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CONCISE REVIEW

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Advances in generating liver cells from pluripotent stem cells as a tool for modeling liver diseases

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Abstract

Developing robust *in vitro* models of the liver is essential for studying the pathogenesis of liver diseases, hepatotoxicity testing, and regenerative medicine. Earlier studies were conducted using cell lines derived from hepatomas. Due to the inherent limitations of cell lines, researchers used primary human hepatocytes (PHHs), which are considered a gold standard for *in vitro* modeling of the liver. However, due to the high cost of PHHs and lack of donors, researchers have sought an alternative source for functional liver cells. Pluripotent stem cells (PSCs) emerged as a viable alternative due to their plasticity and high proliferative capacity. This review gives an overview of the major advances that have been achieved to develop protocols to generate liver cells such as hepatocytes, cholangiocytes, and Küpffer cells from PSCs. We also discuss their application in modeling the pathogenesis of liver diseases such as drug-induced liver injury, acute liver failure, and hepatic steatosis.

KEYWORDS

cholangiocytes, hepatocytes, Küpffer cells, liver, liver diseases, pluripotent stem cells

1 | INTRODUCTION

The liver is a versatile and heterogeneous organ comprised of parenchymal cells (hepatocytes) and non-parenchymal cells (NPCs) such as cholangiocytes, Küpffer cells (KCs), hepatic stellate cells, and liver endothelial cells. Hepatocytes are the major cell type in the liver occupying approximately 80%-90% of the parenchymal mass of an adult organ.[1,2] Hepatocytes perform most of the essential functions in the liver. These include the synthesis of serum proteins such as albumin, detoxification of alcohol, drugs and other chemicals, and removal of inhaled poisons.[3] These cells also play a key role in metabolism, storage of glycogen and lipids. Due to their importance in liver function, the pharmaceutical industry and researchers have invested huge resources to develop protocols to generate liver cells using pluripotent stem cells (PSCs) as a tool for modeling disease, toxicology studies, regenerative medicine, and gene therapy.[2,4-8] This review will describe in detail the recent advances made to generate hepatocytes, cholangiocytes, and KCs using PSCs. We will also discuss the future applications of three-dimensional liver organoids in disease modeling and regenerative medicine.

2 | GENERATION OF LIVER CELLS FROM PSCS

2.1 | Hepatocyte-like cells

Several studies have defined protocols for the generation of hepatocytes from PSCs[1,8-15] and tissue-resident progenitor cells.[3,16,17] The protocols mimic key steps in ontogenic liver development, starting with commitment to definitive endoderm (DE), followed by generation of hepatoblasts, and finally specification to hepatocyte-like cells (HLCs) using defined inductive and repressive signals (Figure 1). Generation of DE is achieved by culturing stem cells in the presence of Activin A, Wnt3a,

bone morphogenic factor 4 (BMP4), fibroblast growth factor 2 (FGF2), and vascular endothelial growth factor (VEGF).[1,7,10,18,19] The resulting DE cells express Sox17, FOXA2, CXCR4, and CERBELUS1.[19] The transition from DE to hepatoblasts cells requires inductive signaling of BMP4 and FGF2, key signals required for liver bud formation in vivo, and repression of Activin.[1,7] The maturation of hepatoblasts to HLCs is achieved by culturing hepatoblasts in media supplemented with hepatic growth factor, oncostatin M, dexamethasone, and other growth factors essential for liver development and functional maturation.[1,7,9,11,12,20,21] The resulting HLCs express key functional markers such as albumin, CYP3A4, and transcription factors like hepatocyte nuclear factor 4 alpha (HNF4α), HNF6, CEBPα, PROX1, and GATA4. Moreover, they have been shown to perform key hepatocyte functions including albumin secretion, glycogen synthesis, urea production, and LDL uptake.[9,11-13,21] The major drawback associated with HLCs is that they retain some of the fetal characteristics like expression of alpha-fetoprotein and limited expression of P450 enzymes compared to primary hepatocytes.[2,8]

Several approaches have been taken to improve the functionality and maturation of hepatocytes. These include the use of vitamin C, 3D coculture, upregulation of c-AMP[2,22] and small molecules.[2,12] A recent study by Ang and colleagues explored increasing the number of steps in the differentiation of PSC to hepatocytes, reasoning that the three-step protocol does not faithfully mirror liver development *in vivo*.[10] They defined a roadmap for differentiation of stem cells to HLCs involving a transition through six consecutive lineage choices and identifying inductive and repressive signals at each juncture. Importantly, they demonstrated a temporally dynamic action of signals to specify one fate early during culturing, and subsequently repressing its formation later[10]; thus, raising caution about the current protocols that heavily rely on providing the same signaling cues for a prolonged period of time. The roadmap involved generation of posterior foregut progenitors by exploiting the activation of retinoic acid, BMP, and

Significance statement

The liver is an essential organ in the body that performs crucial functions such as metabolism. Researchers have invested enormous resources to generate liver cells from induced pluripotent stem cells and to develop robust three-dimensional liver organoids that faithfully resemble the liver. This study reviews in detail advances that have been achieved to develop protocols to generate hepatocytes, cholangiocytes, and Küpffer cells. It then discusses the application of these tools to model liver disease with a specific focus on hepatic steatosis, acute liver failure, and drug-induced liver injury.

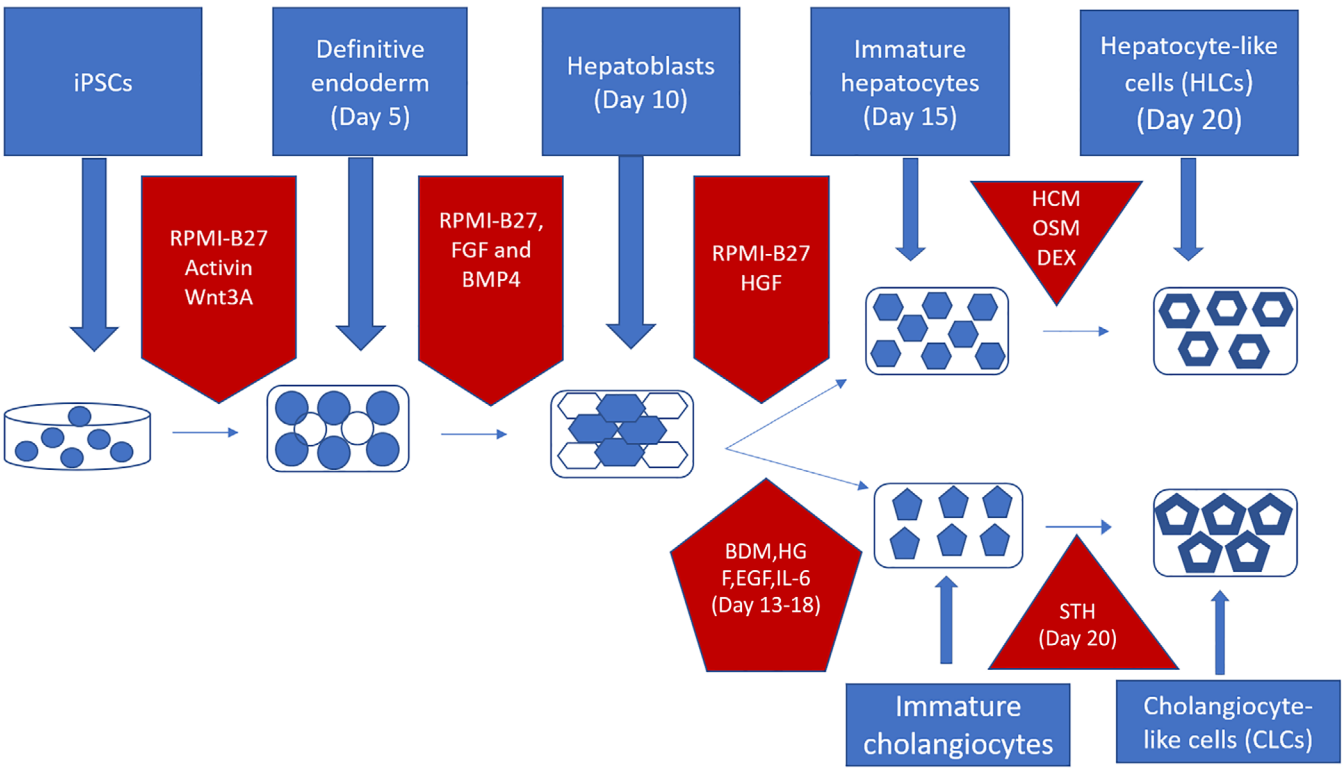

FIGURE 1 Generation of hepatocytes and cholangiocytes from human-induced pluripotent stem cells. Hepatocytes were generated following key differentiation steps that involved induction of definitive endoderm (niche factors; RPMI-B27, 100 ng/mL), Activin A (transforming growth factor-β signaling family), and Wnt3A (Wnt signaling family), hepatoblasts using RPMI-B27, hepatocyte growth factor, and subsequent lineage differentiation into hepatocytes in the presence of hepatocytes conditioned medium, Oncostatin M, and dexamethasone. Cholangiocytes are generated from hepatoblasts that are cultured in biliary differentiation medium supplemented with human growth hormone, interleukin 6, and sodium taurocholate hydrate

FGF pathways while inhibiting transforming growth factor-β (TGF-β) signaling within 24 hours, and subsequently liver bud specification by activation of TGF-β, BMP, and protein kinase A pathways and repression of Wnt pathway.[10] Finally, specification into HLCs was achieved by addition of Notch inhibitor (DAPT), TGF-β inhibitor (A8301), Forskolin (FSK), and ascorbic acid 2-phosphate (AAP). The resulting HLCs expressed all the phenotypic and functional hepatocyte markers, but still displayed limited expression of P450 enzymes compared to primary human hepatocytes (PHHs).[10]

Zhang and colleagues developed a protocol to generate poster gut endoderm cells (PGECs) from stem cells by exploiting FGF, TGF, and Wnt signaling pathways.[19] PGECs were defined by the expression of CDX2 and could be expanded in the presence of epidermal growth factor (EGF), VEGF, Chir99021, and A8301. The expanded $CDX2^+$ PGECs maintained chromosomal stability and were successfully transplanted into immunocompromised (NOD/SCID) mice without inducing teratomas.[19] Culturing $CDX2^+$ PGECs in the presence of endothelial (human umbilical vein endothelial cells, HUVECs) and mesenchymal cells (mesenchymal stem cells, MSCs) generated functional liver bud organoids that rescued mice from acute liver failure (ALF),[19] demonstrating their potential utility in regenerative medicine.

A recent study demonstrated that PHHs can be efficiently converted into hepatic progenitor-like cells (HepLCs) by exploiting relevant developmental cues such as the NAD^+-dependent deacetylase SIRT1 signaling.[23] This study further showed that expanded HepLCs can be converted into mature and metabolically functional HLCs using hepatic maturation medium.[23,24] Additionally, the 3D culture of HepLCs-derived HLCs supported the infection and reactivation of Hepatitis B virus (HBV), demonstrating their potential utility as a model for studying HBV biology and antiviral therapy. Huch and colleagues have also developed a protocol for long-term expansion of adult bile duct-derived bipotent progenitor cells from human liver, showed that these cells initiated organoids formation and maintained genetic stability, a key consideration for generation of cells for regenerative medicine.[3] Importantly, the ductal cells were differentiated into functional hepatocytes *in vitro* using a defined human differentiation medium and small molecules or *in vivo* upon transplantation.[3]

Studies have unveiled the culture and physical conditions required for the formation of 3D liver organoids.[3,16,25] Huch and colleagues showed that long-term expansion of 3D organoids can be achieved by culturing cells in the presence of the extracellular matrix (ECM) provided by Matrigel drops.[3] However, a more recent study by Garnier and colleagues demonstrated that 3D organoids generated in Matrigel suspension exhibited high expression of differentiation markers and albumin expression compared to those embedded in Matrigel drop.[25] Therefore, these studies have highlighted a need to grow 3D organoids in an environment that replicates the tissue niche.

2.2 | Cholangiocyte-like cells

Cholangiocytes also known as biliary epithelial cells (BECs) are the main epithelial cells lining the intra- and extra-hepatic ducts of the biliary tree.[26] These BECs primarily function to modify and transport bile constituents. The biliary excretion route is crucial for the removal of waste products in the body, such as excess cholesterol, bilirubin and hormones, as well as exogenous drugs and toxins from the liver. BECs are like hepatocytes, they have regenerative capacity to restore damaged bile ducts.[26] Several investigators have established protocols for generation of cholangiocytes from hiPSCs, hESCs, and HepRG cells.[27,28] Like hepatocytes, cholangiocytes are derived from hepatoblast (Figure 1), a common progenitor found in the liver bud during the early stages of liver organogenesis.[28-30] Dianat and colleagues successfully generated cholangiocytes-like cells (CLCs) from hepatoblasts generated from hESCs and HepaRG cells that were cultured in defined media supplemented with growth hormones, EGF, interleukin 6 (IL-6), and sodium taurocholate hydrate in the presence of ECM provided by collagen 1.[28] The resulting CLCs expressed cholangiocytes markers such as cytokeratin 18 (CK18), CK19, osteopontin (OPN), Secretin Receptor (SCTR), cystic fibrosis transmembrane conductance regulator (CFTR), apical sodium-dependent bile acid transporter (ASBT), G-protein-coupled bile acid receptor (TGR5), VEGF receptor 2, and transcription factors Sox9, HNF6, and HNF1β.[28] The cells also formed functional cysts and bile ducts with apicobasal polarity.[28]

A study by Sampaziotis has shown that cholangiocytes progenitors (CPs) can be matured by culturing cells in 3D to induce organoid formation.[29] Furthermore, modulating Notch signaling has been demonstrated to enhance the differentiation of CPs into mature cholangiocytes with similar characteristics as primary cells.[29,31] The cells cultured under these conditions proliferate rapidly, self-organize into ring-like structures, and give rise to cystic organoids and branched tubular structures bearing primary cilia.[29] Importantly, they expressed mature cholangiocytes markers such as ASBT, SCTR, CFTR, Somatostatin Receptor 2 (SSTR2), Aquaporin-1 (AQP-1), Anion Exchanger 2 (AE2), and Gamma-Glutamyl Transferase (GGT).[29,31] In addition, the organoids displayed functional multidrug resistance protein-1 (MDR1) activity by secreting rhodamine123, exported bile acid cholyl-lysyl-fluorescein, and responded to acetylcholine and VEGF stimulation.[29] It is worth noting that despite expressing signature markers of cholangiocytes and performing the key function of biliary cells, the cells still expressed fetal markers such as Sox9 as revealed by transcriptomic analysis.[29] Therefore, more research is still required to define the inductive signals and culture conditions that are required to drive the differentiation and maturation of cholangiocytes to resemble adult primary cells.

CLCs derived from patient tissues have been shown to faithfully recapitulate disease *in vitro* and validate drug efficacy. The drug octreotide was shown to reduce the size of organoids generated from hiPSCs derived from a patient with polycystic liver disease.[29] Moreover, CLCs generated from skin fibroblasts from a patient with a homozygous cystic fibrosis mutation displayed the key features of disease and failed to express functional CFTR.[29,31] The experimental drug VX809 increased the expression of CFTR and increased organoid size, validating the therapeutic effect of this drug for cystic fibrosis.[29]

2.3 | Küpffer cells

KCs are liver resident macrophages of monocyte origin. These cells form most of NPCs, representing approximately 15% of the total liver cells and 35% of the NPCs.[1,32] KCs are specialized in performing scavenger and phagocytic functions.[33] KCs also play a role in maintaining liver homeostasis and contribute to the pathogenesis of different liver diseases including liver fibrosis, viral hepatitis, steatohepatitis, and drug-induced liver injury (DILI).[33] KCs exert their effects by direct cell-to-cell contact or a paracrine mechanism involving the release of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and IL-6, growth factors and reactive oxygen species (ROS).[34,35] These pro-inflammatory mediators and ROS can cause injury to hepatocytes and NPCs.[35]

Most studies that have included KCs in models of a liver have utilized cells of animal origin.[34,36,37] The use of human KCs in coculture studies with hepatocytes is impeded by donor availability, and low yield and/or purity of adult primary cells after tedious isolation procedures.[38] Additionally, once human primary KCs are isolated and purified, they lose functionality and fail to thrive in prolonged cultures.[39] Finally, the cost of purchasing human primary cells is a major barrier for the wide utilization in *in vitro* models. Hence, there is a need to develop robust protocols to generate mature KCs from stem cells.

A recent study by Tasnim and colleagues described a protocol for generating KCs using hiPSCs.[39] The protocol involved the differentiation of iPSC into pre-macrophages using a modified protocol from Wilgenburg and colleagues.[40] iPSCs were cultured in mTeSR media supplemented with BMP-4, VEGF, stem cell factor, and ROCK inhibitor to generate embryoid bodies (Figure 2). These cells expressed macrophage markers such as CD14, CD68, CD163, CD11, and CD32.[39] EBs were further cultured in X-VIVO media supplemented with monocyte-colony stimulating factor (M-CSF), interleukin 3 (IL-3), and β-mercapto-ethanol to create pre-macrophages (Figure 2). Finally, pre-macrophages were cultured in a mix of conditioned PHHs media and advanced DMEM to generate induced Küpffer cells (iKCs, Figure 2).[39] iKCs were found to exhibit similar morphology as primary human macrophages. Moreover, iKCs displayed a similar gene expression profile as primary macrophages as shown by microarray and quantitative RT-PCR.[39] Interestingly, iKCs and pKC exhibited a transcriptomic profile that was distinct from bone marrow-derived macrophages, alveolar macrophages, non-liver macrophages as revealed by principal component analysis.[39] Finally, iKCs were able to perform phagocytosis, secrete cytokines, and be cocultured with hepatocytes similarly to primary KC.[39] It can be concluded that iPSCs-derived KCs are like adult human primary KCs and they are mature and liver specific.[39] Therefore, iPSCs-derived KCs and hepatocytes from the same donor could be used in coculture experiments and this will improve the outcomes resulting from mono-hepatocytes cultures for modeling DILI, detection of hepatotoxicity, and modeling other liver diseases. Second, this will also provide a significant platform to enable investigators to perform genetic studies based on patient-derived iKCs.

3 | APPLICATION OF STEM CELL-DERIVED ORGANOIDS IN MODELING LIVER DISEASE

3.1 | Hepatotoxicity and drug-induced liver injury

Hepatotoxicity is the second leading cause of drug failures in human beings, whereas DILI is a frequent cause of liver injury, accounting for approximately 50% of ALF cases.[41] *In vitro* studies have revealed the

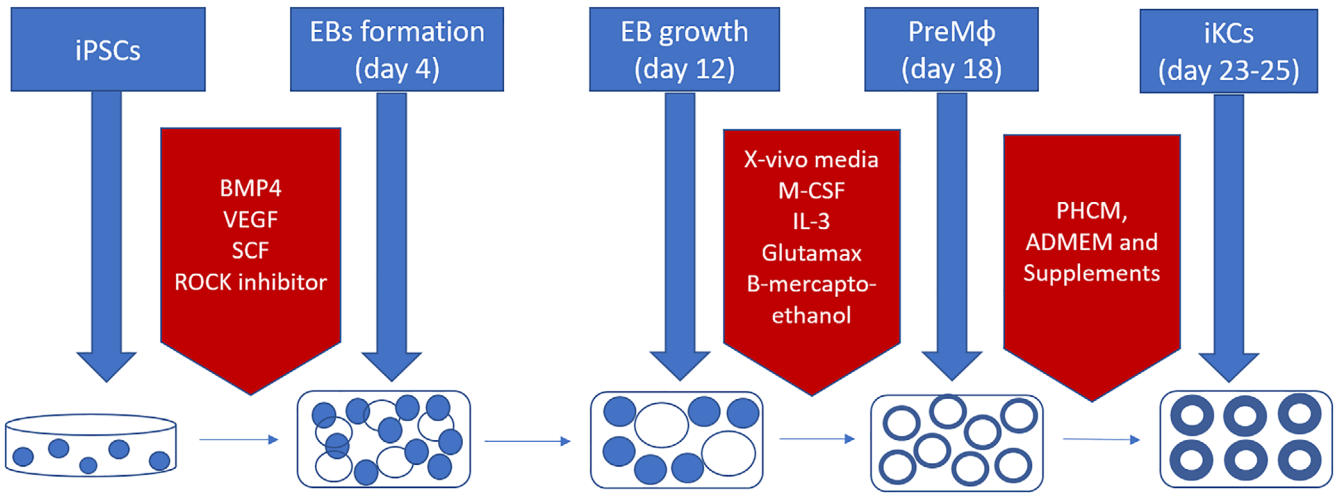

FIGURE 2 Stepwise generation of Küpffer cells from pluripotent stem cells. Pluripotent stem cells are cultured in the presence of niche factors (50 ng/mL bone morphogenic factor 4, 50 ng/mL vascular endothelial growth factor, 20 ng/mL, and 10 μM Rho-associate protein kinase inhibitor) for 4 days, to generate embryoid bodies (EBs). Then, EBs were transferred into X-VIVO media supplemented with 100 ng/mL monocyte-colony stimulating factor, 25 ng/mL interleukin 3, 2 mM Glutamax, and 0.055 mM β-mercaptoethanol to generate precursor macrophages. Precursor macrophages were then given hepatic cues and incubated in primary hepatocytes conditioned media and advanced DMEM plus supplements to generate induced Küpffer cells

potential of using PSCs-derived HLCs to model DILI,[42] and immune-mediated liver toxicity.[34] Additionally, several investigators have explored the potential use of PSCs-derived HLCs for toxicity evaluation. For example, Takebe and colleagues generated a vascularized 3D liver buds resembling adult liver tissue, by coculturing three cell types (endoderm cells, HUVECs, and MSCs) in a 3D structure.[8] These cells grew and aggregated into an organoid that mimicked a functional liver.[8,43] Moreover, transplantation of the liver buds rescued recipient mice from drug-induced liver failure.[8] Another study demonstrated that *in vitro* liver organoids derived from hiPSCs exhibited comparable responses to those observed in PHH after treatment with acetaminophen.[44]

A study by Godoy et al demonstrated that mono-hepatocytes cultures have limited abilities of reproducing hepatotoxic effects observed *in vivo*.[32] Importantly, the toxic responses *in vivo* are mediated by a complex interplay between different cell types; therefore, the predictive performance of hepatocytes alone is limited. Following that, Kostanodiva and colleagues established a human and rat *in vitro* 3D liver coculture system containing both hepatocytes and NPCs.[6] The resulting cells preserved the composition of hepatocytes, stellate cells, KCs, and endothelial cells and maintained liver function for a period of 3 months, as determined by albumin, fibrinogen, urea, and transferrin production.[6] In addition, following incubation of the cells with drugs known to induce idiosyncratic toxicity, the 3D coculture model performed better than monolayer hepatocyte cultures in detecting hepatotoxicity.[6]

A study by Rose and colleagues assessed immune-mediated hepatotoxicity over an extended period of time by coculturing rat donor-matched primary hepatocytes with KCs.[34] Lipopolysaccharide activated hepatocyte and KC coculture performed better than hepatocyte monoculture in detecting trovafloxacin and acetaminophen induced immune-mediated hepatotoxicity, indicated by decreased IL-6 production with concomitant increase in Cyp3A4 activity.[34] A recent study by Tasnim and colleagues showed that hepatocytes-iKCs coculture model was more sensitive at detecting hepatotoxicity induced by acetaminophen, trovafloxacin, and Chlorpromazine-induced cholestasis when compared to hepatocytes alone.[39] These studies have unequivocally demonstrated that this model can provide a more stable and physiologically relevant platform for investigating drug-induced and immune-mediated adverse reactions that could lead to acute hepatotoxic effects.

3.2 | Acute liver failure

Acute liver failure (ALF) is a rare but life-threatening disease, caused by viral infections and DILI.[45] Patients suffering from ALF clinically present with hepatic dysfunction, abnormal liver biochemical metabolite values, and coagulopathy, followed by development of encephalopathy with multiple organ failure.[45] There are limited treatment options for these patients, with transplantation being the most preferred option. However, transplantation presents with its own challenges such as poor access to healthy livers, increased risk of death associated with transplantation of infected tissues, partial grafts, or grafts from donors without matching human leucocytes antigen.[46,47] This has led researchers to sought alternative sources of tissues or cells for transplantation. PHHs have been used for transplantation in patients with liver-based metabolic disorders and ALF, and neonates and children with metabolic disorders.[48] This treatment option is limited by the shortage of donors with matching HLA.[49]

The recent advances in generating 3D liver organoids from stem cells promises to provide an unlimited source of cells or tissues for liver engraftment. A recent study by Nie and colleagues successfully generated single-donor human liver organoids (HLOs) using a 3D micro-well system.[48] Transplantation of the organoids into renal capsule of ALF mice significantly improved survival.[48] Similarly, Takebe and colleagues showed that transplantation of liver buds rescued Alb-Tk-NOG mice from ALF.[8] These studies have demonstrated the potential of organoid transplantation as a novel therapy for ALF.

3.3 | Hepatic steatosis

Steatosis is a benign state of liver injury, defined by the presence of fat in the liver, which accounts for more than 5% of the total weight of the organ.[50,51] Non-alcohol fatty liver disease (NAFLD) is a multifaceted and complicated disease characterized by steatosis. NAFLD patient may develop non-alcoholic steatohepatosis (NASH), which can progress to liver fibrosis and cirrhosis.[51] Generally, NAFLD is caused by interactions between environmental and genetic factors. The precise pathogenesis of NAFLD and transition to NASH is not clearly understood, although the "two hit" model has been suggested as a possible mechanism.[52] The "first hit" is lipid accumulation followed by the "second hit" oxidative stress that increases the production of pro-inflammatory cytokines and DNA damage.[52] Human liver models are required to elucidate the molecular and metabolic alteration involved in the progression of NAFLD.

More recently, a study by Ouchi and colleagues developed an *in vitro* model for steatohepatitis by incubating a multicellular HLO composed of HLCs, hepatic stellate-like cells, and Küpffer-like cells with free fatty acid (FFA) to induce steatosis and fibrosis.[53] The HLO displayed lipid accumulation in a dose dependent manner. Moreover, ELISA revealed that treating HLOs with FFA upregulated the secretion of IL-6, TNF-α, and IL-8 compared to untreated HLOs.[53] Additionally, treating HLOs with FFA induced fibrosis as indicated by increased expression of P3PN, a known marker for enhanced fibrosis. Importantly, the authors also generated Wolman disease patient iPSC-derived organoids and showed that the HLOs phenocopied the clinical features of disease. Finally, they demonstrated that treating patient-derived HLOs with FGF19 improved survival of HLOs in culture, reduced lipid accumulation, reduced ROS production, and reduced stiffening of HLOs.[53] Therefore, this study provided a proof-of-concept for using 3D organoids for modeling NASH and screening of new therapeutic drugs for NASH.

4 | CONCLUSION

Advances in the current protocols used to generate liver organoids hold a great promise for regenerative medicine and therapeutic applications. Researchers have been able to generate functional HLCs from PSCs[1,7-14,21] and patient-derived tissues.[3,16] Researchers have been able to expand and scale the production of cells,[19] and developed tools such as the Omni-well array for mass production of liver buds.[14] Moreover, development of 3D liver organoids,[13,21] coculture of multiple lineages,[8,16,48] and transplantation into immunocompromised mice[8,14] have improved the maturity and functionality of HLCs. It is crucial that challenges associated with genomic instability and formation of teratomas after transplantation of iPSC-derived liver cells are resolved to achieve wide-scale clinical use in regenerative medicine and cell therapy. Finally, liver organoids developed from patient-derived tissues have been shown to faithfully recapitulate disease *in vitro* and could be a useful tools to study disease pathogenesis and screening of novel therapeutic drugs.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

S.W.M., H.N.: conception and design, manuscript writing; H.N.: financial support, administrative support, and final approval of manuscript.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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