liver organoids have been also used to dissect the potential side effects of candidate therapies.

Broutier et al performed a proof-of-principle drug screening using 29 drugs and identified liver PDOs sensitivity to ERK inhibition, which was validated in xenograft models.[40] Chen et al showed that immunosuppressant mycophenolic acid (MPA) effectively inhibited the initiation and growth of mouse liver tumoroids.[62] It has been hypothesized that intratumor genetic heterogeneity may contribute to the high failure rate of chemotherapy. Recently, Li et al evaluated the functional heterogeneity in a cohort of primary human liver cancer organoids and how it might contribute to drug responsiveness. For this purpose, a total of 27 liver cancer organoids were established and tested with 129 cancer drugs. The results suggest that the intratumor drug response heterogeneity does not correlate with molecular profiles variations in these liver cancer organoids.[63] Additional studies identified nine pan-effective drugs from five classes of antineoplastic agents, including histone deacetylase inhibitors, proteasome inhibitors, DNA topoisomerase II inhibitors, protein translation inhibitors, and RNA synthesis inhibitors, as potential drugs for liver cancer treatment.[63] To better understand the limited efficacy of the multikinase inhibitor sorafenib on liver cancer, Lee et al established sorafenib-resistant HCC-PDO lines. They discovered that the Src homology 2 domain-containing phosphatase (SHP2) blocker SHP099 is able to

annul sorafenib resistance by impeding RTK-induced reactivation of the MEK/ERK and AKT signaling pathways. These encouraging results highlighted the potential application of liver tumor organoids for the study of chemoresistance and the development of alternative therapeutic approaches.[64] Another mechanism of resistance to sorafenib treatment by HCC was discovered by Wang et al.[35] The authors demonstrated that CD44-positive HCC PDOs are resistant to sorafenib, and sorafenib increases CD44 levels. A subsequent drug screening conducted by the same group showed that GANT61, a Hedgehog signaling inhibitor, potently suppresses HCC PDO viability. Noticeably, a highly synergistic, antigrowth effect in vitro and in vivo was observed when sorafenib and GANT61 were concomitantly administered to CD44-positive HCC PDOs and cell lines.[35] Together with studies on chemoresistance, investigation has been conducted using liver tumor organoids also to identify molecular markers of chemosensitivity. For instance, Wang et al generated four HCC PDO lines with differential long-chain acyl-CoA dehydrogenase (ACADL) expression and demonstrated that organoids with low expression of ACADL were sensitive to the YAP inhibitor Verteporfin.[36] Saito et al conducted a drug screening among 3 iCCA PDO lines among 339 clinical used medicines and identified 22 compounds able to suppress iCCA organoid growth. Among these compounds, anticancer agents such as antimicrotubule agents, tyrosine kinase inhibitors, antimetabolite agents, mammalian target of rapamycin (mTOR) inhibitors, and proteasome inhibitors were found. Intriguingly, they also proved two antifungal compounds (amorolfine and fenticonazole) as promising drugs against iCCA.[65] More recently, Antonia et al applied a high-throughput drug screening platform with AI-enhanced robotics to iCCA PDOs, reporting responsiveness to the mTOR inhibitor Sapanisertib (INK128) by all iCCA PDO lines. Intriguingly, INK128 was synergistic with gemcitabine in PDOs derived from one patient.[66]

Another prospective direction of applying organoids to the study of liver cancer is using omics profiling data gathered from organoid to indirectly guide drug repurpose. For instance, Guan et al performed single-cell RNA-sequencing and metabolomic analysis on developing organoid cultures at the iPSC, hepatoblast, and mature organoid stage. Data analysis indicated that early hepatoblasts are selectively sensitive to the cytotoxic effect of the antihistamine drug meclizine. Importantly, administration of meclizine in combination with the glycolysis inhibitor PFK158 suppressed liver carcinoma cell line growth in vitro and in a xenotransplantation model without major side effects, supporting the clinical efficacy of meclizine as a repurposed anticancer drug when used in combination therapy.[67]

Omics Profiling and Biomarker Discovery

Biomarkers have a pivotal function in assisting diagnosis and providing guidance in precision medicine. Indeed, the identification of reliable biomarkers able to accurately predict drug responsiveness is the key for effective cancer treatments. By using molecular profiling approaches, including transcriptomics, proteomics, and metabolomics, several studies revealed the promising application of liver PDOs as a biomarker discovery tool. Using liver PDOs in combination with genome-wide RNAseq analysis, Broutier et al reported C19ORF48, UBE2S, DTYMK (for HCC), and C1QBP and STMN1 (for iCCA) as biomarkers associated with poor prognosis for PLC.[27] Moreover, Li et al demonstrated that liver tumor organoids generated from high-grade tumors exhibited

significantly increased extracellular vesicle secretion, suggesting that the identification of tumor-specific extracellular vesicle proteins in plasma might be a promising tool for liver cancer detection.[52]

Discussion and Future Directions

Similar to that described in other organs and tumor types, organoid technologies are emerging as a powerful tool to model liver diseases, elucidate molecular mechanisms underlying hepatocarcinogenesis, and screen for drug sensitivity for liver cancer treatment. Despite the encouraging results reached using this technology to date, however, important limitations are still present and should be circumvented (►Table 2). In the setting of translational research, large sample sizes are required to ensure feasible resemblance of distinct genetic characteristics among tumors, to provide reliable drug screening results. Several large collections of pancreatic[68] and colorectal cancer[69] PDOs have been established for omics profiling, allowing to link genetic and transcriptional information to drug responsiveness. However, current high-throughput screening results for liver PDOs are still limited by the small sample sizes.[38] Therefore, the generation of liver tumor organoid biobanks is highly necessary. Another concern for applying organoid based drug screening results to the clinical setting is whether the in vitro outcomes truly represent patient responsiveness. Thus, clinical trials comparing the predictive value of the organoid treatment response to the clinical outcome of the corresponding liver cancer patients should be conducted.

Liver organoids are able to perform most hepatic functions, including albumin secretion, glycogen storage, and drug metabolism.[70] However, the capacity of liver organoids in terms of hepatic function is not comparable with that of the mature adult liver. Indeed, transcriptomic analysis showed that liver organoids are molecularly similar to fetal liver or hepatoblasts rather than to the adult liver.[16] Thus, the current in vitro technologies require further improvements to promote a complete maturation and performance of liver organoids comparable to those of the adult liver tissues. One important reason responsible for the different functionality between normal liver and organoids might be that liver organoids fail to recapitulate the complex network of the normal liver. Emerging studies have implied the possibility to integrate the microengineered miniorganoid systems (also called as "organoid-on-a chip") for liver organoid studies.[71,72] These novel technologies may provide new tools for improved engineered liver organoids. Furthermore, development of more comprehensive liver organoids including mesenchymal, vascular, and nervous components will presumably allow a better understanding of the hepatocyte-stroma crosstalk and the effects of their interplay. This approach could be highly relevant also to dissect out the functional interaction between liver cancer cells and the tumor microenvironment.

Recent advances in immunotherapy suggest that this treatment approach might become the first-line therapy for multiple tumor types, including HCC and iCCA.[73,74] One of the major limitations of liver tumor organoids is the lack of tumor infiltrating immune cells and, therefore, they may have limited value in studying immunotherapy or provide guidance in terms of immunotherapy effectiveness. In a recent study, it has been shown that tumor organoids can be reconstituted with native embedded infiltration of immune cells,

including T cells, B cells, NK cells, and macrophages.[75] Importantly, these reconstituted tumor organoids were able to accurately model the therapeutic features and effects of anti-PD1 and/or anti-PD-L1 antibodies.[75] Although additional studies supported the use of immune-enhanced tumor organoids for immunotherapy testing,[76,77] further investigation is needed on this topic.

Overall, liver organoids are an invaluable system to investigate and provide critical insights into liver cancer molecular pathogenesis, tumor diagnosis and prognosis, as well as preclinical drug testing.

Funding

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Main Concepts and Learning Points

- Liver organoids can be generated from induced pluripotent stem cells or liver tissue originated progenitor cells.

- HCC and iCCA organoids can be induced in normal liver organoids upon transfection with oncogenes or be isolated and cultured from human or murine liver tumor tissues.

- Liver organoids as well as liver tumor organoids are valuable systems to elucidate the molecular mechanisms of hepatic carcinogenesis and investigate the therapeutic strategies against liver cancers.
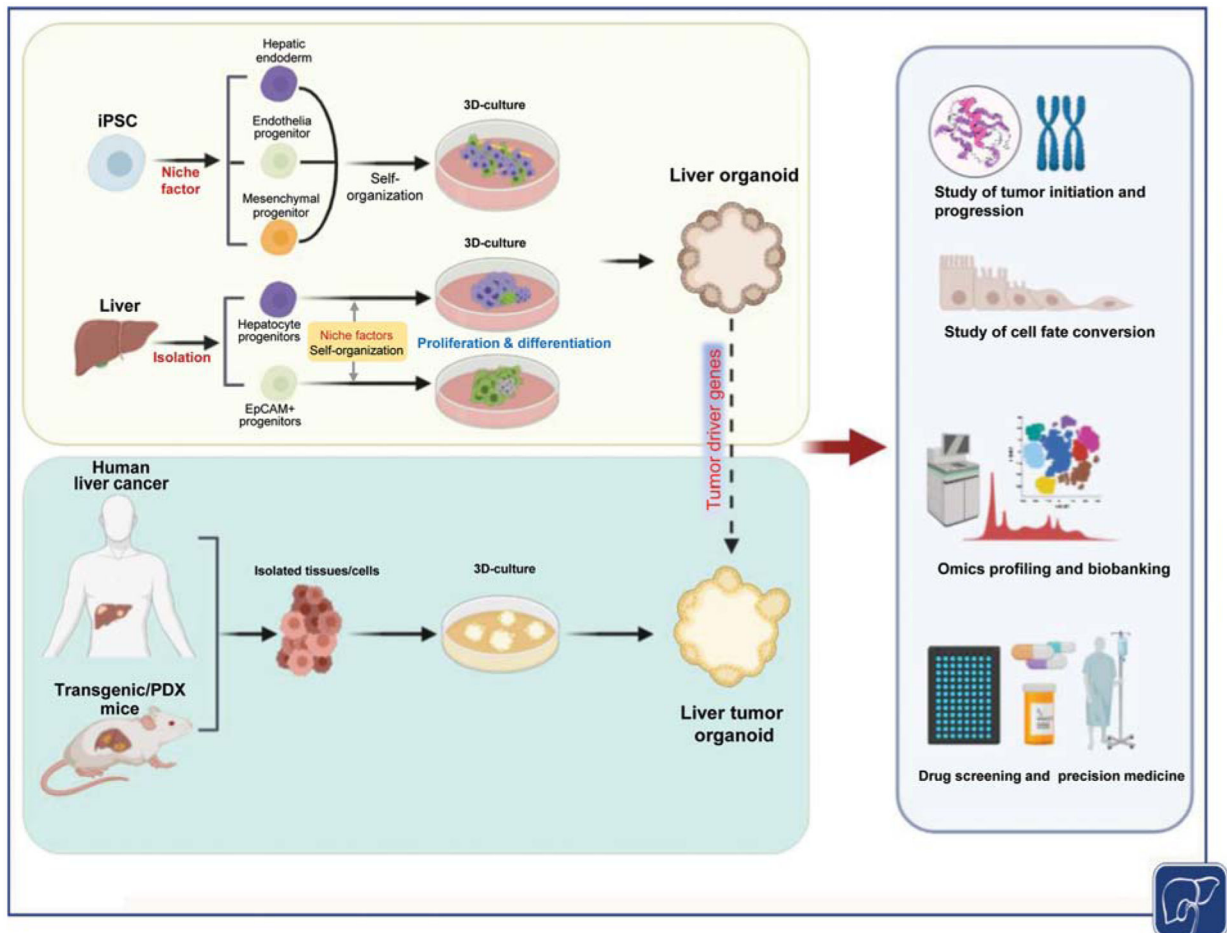
Fig. 1.
Establishment and applications of liver tumor organoids. Liver organoids can be generated
from induced pluripotent stem cells (iPSC) or liver tissue originated progenitor cells. Liver
tumor organoids can be induced in normal liver organoids upon transfection with tumor
driver genes or from isolated human or murine liver tumor cells. Liver tumor organoids can
be used for mechanistic study of tumor development, cell fate conversion, omics profiling,
biobanking, drug screening, and precision medicine.

Table 1

Summary of HCC and iCCA organoids for the study of liver cancer

Cancer type	Cell/tissue of origin	Key factors for derivation	Applications
HCC	Human HCC specimens[27,28]	Matrigel-based liver organoid culturing system; dexamethasone and Y27632; removal of R-spondin-1, Noggin and Wnt3a	• Omics profiling • Biobanking • Drug screening
	Primary human hepatocytes[25]	FOXA3, HNF1A, and HNF4A; SV40LT that inactivates p53 and RB; c-MYC overexpression	• Study of HCC initiation and progression
	HCC-PDX cells[37]	3D bioengineered sponge system	• Omics profiling • Biobanking • Drug screening
	Human HCC cell lines[41]	Matrigel based; HCC cells and nonparenchymal cells; assembly culture medium	• Study of tumor microenvironment • Drug testing
	DEN-induced murine liver tumor tissues[38]	Matrigel-based culturing system	• Mechanistic study of liver tumor progression
CCA	Human iCCA specimens[27,28]	Matrigel-based culturing system	• Omics profiling • Biobanking • Drug screening
	Primary human hepatocytes[25]	FOXA3, HNF1A, and HNF4A; SV40LT that inactivates p53 and RB; RAS overexpression	• Mechanistic study of CCA initiation and progression • Drug testing
	$Kras^{G12D}/Tp53^{-/-}$ murine iCCA tumor[40]	Matrigel-based culturing system	• Mechanistic study of CCA progression

Abbreviations: HCC, hepatocellular carcinoma; iCCA, intrahepatic cholangiocarcinoma; FOXA3, forkhead box A3; HNF1A, HNF1 homeobox A; HNF4A, HNF4 homeobox A; SV40LT, SV40 large T antigen; PDX, patient derived xenograft.

Table 2

Summary of advantages and current limitations of liver organoids for the study of liver cancer

Advantages	Limitations
• Three-dimensional spatial organization to mimic liver structure	• Small sample sizes and need for invasive methods
• In vivo model for the study of liver tumor initiation and tumor microenvironment	• Fail to recapitulate the complex network of the normal liver
• Practical time to generate liver tumor organoids from the source and capable of mass production	• Fail to resemble the vascular cells and tumor infiltrating immune cells of the liver
• Maintenance of genetic features and cellular heterogeneity of the original tumors • Absence of teratoma generation	• Low success rate for generating patient-derived liver tumoroids
• Patient-specific responses for personalized therapy and in vivo drug response predictor • Possibility of gene editing	• Medium-high cost
• Applicable to omics profiling, biobanking and high-throughput drug screening	