REVIEW

Hepatitis B virus infection modeling using multi-cellular organoids derived from human induced pluripotent stem cells

Di Cao, Jian-Yun Ge, Yun Wang, Tatsuya Oda, Yun-Wen Zheng

ORCID number: Di Cao 0000-0002-9711-5001; Jian-Yun Ge 0000-0002-4890-0628; Yun Wang 0000-0002-9281-0048; Tatsuya Oda 0000-0001-6115-0158; Yun-Wen Zheng 0000-0001-9002-3190.

Di Cao, Yun Wang, Yun-Wen Zheng, Institute of Regenerative Medicine and Affiliated Hospital of Jiangsu University, Jiangsu University, Zhenjiang 212001, Jiangsu Province, China

Jian-Yun Ge, Tatsuya Oda, Yun-Wen Zheng, Department of Gastrointestinal and Hepato-Biliary-Pancreatic Surgery, Faculty of Medicine, University of Tsukuba, Tsukuba 305-8575, Ibaraki, Japan

Jian-Yun Ge, Yun-Wen Zheng, Guangdong Provincial Key Laboratory of Large Animal Models for Biomedicine, and School of Biotechnology and Heath Sciences, Wuyi University, Jiangmen 529020, Guangdong Province, China

Yun-Wen Zheng, School of Medicine, Yokohama City University, Yokohama 234-0006, Kanagawa, Japan

Corresponding author: Yun-Wen Zheng, PhD, Associate Professor, Department of Gastrointestinal and Hepato-Biliary-Pancreatic Surgery, Faculty of Medicine, University of Tsukuba, Tennodai 1-1-1, Tsukuba 305-8575, Ibaraki, Japan. ywzheng@md.tsukuba.ac.jp

Abstract

Chronic infection with hepatitis B virus (HBV) remains a global health concern despite the availability of vaccines. To date, the development of effective treatments has been severely hampered by the lack of reliable, reproducible, and scalable *in vitro* modeling systems that precisely recapitulate the virus life cycle and represent virus-host interactions. With the progressive understanding of liver organogenesis mechanisms, the development of human induced pluripotent stem cell (iPSC)-derived hepatic sources and stromal cellular compositions provides novel strategies for personalized modeling and treatment of liver disease. Further, advancements in three-dimensional culture of self-organized liver-like organoids considerably promote *in vitro* modeling of intact human liver tissue, in terms of both hepatic function and other physiological characteristics. Combined with our experiences in the investigation of HBV infections using liver organoids, we have summarized the advances in modeling reported thus far and discussed the limitations and ongoing challenges in the application of liver organoids, particularly those with multi-cellular components derived from human iPSCs. This review provides general guidelines for establishing clinical-grade iPSC-derived multi-cellular organoids in modeling personalized hepatitis virus infection and other liver diseases, as well as drug testing and transplantation therapy.

Core Tip: The development of an effective treatment for hepatitis B virus relies on reliable and reproducible *in vitro* modeling systems. Recently, three-dimensional multi-cellular organoid systems have attracted considerable attention owing to their superior susceptibility and capability to precisely recapitulate the virus life cycle. Recent advances in organoid-generating strategies, particularly those derived from human induced pluripotent stem cells, together with future improvements in genetic modification and scalability, will undoubtedly promote personalized disease modeling and drug development.

INTRODUCTION

Hepatitis B virus (HBV), a highly prevalent global health concern, is among the most common causes of advanced chronic liver disease development. Globally, 292 million people (approximately one-third patients in China) reportedly present with chronic HBV infection[1], which is associated with a wide range of clinical manifestations, including liver cirrhosis, liver failure, and hepatocellular carcinoma[2]. Despite the availability of approved vaccines, currently, HBV treatment is mainly restricted to interferon (IFN) and nucleoside analogs, which rarely achieve absolute hepatitis elimination[3]. Notably, clinical treatment usually results in different responses and outcomes due to viral genotype diversity and patient genetic susceptibility, which further increases treatment difficulties[4]. To date, *in vitro* culture systems have been utilized to determine the characteristics and life cycle of hepatitis C virus. Furthermore, these systems have been proven to substantially contribute to our understanding of the genomic replication subsystems that ultimately led to productive viral infection, as well as successful identification of small molecules having effective activity against various hepatitis C viral enzymes[5]. Therefore, to better understand the HBV life cycle and to promote drug development, a robust personalized *in vitro* HBV modeling platform is urgently needed to recapitulate the entire HBV replication cycle, especially that of covalently closed circular DNA (cccDNA) infection and formation. Several *in vitro* modeling systems have been developed, mostly based on *in vitro* culture systems using primary hepatocytes or hepatic cells from other sources[6-9]. Besides, sodium taurocholate cotransporting polypeptide (NTCP)-transformed hepatocellular carcinoma cell lines are also available. No one system fits all studies, but findings from different systems may be complementary. Maintenance of hepatic function and increase of susceptibility to HBV infection *in vitro* are the principal focus for most established systems. In this regard, numerous culture systems have also been developed to better fit the in *vivo* microenvironment.

Considering the demand for clinical applications, the reproducibility and scalability of modeling systems have generated interest in recent years. Accordingly, as a theoretically unlimited source of stem cells without ethical implications, pluripotent stem cells [induced pluripotent stem cell (iPSC)] represent the most promising source that can be used to yield substantial quantities of homogeneous and reproducible cellular components of the liver. Moreover, with a patient-specific genetic background, iPSC-derived hepatocyte-like cells (HLCs) are optimal for individualized disease modeling, which may remarkably benefit mechanistic studies and drug development for hereditary diseases and diseases closely related to the host genetic background, such as HBV infection[10-12]. With considerable expression of major mature hepatocyte markers, as well as host factors required for HBV infection, including the NTCP, iPSC-

HLCs could support robust production of HBV particles and viral RNAs[7,13]. More importantly, significant inhibition of HBV infection was detected upon anti-HBV agent treatment, suggesting that iPSC-HLCs could be utilized as a novel HBV infection model for drug testing[14].

However, the drawbacks in maturation and difficulty in maintenance of iPSC-HLCs have raised considerable concerns. Liver organoids (LOs) can be maintained for a longer time, are more susceptible to HBV infection, and exhibit enhanced liver function[3]. Multi-cellular three-dimensional (3D) organoid culture systems have recently become an effective strategy to compensate the deficiencies and also extend the potential modeling duration. With supportive mesenchymal and endothelial cells in the 3D microenvironment, HBV susceptibility was greatly enhanced compared to monolayer culture system, while the duration for HBV propagation and virus production was also prolonged[15]. HBV-infected LOs could serve as a reliable and viable *ex vivo* infection model for hepatitis research to investigate the role of host genetic background in HBV infection and individual prognosis of infection, enabling personalized hepatitis treatment[15]. To further enhance the reproducibility and scalability, an all-iPSC-based strategy has been adopted most recently in generating multi-cellular LOs with improved hepatic functions almost equivalent to those of adult hepatocytes, although their advantages in HBV modeling remains to be confirmed in future studies[16]. With the development of 3D expansion strategies, together with advances in direct programming or induction of liver fate, novel engineered LOs are expected to be established on a large scale for broad applications in disease modeling, drug screening, and transplantation.

Here, we summarized the recent advances in *in vitro* modeling systems for HBV infection, especially using multi-cellular LOs. Additionally, we discussed the current and future challenges in the application of advanced organoid generation platforms in terms of efficiency, reproducibility, and scalability. Finally, we explored the future applications of multi-cellular LOs in personalized and precise treatments.

ADVANCES AND LIMITATIONS IN MODELING HEPATITIS B INFECTION

Cell sources
HBV only efficiently infects fully differentiated hepatic cells. Until now, primary human hepatocytes (PHHs) were the only permissive agents used for the study of HBV infection *in vitro*[17]. This system remains the gold standard and is utilized to model HBV infections *in vitro*[18]. However, fresh PHHs have markedly limited availability and unpredictable variability among donors. Furthermore, PHHs have extremely limited replication ability in conventional culture systems and cannot be easily subjected to genetic manipulation, which hinders scaled-up manufacturing and targeted gene therapy. Moreover, PHHs show rapid loss of their mature hepatic functions and infection susceptibility after plating. Thus, these cells are unsuitable for utilization as a stable source for long-term modeling and testing[19]. For a reliable and practical platform for long-term disease modeling and drug development, a new gold standard should be established.

To overcome restrictions in the use of PHHs, especially source availability and cost, researchers have shifted their attention to immortalized tumor-derived or transformed hepatocytes and hepatocellular carcinoma cell lines, such as Huh7, HepG2, HepG-2.2.15, HepAD38, HepaRG, HepDE19, HepBHAe82, and HepG2-NTCP cells[3,20,21]. HepG2.2.15 cells can stably express HBV viral gene products. However, HBV viral particles are derived from chromosomally integrated DNA rather than cccDNA during *in vivo* processing. Later, a highly HBV-permissive cell clone of HepAD38 cells was created, which could produce more robust viruses. However, this cell line failed to recapitulate the complete viral replication process[20,21]. In this regard, the liver progenitor cell line HepaRG is a more attractive source for modeling, since these cells show morphological and functional features similar to those of primary hepatocytes. More importantly, HepaRG cells are susceptible to HBV infection upon supplementation with corticoids and DMSO, which maintains the cellular maturation state [6]. However, this infection system has a relatively low infection efficiency, even under the strict culture conditions necessary to prevent dedifferentiation. Additionally, cccDNA amplification did not occur in this system[7]. Subsequently, to meet the requirements for high-throughput screening of cccDNA-targeting drugs, a HepDE19 cell line was developed in which the expression of HBV e antigen (HBeAg) was derived from cccDNA. However, the ELISA antigen shows cross-reactivity with viral HBeAg homologues, which dramatically diminishes the assay specificity[22,23]. To

address this problem, the HepBHAe82 cccDNA reporter cell line was developed based on similar principles. Importantly, this line produced high levels of cccDNA-dependent HBeAg with high, specific readout signals and did not disrupt any cis-elements that were essential for HBV replication and HBeAg secretion[21]. As a potential tool for cccDNA-targeted drug screening and testing, it remains to be determined whether cccDNA formation, stability, and transcription are recapitulated in this system. To further achieve high susceptibility to HBV infection, NTCP, an essential receptor for HBV infection[24] was introduced into liver cancer cell lines. Establishment of susceptible cell lines, such as HepG2-NTCP, permitted highly efficient HBV infection and enabled the identification of key events or processes in the viral life cycle, although this system could not be used to elucidate the entire HBV life cycle[25]. Furthermore, the physiological and functional characteristics of tumor-derived or transformed hepatoma cell lines are distinct from those of normal hepatocytes. Comparing the HepG2 transcriptome to cells obtained from liver tissue biopsies showed that most genes with upregulated expression in HepG2 cells were associated with carcinogenesis, while those with upregulated expression in the liver were enriched in heterologous biometabolism[26], thereby implying a fundamental deficiency of hepatocellular carcinoma cell lines for reliable and precise modeling. Besides, a non-hepatic cell line was recently developed for HBV modeling. Constructed by exogenous expression of human NTCP, HNF4α, RXRα, and PPARα, the 293T cell line (293T-NE-3NR) was able to support HBV entry, transcription, and replication, although the HBV production (HBV DNA, cccDNA, and pgRNA) in 293T-NE-3NR remained lower than that in the HepG2 cell line. To date, the use of a non-hepatic model is quite a new concept; however, it may serve as a beneficial complement to the current hepatocellular carcinoma cell models. Without intrinsic liver-related host factors, an exogenous construction strategy may help discover the key factors involved in HBV infection[27].

To achieve functional hepatocytes that closely resemble primary hepatocytes, HLCs differentiated from pluripotent stem cells have attracted considerable attention as a novel cell source. Since the use of embryonic stem cells is markedly hindered due to ethical issues, iPSCs induced by the ectopic expression of defined transcription factors (*e.g.*, *Oct4*, *Sox2*, *Klf4*, and *c-Myc*) in patient fibroblasts or other somatic cells have emerged as the most promising source for generating HLCs[28-30]. After conduction of stepwise hepatic differentiation[31-33], iPSC-derived HLCs could support effective HBV infection and last for a long time[14,34]. Together with their infinite expansion capability, iPSCs have enabled the provision of numerous hepatic cell sources necessary for stable and scalable modeling with less variability than PHHs[25]. Meanwhile, the epigenetic effects in human iPSCs are minimal and the genetic differences between individual donors contribute to the major heterogeneity between different iPSC lines[35]. In this regard, iPSCs may serve as stable, reliable, and powerful platforms for the precise analysis of the hepatitis virus in specific genotypes and for modeling infection in individuals with different genetic backgrounds[29,36, 37]. Additionally, induced hepatocytes (iHeps) generated by direct reprogramming from somatic cells share similar hepatic features and may be potentially used to support productive HBV infection[34].

Despite the progressive improvements reported thus far, *in vitro* hepatocyte induction with transcription factors, growth factors, or chemicals cannot fully represent the complete phenotypic spectrum of PHHs. Further, inhibition of the innate immune response remains an obstacle to achieve efficient infection[38]. Thus, improvements including optimized induction strategies and niche signaling are warranted to generate transcriptional/functional and hepatitis virus-friendly hepatic sources to recapitulate *in vivo* virus-host recognition.

In vitro microenvironment

The biological characteristics and functions of primary hepatocytes depend considerably on the *in situ* liver microenvironment[39,40]. Simply covering hepatocytes with multiple layers of collagen gel can markedly extend the culture time and help retain cellular functions[41]. Nevertheless, utilization of the conventional *in vitro* culture systems results in a rapid loss of hepatocyte polarity and associated liver function[42], innate immune responses, and susceptibility to HBV infection due to the unsatisfied intrinsic requirements of the liver microenvironment. Consequently, most of these HBV infection systems require the addition of DMSO, PEG[43], or immunomodulatory agents to achieve essential susceptibility, which is not feasible for practical considerations[34]. *In vivo*, however, HBV can infect more efficiently[44], which highlights that the native hepatic microenvironment, including the physiological liver architecture and niche signaling, markedly impacts productive HBV infection.

As the most abundant non-parenchymal hepatic cell population, liver sinusoidal endothelial cells (LSECs) can efficiently enhance HBV infection by secreting epidermal growth factor (EGF)[45] and can lead to the development of liver fibrosis by constitutively expressing the major histocompatibility complex I-restricted antigens and co-stimulatory molecules, which shifts the immune balance of the liver toward tolerance[46]. Additionally, hepatic stellate cells (HSCs) maintain hepatocyte function *via* cell signaling and secretion of the necessary factors[47]. Furthermore, HSCs are actively involved in the development of hepatitis virus-induced hepatic fibrosis[48]. Kupffer cells (KCs) and Ly6C+ monocytes are closely associated with host defense in the liver and play crucial roles in the development of anti-HBV immunity. Particularly, KCs are involved in hepatitis virus recognition, suppression of infection, immune cell recruitment and activation, and are correlated with HBV progression and outcomes [49,50]. The integration of multi-cellular interactions as a whole requires precise imitation of the native liver niche. Conventionally, monolayer co-culture systems are used as a simple tool to recapitulate cell-cell interactions and signaling. However, these systems provide non-natural physiological conditions, in which only parts of the hepatocyte surface establish communication with the adjacent cells or the extracellular matrix (ECM), leaving the rest of the cell exposed to the culture medium. Although certain aspects of hepatitis or other viral infections may be elucidated using this system, there remain major concerns regarding a physiologically intact host for virus recognition and replication. Cells assembled in 3D aggregates are more similar to cells *in vivo*[15]. PHHs cultured in 3D systems preserve certain metabolic functions and permit more accurate hepatotoxicity prediction during *in vitro* modeling[42]. However, the liver microenvironment includes the 3D mass of hepatocytes and an organized architecture consisting of numerous cell types, which synergistically regulate liver-specific physiology. Therefore, reconstruction of these interactions will theoretically help in the development of a more reliable system for the initiation and long-term maintenance of the hepatitis infection cycle. Recent achievements in generating multi-cellular organoids have paved the way for the establishment of an accurate model for obtaining fundamental knowledge on disease progression, including HBV infection [15], particularly when single patient-derived iPSCs contribute to the cellular composition of desired organoids[51]. iPSC-derived multi-cellular LOs maintain mature hepatic phenotypes and functions to levels comparable to those of PHHs. More importantly, LOs are more susceptible to HBV infection than iPSC-HLCs and maintain long-term HBV propagation while producing infectious viruses. Together with the genetic characteristics inherited from the donor iPSCs, LOs help in the achievement of a promising individualized infection model[15].

In static culture systems, gas exchange, nutrient supply, and waste removal remain significant challenges. The emergence of microfluidic technology has enabled the control of the culture system with optimized temperature, pH, nutrients, and gas exchange, while providing microscale structures and parameters that may help to obtain an approximate simulation of the *in vivo* microenvironment[52,53]. Thus, the application of these systems permits organoid maintenance in *in vivo*-like physiological states or even in disease-like conditions in a controlled manner. Notably, improved drug sensitivity can be obtained using microfluidic 3D systems compared to static culture systems. With the utilization of further engineering approaches to control the initial cellular composition, shape, and size of cell aggregates; cell-cell and cell-ECM[54] interactions; and biochemical gradients similar to *in vivo* microenvironment, the incoming generation of organoids-on-a-chip has considerable potential for large-scale applications in high-throughput drug testing and screening.

Micropatterning

Highly structured organs and tissues provide a fundamental *in vivo* microenvironment for cells, in which cell polarity and functions are consistently influenced by the specific boundaries imposed by the neighboring cells and the ECM. The mechanical and spatial properties of the microenvironment are tightly correlated with intracellular signaling pathways and affect cell transcriptome status and function. In the past decade, numerous studies have been conducted and have provided insights into the geometrical modeling of the *in vitro* culture microenvironment by developing engineered substrates that precisely mimic the composition, structure, and mechanical properties of a specific organ or tissue.

To date, accumulative *in vitro* studies show that cell differentiation can be directed by using micropatterned substrates. The micropattern features on the spreading area and the aspect ratio determine the differentiation status of human mesenchymal stem cells (MSCs)[55]. Moreover, hydroxyapatite bioceramic-based hybrid structures stimulate osteogenic differentiation of MSCs by activating integrins, the BMP2

signaling pathway, and cell-cell communication. The width of micropattern stripes regulates vascular smooth muscle cell orientation by regulating cell elongation[56]. Similarly, mechanical and spatial control was observed during pluripotent stem cell differentiation. When these cells are differentiated within geometrically uniform and circular micropatterns, the patterning of regionalized cell fate can be reproducibly recapitulated[57,58]. Moreover, recent evidence shows that a decreased patterning width promotes iPSC differentiation efficiency, suggesting that stricter topographies direct cell fate specification[59]. Interestingly, the micropattern stiffness, degradability, and biochemical composition can even promote pluripotency reprogramming, thus emphasizing the critical role of micropatterns in morphogenetic and functional remodeling[60].

Although micropatterning strategies are increasingly applied in cell-based modeling and testing, they pose challenges in ensuring compatibility with rapidly advancing organoid technology that requires the use of non-adhesive substrates and extensive cell-cell interactions for self-organization. To this end, scaffold-dependent micropatterning strategies were adopted in recent studies conducted on distinct epithelial organoid generation. Meanwhile, structural and functional improvements have been increasingly reported. For example, a microfilament-based floating scaffold was developed to perform micropatterning and to guide brain organoid self-organization with increased reproducibility and improved tissue architecture[61]. Additionally, a spatially confined hydrogel scaffold was developed to guide self-organization of tube-shaped functional intestinal organoids with an accessible lumen and a physiologically relevant spatial arrangement of crypt- and villus-like domains[62]. Similarly, in terms of LOs, both colloidal crystal and hydrogel-based scaffolds were found to support advanced liver functions[63,64]. However, to date, the mechanism of spatial microenvironmental control of organ/tissue-specific cell fate specification remains undetermined. Once identified, scaffold-based micropatterning systems are expected to offer a potential platform for generating liver-specific and regionalized cell types simultaneously from a single iPSC. These platforms will be of remarkable importance for constructing multi-cellular organoids with native spatial composition, and will help to elucidate the mechanisms by which intra- or intercellular signals regulate distinct cellular identities and maturation patterns, and will fundamentally mimic the *in vivo* physiological features and hepatic functions for precise hepatitis modeling.

CURRENT CHALLENGES IN GENERATING MULTI-CELLULAR LIVER ORGANOIDS

Liver-like cellular organoid composition and reconstitution

To better mimic an *in vivo* niche resembling liver-specific function and physiology for disease modeling, vascularized functional multi-cellular LOs were first developed in 2013[65]. However, in this study, human MSCs and umbilical vein endothelial cells (HUVECs) were used to substitute the liver intrinsic stromal cellular components, including HSCs and LSECs, which remain major obstacles for future clinical applications. To address this problem, a complete all-iPSC-based strategy was established, in which hepatic functions were promoted by using iPSC-septum transversum mesenchymal and iPSC-endothelial progenitor cells. In both global transcriptome and function, the new system was more advanced than the HUVEC/MSC-based strategy and was comparable to primary adult hepatocytes[16]. However, this system remains deficient in recapitulating the immune response and inflammation due to the lack of an essential liver component, KCs. Recently, KCs, hepatocytes, stellate cells, and biliary cells were successfully induced synchronously in a multi-cellular system by facilitating co-differentiation from iPSCs, which represents a real and more complex liver-like tissue system that may be used for modeling inflammatory and fibrotic responses[51]. With advanced integration and optimization of cellular composition (Table 1), multi-cellular LOs are expected to support precise and more complex disease modeling.

With the progressive understanding of liver organogenesis and technical advancements in iPSC differentiation methods toward tissue-specific cellular compositions, the following aspects remain as future challenges: (1) The procedures that can be used to remodel the intrinsic cellular composition proportion and distribution in LOs; (2) Their specific roles in liver development and disease processes; (3) Clarification of biochemical signatures of liver-specific ECM; and (4) The use of clinically compatible ECM hydrogels or microparticles. Overcoming these challenges will be beneficial for maintaining LO physiological characteristics during modeling.

Novel strategies for synchronous induction

Before the all-iPSC-based strategy was adopted, the development of multi-cellular organoids included the collection and separate preparation of each cell type and subsequent self-organization into a 3D structure. Although the emergence of all-iPSC-based strategies facilitated the establishment of a reproducible and stable source for clinical and pharmaceutical applications, the procedure for diverse cell co-culture remains time-consuming, labor-intensive, inefficient, and cannot meet the requirements for large-scale production and practical applications. Moreover, selection of the specific culture medium and ECM to simultaneously maintain multiple cell lineages remains challenging.

To address these limitations, a stepwise organoid induction and maintenance platform was developed, which involved sequential stimulation with the addition of growth factor cocktails into the culture medium. Following the induction of foregut spheroids by retinoic acid (RA) treatment and subsequent hepatic maturation, iPSCs were simultaneously differentiated into multi-cellular LOs. However, this approach lacks precise control over multi-lineage specification and physiological functions. Furthermore, the optimization of medium and growth factors that are used to direct and maintain distinct cell specificity remains an obstacle. In this regard, systematic probing of the molecular pathways and transcriptional networks has emerged as a more precise method for direct organogenesis *in vitro*. *PROX1* and *ATF5* overexpression combined with *CYP3A4* activation enables gene regulatory network (GRN)-based engineering and facilitates direct programming or induction and the development of iPSC-derived multi-cellular LOs. Importantly, compared to primary mature hepatocytes, the established GRNs not only showed major similarities in natural liver functions, but were also responsive to perturbation and feedback regulation, and thus were superior to immature HLCs and fetal LOs[66]. Although this new method enabled the development of multi-cellular systems *via* synthetic genetic control, it is difficult to reflect every facet of human liver physiology and function, such as relatively low levels of urea production and CYP2C19 activity. Further global assessment of GRNs may be necessary to identify critical regulatory signatures for advanced liver fate. Additionally, it is important to carefully determine and avoid potential tumorigenesis by transcription factor-based genetic programming and delocalization processes using guide RNAs (gRNAs) to achieve clinical safety standards.

Taken together, synchronous induction or programming systems may substantially improve the efficiency of traditional multi-lineage differentiation, which is a promising strategy for practical applications (Figure 1). However, the lack of maturation, purity, and batch-to-batch variability remains a major challenge. Further optimization of stepwise induction or programming strategies, including medium, supplements, timing, and application of tissue scaffolding and microfluidic devices, is warranted to precisely recapitulate the intrinsic multi-cellular organ system *in vivo*.

Scaling up

The possibility of scaled-up applications is a major advantage of using *in vitro* organoid systems over animal models. The increasing biomedical and preclinical demands for high-throughput disease modeling, drug testing, and screening, have led to the identification of critical bottleneck of scaling up, which prevents their application to provide more reliable, rapid, and cost-effective modeling.

Using chemical induction, a limited expansion capability of human hepatocytes was successfully induced in 2D monolayer culture systems[67,68]. However, expansion in a 3D system is subjected to complicated factors, such as nutrient/gas exchange, contact inhibition, and complex cell-cell signaling. Although long-term expansion of organoids from human adult biliary epithelial-derived progenitor cells, fetal/adult hepatocytes, and pluripotent stem cell-derived hepatocytes has been successfully established, the expansion efficiency remains markedly lower than that observed with the application of 2D systems[69-73] (Table 2). Recently, a rotating flask-based method was developed for the large-scale expansion of human LGR5-positive liver stem cell organoids. In this system, organoids were subjected to continuous passage and stable maintenance for at least 6 wk, which was mostly achieved with improved oxygenation[74].

Scaling up multi-cellular systems is another major challenge. By developing an omni-well array culture platform, Takebe *et al*[16] adopted an all-iPSC strategy for the large-scale production of homogenized and vascularized LOs in a clinical setting and achieved a 10^8-cell grade, which seemed to be feasible for human therapeutic applications. However, this platform involved the performance of labor-intensive work, including separate preparation of diverse cell sources for co-culture, thus limiting the efficacy of scaling. Later, the same group established a synchronous co-differentiation

Table 1 Current modeling strategies and applications of human multi-cellular liver organoids

Model/application	Multiple cells (ratio)	Culture system	Advances/significance	Limitations	Ref.
ALF	iPSC-HEs, HUVECs, BM-MSCs (10:8:2)	3D, Matrigel	Multi-cellular LOs with vascularization	Low reproducibility; Time-consuming	2013[65]
ALF	HEs, MCs, ECs (all from iPSCs) (10:8:2)	3D, ULA	All-iPSC-based strategy	Time-consuming; High cost	2017[16]
ALF	iPSC endoderm cells, HUVECs, UC-MSCs (10:7:1)	3D, ULA	LOs generated from single donor-derived cells	Low reproducibility; Time-consuming	2018[75]
ALF	iPSCs, HAMECs (3:1)	EB, Agarose	HAMECs improved hepatic functions	Unable to reflect the nature cellular composition of liver	2019[102]
Liver fibrosis	HepaRG, THP-1, hTERT-HSC	3D, Hanging drop	LOs derived from cell lines	Functional deficiency	2017[103]
Liver fibrosis and steatohepatitis	PHHs, KCs, HSCs, SECs (16:2:1:1)	3D, ULA	LOs derived from primary cell sources	Low reproducibility; High cost	2018[104]
Steatohepatitis	Hepatocytes, HSCs, BCs, KCs (all from iPSC)	3D, ULA	Co-differentiation of multiple cell lineages for iPSC- LOs	Functions undetermined; Potential inter/intra-batch variability	2019[51]
HBV infection *ex vivo*	iPSC endoderm cells, HUVECs, BM- MSCs (10:7:1)	3D, ULA	Validation of advantages of iPSC-LOs in HBV modeling	Low reproducibility; Time-consuming	2018[15]
Hepatic differentiation	iPSC-HEs, MSCs, HUVECs (10:2:7)	3D, Matrigel	Platform to identify developmental paracrine signals involved in hepatocyte differentiation	Low reproducibility; Time-consuming	2017[105]
Hepatic differentiation	iHEPs, ECs, HSCs, cholangiocytes (10:7:2:1)	3D, ULA	Cholangiocytes impaired the hepatic functions in LOs and were associated with the liver disease relevant phenotype	Cholangiocyte activation in LOs was unclear	2019[106]
Liver development and angiogenesis	Hepatocytes, BCs, ECs, HSCs (all from iPSC)	3D, Matrigel	Engineered iPSC-LOs by programming of the gene regulatory network	Not completed for liver functions	2021[66]

ALF: Acute liver failure; BCs: Biliary cells; BM: Bone marrow; EB: Embryoid body; ECs: Endothelial cells; HAMECs: Human adipose microvascular endothelial cells; HBV: Hepatitis B virus; HEs: Hepatic endoderm cells; HSCs: Hepatic stellate cells; HUVECs: Human umbilical vein endothelial cells; iHEPs: Induced hepatocytes; KCs: Kupffer cells; Los: Liver organoids; MCs: Mesenchymal cells; MSCs: Mesenchymal stem cells; NPCs: Non-parenchymal cells; PHHs: Primary human hepatocytes; SECs: Sinusoidal endothelial cells; UC: Umbilical cord; ULA: Ultralow adhesion microwell plate.

method that facilitated the generation of multi-lineage organoids in a preset growth factor administration strategy, thereby saving substantial time and costs for co-culture procedures[51]. Most recently, organoid programing has emerged as a promising strategy for establishing both functionality and scalability. A tissue-specific set of factors were identified using computational analyses. These factors were then genetically introduced into LOs, and after manipulation of GRNs, multi-lineage tissue fate could be directed to develop multi-cellular systems. Notably, these LOs can be passaged and cryopreserved while retaining their vascular networks[66]. This scalable organoid production system may pave the way for personalized disease modeling, drug testing, and even transplantation. However, there remain challenges in the identification of liver-specific GRNs to directly re-establish the entire profile of human liver physiology and function. Other tissue engineering techniques, such as pinning bioreactors, microfluidics, and bioprinting may be developed before high-throughput and highly efficient platforms can be established to meet the practical demands of pharmaceutical and clinical applications.

Transplantation and gene editing

In addition to demonstrating utility as an *in vitro* tool in the modeling of a structural and functional organ unit, organoids are also potential transplantation substitutes for organ donors. Indeed, organoids exhibit various advantages over conventional cell-based strategies, as mentioned elsewhere[69,75]. By mimicking liver regeneration after injury, an advanced *in vitro* system was recently developed for the long-term expansion of functional LOs. Particularly, LOs generated from primary liver sources have demonstrated genetic stability after long-term culture[69,70], suggesting that these

Table 2 Up-to-date methods for obtaining expandable human liver organoids

Initial cells	Expansion systems (substrate for embedding)	Medium	Expansion capability (Split ratio/passage days/expansion duration)	Ref.
Fetal and adult hepatocytes	3D, Matrigel	AdDMEM/F12, B27, N-Acetylcysteine, gastrin, RSPO1, Noggin, Wnt, EGF, FGF7, FGF10, HGF, TGFa, Nicotinamide, A83-01, CHIR99021 and Y27632	1:3/7-8 d/> 16 passages	2018[70]
EpCAM+ bile duct cells	3D, Matrigel or BME gel	AdDMEM/F12, N2, B27, N-Acetylcysteine, gastrin, RSPO1, Noggin (d0-3), Wnt (d0-3), EGF, FGF10, FGF19, HGF, Nicotinamide, A83-01, FSK, and Y27632 (d0-3)	1:4-1:8/7-10 d/6 mo	2015[69]
EpCAM+ bile duct cells	3D, BME gel	AdDMEM/F12, B27, N-Acetylcysteine, gastrin, RSPO1, Noggin (d0-3), Wnt (d0-3), EGF, FGF10, FGF19, HGF, Nicotinamide, A83-01, FSK, and Y27632 (d0-3)	1:5/7-10 d/> 6 mo	2019 [107]
iPSC derived EpCAM+ hepatic progenitors	3D, Matrigel	AdDMEM/F12, B27, N-Acetylcysteine, gastrin, RSPO1, Noggin (d0-3), Wnt (d0-3), EGF, FGF10, HGF, Nicotinamide, A83-01, FSK and Y27632 (d0-3)	1:4-1:8/7-10 d/9-12 mo	2019[71]
PSC-derived hepatocytes	3D, Matrigel	AdDMEM/F12, N2, B27, N-Acetylcysteine, gastrin, RSPO1, EGF, FGF10, HGF, Nicotinamide, A83-01, FSK	1:3-1:10/7 d/3 mo	2019[72]
PSCs transduced with *PROX1*, *ATF5* and *CYP3A4*	3D, Matrigel	APEL medium	NA/10 d/17 d	2021[66]

APEL: A commercial medium from Stem Cell Technologies; BME: Basement membrane extract; bFGF: Basic fibroblast growth factor; BME: Basement membrane extract; EGF: Epidermal growth factor; FGF: Fibroblast growth factor; FSK: Forskolin; HGF: Hepatocyte growth factor; iPSCs: Induced pluripotent stem cells; PSCs: Including embryonic stem cells and induced pluripotent stem cells; RSPO1: R-spodin1.

organoids are a safe transplantation source for clinical applications. Alternatively, autologous transplantation of iPSC-derived LOs is expected to be promising for the realization of reproducible and personalized liver disease treatment. As a highly prevalent disease, HBV infection remains a strong risk factor for developing liver cirrhosis, hepatocellular carcinoma, or other end-stage liver diseases, thus posing a major threat to health worldwide[76]. To this end, there exists a necessity of developing effective treatments for chronic HBV infection. Currently, the standard therapy for chronic hepatitis B infection includes two major agents. First, nucleotide or nucleoside analogs such as tenofovir and entecavir interfere with viral replication by suppressing the synthesis of reverse transcriptase; second, IFNs such as IFNα induce long-lasting immunological control[76]. However, available drugs show side effects or high response variability, and drug resistance leads to reduced long-term effectiveness. Currently, HBV infection cannot be absolutely cured[77]. Combined with the CRISPR/Cas9 genome editing technology, patient iPSC-derived organoid transplantation may provide a new therapeutic strategy for realizing a highly specific HBV treatment. As the functional regions in key HBV receptors (*e.g.*, NTCP) for HBV binding and post-binding entry have been identified, specific genetic knock-out or modification may markedly change HBV infection susceptibility, thereby permitting the development of transplanted organoids that are resistant to HBV infection. More importantly, the risk of HBV recurrence may be permanently avoided, which remains a major concern after liver donor transplantation and is associated with graft dysfunction or failure and cirrhosis progression[78].

Nevertheless, current research on organoids is in the preclinical stages, and clinical safety and efficacy remain to be established for adult or iPSC-derived LO transplantation. Moreover, the potential risks associated with genetic modification, tumorigenesis, and transplantation techniques should be emphasized in light of individual and societal values. In the context of continuing investigation regarding clinical concerns[79,80], quality-controlled and personalized LOs from authorized patient-derived cell banks are expected to be used routinely in the future.
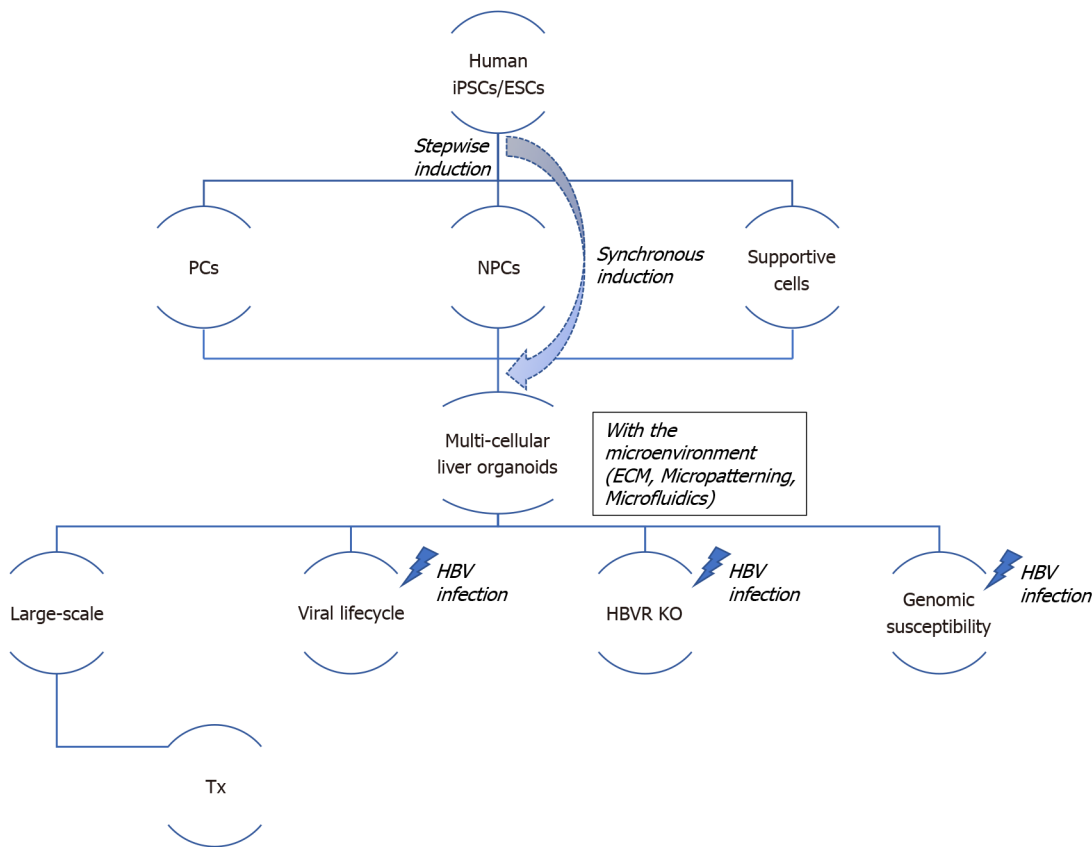
Figure 1 Schema to generate human liver organoids and strategy regarding their application potential and hepatocyte B virus modeling.
Multi-cellular liver organoids are generated and self-organized with parenchyma cells, non-parenchyma cells, and other hematopoietic or/and neuronal supportive cells after stepwise induction from human induced pluripotent stem cells/embryonic stem cells; in contrast, synchronous induction using a CRISPR-based strategy markedly enhances their hepatic functions and improves their reproducibility and scalability. The personalized and population organoid system may provide a reliable platform for high-throughput hepatocyte B virus (HBV) drug screening, allowing to understand novel key points of the HBV lifecycle, gene editing to knock-out the HBV receptor, and distinguish genomic susceptibility in a large population. Transplantable multi-cellular organoids without the HBV receptor have much potential for future applications. ECM: Extracellular matrix; ESCs: Embryonic stem cells; HBV: Hepatocyte B virus; HBVR: Hepatocyte B virus receptor; iPSCs: Induced pluripotent stem cells; KO: Knock-out; NPCs: Non-parenchyma cells, including Kupffer cells, hepatic stellate cells, and liver sinusoidal endothelial cells; Supportive cells: Such as neuronal cells, hematopoietic cells, and vascular endothelial cells; PCs: Parenchyma cells such as hepatocytes; Tx: Transplantation.

PERSPECTIVES

Automated and large-scale cultural systems

Robust, consistent, and cost-effective manufacturing is extremely important for facilitating the practical applications of LOs for high-throughput disease modeling, drug testing, and therapeutic purposes. Similarly, automatic LO generation and culture systems may provide promising opportunities to meet critical requirements, especially in reproducibility and scalability.

In recent years, automated culture systems have garnered considerable attention, particularly in the maintenance and differentiation of iPSCs. Since the quality of iPSCs is dependent on the technical skills applied during culture and manipulation[81], automated culture systems can decrease technical variability. With the development of fully automated cell culture systems that have revolutionized cell seeding, medium changing, imaging, harvesting, and analysis, human iPSCs can be maintained in an undifferentiated state for a long period[82]. Additionally, reprogramming and differentiation of cardiomyocytes, hepatocytes, mesenchymal cells, neural stem cells, and retinal pigment epithelial cells have also been successfully performed using automated systems, with high quality and consistency[83-86]. Recently, an automated system was first used to produce LOs. Although the differentiation process was not fully automated, automatic LO self-assembly and maintenance substantially permitted the obtainment of reliable and reproducible results, suggesting potential applications in applied research and industry[87]. With the development of differentiation strategies and medium optimization for iPSC-derived parenchymal and non-parenchymal liver cells, the integration of automated cell production and maintenance systems is expected to further promote a completely standardized high-throughput workflow for

LO production. These workflows will enable downstream applications such as HBV and liver cancer modeling, drug screening, and regenerative medicine.

Genetic manipulation

Genetic manipulation seems extremely promising in the revelation of the biological functions of specific genes, their regulation networks, and their relevance to disease progression[88]. Moreover, genetic manipulation facilitates drug testing in various disease states with different genetic backgrounds. In recent years, the CRISPR/Cas9 technology has markedly simplified genetic engineering because of its versatility and broad application potential. Combined with the development of organoid generation and culture technology, CRISPR has enabled the establishment of multiple disease models using genetically engineered tissue-specific organoids. For example, colorectal cancer models were derived from intestinal organoids with mutations in *APC*, *SMAD4*, *TP53*, and *KRAS*[89]; injury models were derived from kidney organoids *via* podocalyxin knock-out[90]; brain tumor models were derived from cerebral organoids with mutations in *MYC* and mutations commonly found in glioblastoma[91]; and liver cancer models including hepatocellular carcinomas and intrahepatic cholangiocar-cinomas were derived from LOs with mutations in *c-Myc* or *RAS*[92]. Notably, genetically modified iPSC-derived organoids may permit broader practical applic-ations in modeling genetic diseases once consistent patient-derived organoids are developed, such as those with A1AT deficiency or Allagile syndrome. Although not reported, iPSC-derived LOs are expected to acquire altered susceptibility to HBV infection with mutated NTCP expression, which is critical in studying viral replication.

In addition to demonstrating versatility as an *in vitro* tool used for the elucidation of substantial clues to diseases, gene correction in organoid models provides a potential strategy for future gene therapy. Using the CRISPR/Cas9 genome editing system, the mutation of cystic fibrosis transmembrane conductor receptor was corrected in intestinal organoids derived from cystic fibrosis patients, which restored normal function[93]. Subsequently, mutation correction was achieved in retinal organoids derived from retinitis pigmentosa patients using a similar strategy[94]. With increasing interest in the genetic manipulation of various tissue-specific organoids, the deve-lopment of a robust genome editing strategy is necessary to further enhance organoid-based disease modeling and related gene correction. Recently, CRISPR–Cas9-mediated homology-independent organoid transgenesis (CRISPR-HOT) was developed to efficiently generate knock-in human organoids[95], thus providing a powerful plat-form for obtaining reliable and scalable applications in this field.

However, to date, no gene editing has been performed on multi-cellular organoids, partially because of the technical difficulties in targeting specific cell types within the system. Additionally, there are several issues that are encountered with gene therapy, including off-target mutations, delivery difficulties, and the lack of standardized tests to assess anti-HBV gene therapy[96,97]. Undoubtedly, new innovations in the rapidly evolving field of the CRISPR technology may provide exciting possibilities for organoid-based clinical applications.

Susceptibility to HBV infection

The identification of NTCP, the specific receptor for HBV[98], paved the way to construct NTCP-expressing hepatoma cell lines that were originally not susceptible to HBV. Exogenous NTCP expression may render these cell lines vulnerable to HBV infection, providing a more reliable system for studying the HBV life cycle. However, this system does not help elucidate the entire HBV life cycle and permits limited viral spread, with only moderate amounts of detectable cccDNA, partially due to intrinsic deficiencies of these cell lines. As a result, an extremely high multiplicity of infection is necessary to achieve substantial infection, and the addition of polyethylene glycol is needed to enhance infection in most reported cases[99]. Meanwhile, hepatoma cells are physiologically impaired in several intracellular signaling pathways and functions after forced NTBC expression, which limits their use for studying virus-host inter-actions[38].

In addition to NTCP, HBV infection may also depend on other receptors, such as the sialic acid glycoprotein receptor (ASGPR)[100,101]. A comparison of iPSC-derived hepatic progenitors and HLCs revealed a significant increase in ASGPR expression in iPSC-HLCs, suggesting that ASGPR might be considered a candidate receptor for hepatitis virus infection in mature hepatic cells and might mediate HBV entry into cells. To date, NTCP expression is considered the most important factor for HBV infection in host cells[13]. However, it remains necessary to identify other unknown receptors or media that contribute to hepatitis virus infection susceptibility. The emergence of 3D multi-cellular LOs, which mimic the native liver microenvironment,

has attracted substantial attention owing to its superior HBV susceptibility compared to 2D culture[15]. Although the underlying mechanism remains unclear, these organoids may provide an opportunity for the investigation of potential receptors and related pathways involved in viral infection. Computational analyses, followed by sequential knock-out of the candidate targets in engineered multi-cellular LOs, may enable the complete understanding of the key genes contributing to HBV susceptibility. Furthermore, the role of ethnic genotypes in viral susceptibility may be studied using this system. Individuals of different races present with different susceptibilities to hepatitis virus. For example, Chinese populations have a high susceptibility to hepatitis B, while European and American populations are prone to hepatitis C infection. It would be beneficial to use iPSC-derived LOs from different genomic backgrounds to compare HBV infection in different populations with the same risk factors, or to compare susceptibility in the same population with different risk factors. In this regard, drugs, which may account for disparities in different populations and individuals, may also be developed.

CONCLUSION

With the progressive understanding of liver organogenesis, the development of 3D organoid culture systems has helped in the establishment of a novel platform for precise and personalized liver disease modeling. However, it remains particularly challenging to promote the efficiency, reproducibility, and scalability of organoid reconstitution and maintenance. It is expected that future advances in genetical engineering and automated culture system will put 3D multi-cellular organoids into a variety of practical uses, including hepatitis infection modeling and related drug development. Furthermore, the proof of concept for clinical-grade patient iPSC-derived multi-cellular LOs is expected and will contribute much to modeling and treatment of hepatitis virus infection and other liver diseases.

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