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REVIEW

Engineered Platforms for Maturing Pluripotent Stem Cell–Derived Liver Cells for Disease Modeling

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SUMMARY

This review summarizes the design features and characterization of advanced/engineered culture platforms useful to enhance the differentiation/maturation of stem cell–derived liver cells in vitro. The use of such platforms for modeling various liver diseases and the desirable advances to move this field forward are also discussed.

Several liver diseases (eg, hepatitis B/C viruses, alcoholic/nonalcoholic fatty liver, malaria, monogenic diseases, and drug-induced liver injury) significantly impact global mortality and morbidity. Species-specific differences in liver functions limit the use of animals to fully elucidate/predict human outcomes; therefore, in vitro human liver models are used for basic and translational research to complement animal studies. However, primary human liver cells are in short supply and display donor-to-donor variability in viability/quality. In contrast, human hepatocyte-like cells (HLCs) differentiated from induced pluripotent stem cells and embryonic stem cells are a near infinite cell resource that retains the patient/donor's genetic background; however, conventional protocols yield immature phenotypes. HLC maturation can be significantly improved using advanced techniques, such as protein micropatterning to precisely control cell-cell interactions, controlled sized spheroids, organoids with multiple cell types and layers, 3-dimensional bioprinting to spatially control cell populations, microfluidic devices for automated nutrient exchange and to induce liver zonation via soluble factor gradients, and synthetic biology to genetically modify the HLCs to accelerate and enhance maturation. Here, we present design features and characterization for representative advanced HLC maturation platforms and then discuss HLC use for modeling various liver diseases. Lastly, we discuss desirable advances to move this field forward. We anticipate that with continued advances in this space, pluripotent stem cell–derived liver models will provide human-relevant data much earlier in preclinical drug development and reduce animal usage, help elucidate liver disease mechanisms for the discovery of efficacious and safe therapeutics, and be useful as cell-based therapies for patients suffering from end-stage liver failure. *(Cell Mol Gastroenterol Hepatol 2023;15:1147–1160; https://doi.org/10.1016/j.jcmgh.2023.01.013)*

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The liver performs various essential functions, such as protein synthesis, carbohydrate and fat metabolism, bile production, and the detoxification of drugs and industrial chemicals. These functions can be compromised by several diseases, notably pathogen infections, nonalcoholic fatty liver disease (NAFLD), and hepatocellular carcinoma (HCC). Hepatitis B virus (HBV) and hepatitis C virus (HCV) have infected more than 354 million people globally (World Health Organization), while the prevalence rate of NAFLD in North America is 21%–24.7% and rising

Abbreviations used in this paper: 2D, 2-dimensional; 3D, 3-dimensional; AAT, α-antitrypsin; ADSC, adipose-derived stromal cells; AFLD, alcohol fatty liver disease; AFM, atomic force microscopy; AFP, α-fetal protein; ALB, albumin; ALD, alcohol liver disease; ALT, alanine aminotransferase; ASGPR1, asialoglycoprotein receptor 1; AST, aspartate aminotransferase; ATAC, assay for transposase-accessible chromatin; ATD, α-antitrypsin deficiency; ATF5, activating transcription factor 5; ATZ, α-antitrypsin Z; BMP4/7, bone morphogenetic protein 4/7; cAMP, adenosine 3',5'-cyclic monophosphate; CEBPA, CCAAT enhancer binding protein alpha; CK18/19, keratin 18/19; CLDN1, claudin 1; CRISPRa, clustered regularly interspaced short palindromic repeat (CRISPR)-based transcriptional activation; CXCR4, C-X-C motif chemokine receptor 4; CYP450, cytochrome P-450; DE, definitive endoderm; Dex, dexamethasone; DILI, drug-induced liver injury; DMSO, dimethyl sulfoxide; ECAD, E-Cadherin; ECM, extracellular matrix; EGF, epidermal growth factor; ER, endoplasmic reticulum; FA, fatty acid; FeLO, fetal liver organoids; FGF, fibroblast growth factor; FGF15, fibroblast growth factor 15; FH, familial hypercholesterolemia IIA; FOXA2, Forkhead box A2; GATA6, GATA binding protein 6; GelMA, gelatin-methylacryloyl; GMHA, glycidal methacrylate-hyaluronic acid; GSD1a, glycogen storage disease type Ia; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; hESC, human embryonic stem cell; hFLMCs, human fetal liver mesenchymal cells; HGF, hepatocyte growth factor; hiPSC-LO, human iPSC-derived liver organoid; HLC, hepatocyte-like cell; HNF1B, hepatocyte nuclear factor-1 β; HNF4α, hepatocyte nuclear factor-4 α; hPSC, human pluripotent stem cell; HSC, hepatic stellate cells; HUVEC, human umbilical vein endothelial cells; iHLC, iPSC-derived hepatocyte-like cell; IL, interleukin; iMPCC, micropatterned iPSC-derived hepatocyte-like cell with 3T3-J2 coculture; iPSC, induced pluripotent stem cell; iPSC-LB, induced pluripotent stem cell–derived liver bud; KC, Kupffer cells; LDH, lactate dehydrogenase; LDLR, low-density lipoprotein receptor; LEC, liver endothelial cells; LPC, liver progenitor cell; M-CSF, macrophage colony stimulating factor; MEF, mouse embryonic fibroblasts; MRP-2, multidrug resistant protein-2; MSC, mesenchymal stem cell; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NPC, nonparenchymal cell; NTCP, sodium taurocholate cotransporting polypeptide; OCLN, occludin; OSM, oncostatin M; PCL, polycaprolactone; PEG, polyethylene glycol; PEGDA, polyethylene glycol-discrylate; PHH, primary human hepatocyte; PLIN2, peripilin 1; PPAR, peroxisome proliferator activated receptor; PROX1, Prospero homeobox 1; RA, retinoic acid; RXRa, Retinoid X Receptor alpha; SLA, stereolithography; SR-B1, Scavenger receptor class B member 1; TLR, Toll-like receptor; VEGF, vascular endothelial growth factor; VP, Vat polymerization; ZO1, Zona occludens 1.

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because of the epidemic of obesity.[1] Patients with such chronic liver diseases can develop liver cirrhosis and HCC, which is poised to become the third leading cause of cancer-related deaths in the United States by 2030.[2]

Although a vaccine and antiviral drugs are available for HBV, there is no permanent cure because lifetime drug therapy is typically necessary to keep virus proliferation in check. Similarly, no long-term effective drugs for NAFLD or HCC have yet to enter the marketplace. Although animal models have provided important insights into the mechanisms of the previously mentioned diseases,[3] they do not fully capture human-relevant disease outcomes nor the diversity of such outcomes in human patients.[4–7] Thus, investigators across academia and industry are now increasingly using in vitro models of the human liver that can be used to complement animal studies for recapitulating critical features of human liver diseases and aid in the development of efficacious and safe therapeutics.

Although primary human hepatocytes (PHHs) are the gold standard cell type to study liver physiology and disease in vitro, these cells are in short supply, present donor-donor variability in viability and quality for in vitro use,[8] and cannot always be used to understand the genotype-phenotype relationships of liver diseases.[9,10] However, although hepatoma cell lines (eg, HepG2, HepaRG, and Huh7) provide an abundant and cost-effective cell source for drug screening, they suffer from very low liver functions because of their proliferative/abnormal state.[11–13] In contrast, human hepatocyte-like cells (HLCs) differentiated from human pluripotent stem cells, including induced pluripotent stem cells (iPSCs) and human embryonic stem cells (hESCs), can mitigate the previously mentioned limitations of PHHs and hepatoma cell lines; however, HLCs exhibit immature functions following the application of conventional multistep differentiation protocols. In this review, we first present different culture strategies/platforms to further mature HLCs closer to the adult PHH phenotype and then discuss ways in which differentiated HLCs are being used to successfully model key features of major liver diseases in vitro despite their non-adult-like state (Figure 1*A*). We end this review with advances that will likely be necessary to fully realize the potential of HLCs for understanding the mechanisms underlying human liver diseases and to develop novel therapeutics.

Monolayer Approaches

The hepatic differentiation in vitro follows liver development in vivo.[17] iPSCs first form definitive endoderm (DE), then specified hepatic endoderm, hepatoblasts, and finally HLCs. Several growth factors and small molecules have been used to improve protocols to generate HLCs. For instance, activin A is the key driver for definitive endoderm induction that can activate the Activin-nodal pathway.[18] Addition of small molecules, such as LY294002, which is a chemical inhibitor of PI3K, result in a significant increase in the expression of endoderm markers.[19] FGF10 and retinoic acid (RA) promote hepatic progenitor cells' differentiation and proliferation. Specifically, RA synergizes with FGF10 and Wnt signaling to promote hepatic specification.[19] FH1 and FPH1 small molecules were able to further differentiate HLCs, enabling prototypical hepatocyte morphology, including polygonal cell shape, visible nuclei, and bile canaliculi formation between hepatocytes.[20] In addition, cytochrome P-450 3A4 (CYP3A4) activity was found to increase by 16- and 45-fold following treatment with FH1 and FPH1, respectively.[20] The cytokine oncostatin M (OSM) belongs to the interleukin-6 family and plays a crucial role in hepatoblast proliferation and HLC differentiation, potentially caused by the downregulation of SOX9 and the activation of STAT3/K-RAS pathways for the formation of adherens junctions in hepatocytes.[21–23] Additionally, dexamethasone (Dex) can induce the expression of hepatocyte nuclear factor 4-alpha (HNF4A) and CCAAT/EBPa (CEBPα), which are key transcription factors that drive HLC differentiation.[24,25] More recently, Boon et al[26] found that elevating the concentration of amino acids in the culture medium significantly induced hepatic maturation, such that higher concentrations of glycine, serine, alanine, or leucine activated CYP3A4 activity; such findings indicate that modulating the availability of nutrients and their building blocks can be a complementary tool to growth factor or small molecule treatment for HLC maturation.

Different monoculture and coculture models have been used for HLC maturation. For instance, Berger et al[27] cultured iPSC-derived HLCs onto micropatterned collagen domains and then surrounded them with 3T3-J2 murine embryonic fibroblasts (Figure 2*Ai*), which have been shown to also support PHH functions. Nearly all of the HLCs that attached onto the micropatterned collagen domains displayed glycogen and albumin (ALB) staining shortly after plating, suggesting a homogenous phenotype (Figure 2*Aii*). Coculturing with the fibroblasts in the micropatterned cocultures further enhanced HLC polarity and functional maturity with significantly higher ALB secretion (Figure 2*Aiii*), urea synthesis, and activity of drug metabolism enzymes than in conventional confluent HLC monocultures. The J2 fibroblast subclone of 3T3 fibroblasts produces higher levels of decorin and truncated-cadherin, which are present in the liver and are known to induce hepatic functionality.[28,29] In another study, Freyer et al[30] cocultured iPSC-derived DE with human umbilical vein endothelial cells (HUVECs) with an optimized culture medium so that both cell types could survive and display prototypical functionality; however, cocultivation with HUVECs did not further improve the downstream HLC differentiation, potentially because liver-specific endothelia may be more suitable for such cocultures than HUVECs. Lastly, Dao Thi et al[31] found that when HLCs were cultured on Transwell™ inserts, HLCs displayed columnar polarized phenotype with clearly defined basolateral and apical membranes. Although monolayer approaches are suitable for high-throughput screening, they cannot fully recapitulate the complex 3-dimensional (3D) cell-cell and cell-matrix interactions in the liver.

Spheroids and Organoids

Spheroids and organoids are either derived from self-organized stem cells, progenitor cells, and diverse types of

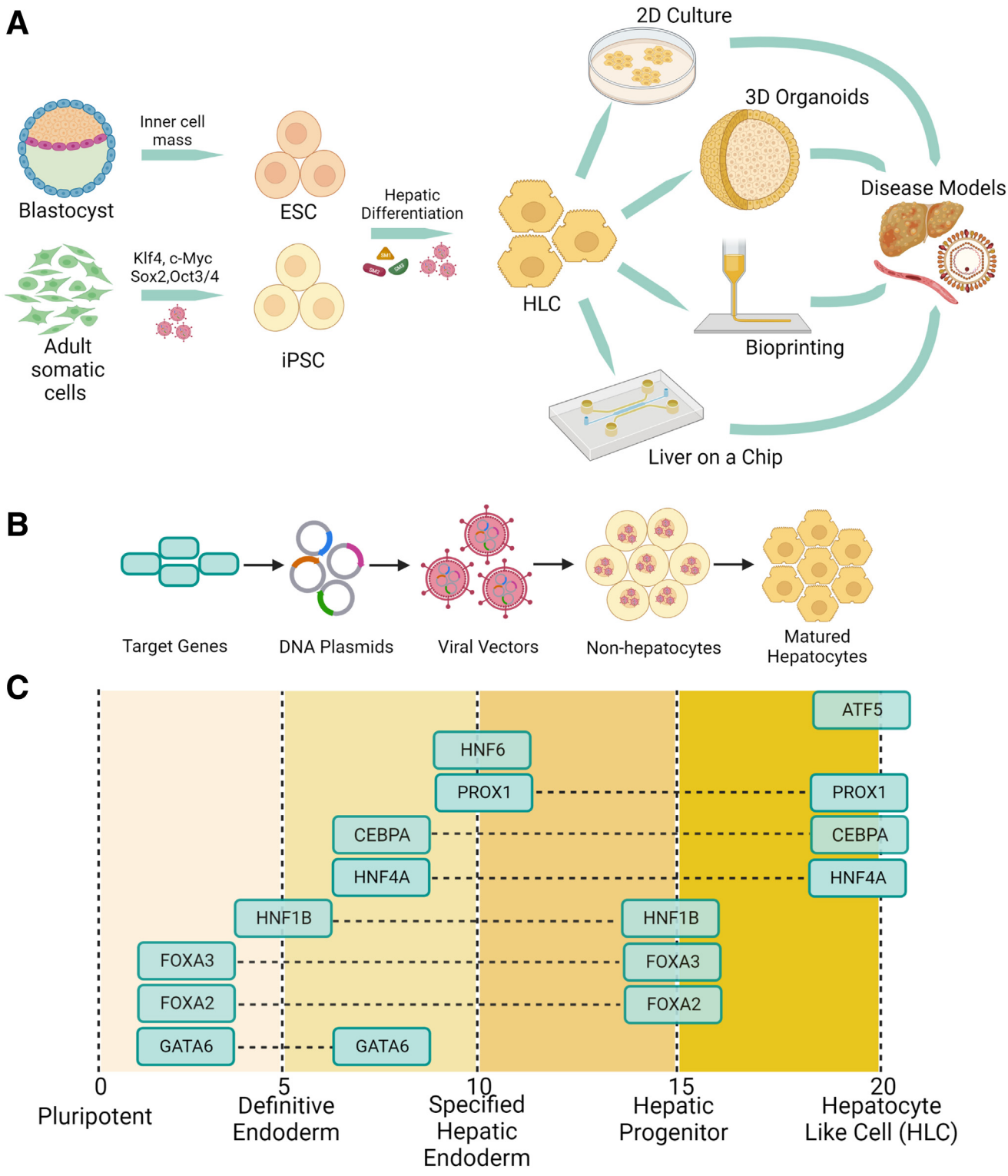

Figure 1. HLC maturation platforms/strategies with applications in disease modeling. (*A*) Cells sources considered include ESCs derived from the inner cell mass of a human blastocyst, iPSCs derived from reprogrammed adult somatic cells, and bipotent stem cells derived from adult human livers. These cells are then differentiated into HLCs via growth factors, small molecules, and/or genetic manipulation. These HLCs are integrated into various culture systems including 2D culture, 3D organoids or spheroids, bioprinted tissue constructs, and liver-on-a-chip (microfluidic) devices. The culture systems can then be used to model various liver diseases including HBV, HCV, malaria, NAFLD, ALD, and monogenic diseases. (*B*) Schematic of synthetic biology procedure to promote maturation of HLCs, first via the identification of target genes (often transcription factors), creation of DNA plasmids for each gene, insertion of those plasmids into a viral vector, and finally transduction into the nonhepatocyte cell to get more functionally mature hepatocytes through the activation of hepatocyte-related genes. (*C*) An approximate timeline of peak transcription factor expression in hepatocyte differentiation.[14–16]

mature cells or spatially patterned cells that can better mimic counterpart tissues in vivo. They are promising tools to study stem cell development, tissue regeneration, and for cell-based therapy. Several studies have shown greater functional maturation of HLCs in spheroids or organoids. For instance, Ogawa et al[36] demonstrated that adenosine 3',5'-cyclic monophosphate (cAMP) signaling enhanced the maturation of hPSC-derived hepatoblasts to a hepatocyte-like population in HLC aggregates, as assessed by higher albumin expression and CYP1A2, CYP2B6, and UGT1A1 enzyme activities. In follow-up work, these researchers showed that the addition of cAMP and Dex to the culture medium increased gluconeogenesis in HLCs.[37] Huch et al[38] created a protocol to expand and differentiate adult bile duct–derived bipotent progenitor cells. These cells showed good chromosomal stability with low rates of single base changes and displayed 10-fold fewer base substitutions than with iPSC reprogramming. The researchers found that Wnt stimulation, cAMP activation, and TGF-β inhibition were important for HLC maturation. The TGF-β inhibitor A8301, Notch inhibitor DAPT, FGF19, and Dex promoted hepatocyte maturation, as assessed by glycogen accumulation, albumin secretion, CYP3A4 activity, and ammonia detoxification. The addition of BMP7 during progenitor cell expansion and differentiation also facilitated the expression of albumin and CYP3A4. Additionally, the differentiated liver organoids were functional in vivo for 2 months and successfully mirrored genetic liver disease (α1-antitrypsin deficiency; Alagille syndrome) pathology. The longest lasting 3D HLC tissues to our knowledge were generated by the Hay laboratory, which exhibited a stable phenotype for more than 1 year, as assessed by HNF4A, E-Cadherin (ECAD), zona occludens 1 (ZO1), albumin expression, and CYP3A4 activity (Figure 2*B*).[32] In this study, HLCs were also cultured on a biocompatible polycaprolactone (PCL) nanofiber scaffold and maintained hepatic function (eg, CYP3A4 activity) for 16 days. The 3D HLC-loaded scaffolds were successfully transplanted into immunocompetent mice and immunodeficient mice, in which the HLC scaffolds were tolerated and vascularized. Moreover, the HLC tissues rescued the mice with liver injury showing downregulated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) secretions in the serum.

When iPSC-derived hepatic cells were mixed with HUVECs and mesenchymal stem cells (MSCs) at a ratio of 10:7:2 in Matrigel, an iPSC-derived liver bud (iPSC-LB) was successfully generated.[39] After transplantation into mice, iPSC-LBs could be further matured and proliferated with the expression of tight junction protein ZO1, asialoglycoprotein receptor 1 (ASGR1), albumin, and collagen IV. Of note, iPSC-LBs were connected to the host vessels within 48 hours after transplantation, which indicated that endothelial cells in the iPSC-LBs could vascularize to stimulate liver organogenesis and regeneration. An advanced protocol was generated by the Takebe laboratory in which multiple liver cell types spontaneously developed in the organoids without the addition of any exogenous cell types.[33] Specifically, five key factors (FGF2, epidermal growth factor [EGF], vascular endothelial growth factor [VEGF], Chir99021, and A83-01) were crucial for organoid expansion, and RA was used to enhance the formation of bile canaliculi (Figure 2*Ci*). Single-cell RNA sequencing revealed that the organoids were 74.41% parenchymal cells and 25.59% nonparenchymal cells; furthermore, pericentral- and periportal-like HLCs were observed (Figure 2*Cii*). Additionally, the organoids produced bile acids and exhibited bile transport properties. Lastly, organoids were placed in a 384-well plate and used for high-throughput drug screening via high content imaging readouts of hepatotoxicity and cholestasis.

Wu et al[40] generated a protocol that uses a "cholesterol$^+$ mix" at the hepatocyte and cholangiocyte differentiation stage to generate functional hepatobiliary organoids. The organoids displayed various hepatic functions, such as the uptake of indocyanine green, lipid accumulation, gluconeogenesis, albumin and urea secretions, and CYP3A4 activity; the biliary system could efflux rhodamine, store bile acid, and displayed γ-glutamyltransferase activity. Furthermore, hepatobiliary organoids survived for more than 8 weeks in vivo within immunodeficient mice as indicated by the presence of CK19, hCFTR, and Ac-a-tubulin staining patterns. To our knowledge, this is the first organoid derived from iPSCs with a well-established hepatobiliary structure and holds great potential for regenerative medicine and to dissect the molecular mechanisms underlying liver organogenesis.

3D Bioprinting

Although self-assembled spheroids and organoids can recapitulate cell-cell and cell-ECM interactions, it is difficult to control the size of the organoids and spatial localization of different cell populations. In contrast, 3D bioprinting can mitigate the previously mentioned limitations inherent with self-assembled spheroids and organoids. Current techniques used for iPSC printing rely on extrusion, stereolithography (SLA), laser-assisted printing, and drop-on-demand bioprinting.[41,42] Extrusion bioprinting is used to print iPSCs and iPSC-derived cells. Cells embedded in a hydrogel bioink can be extruded through the nozzle orifice by pneumatic or mechanical force, allowing for the generation of spatially organized and layered tissue constructs.[43] However, one disadvantage of extrusion-based printing is that the cells experience high shear stress during the process, which can damage cell membranes. In contrast, vat polymerization (VP)-based bioprinting can polymerize biomaterials through a UV laser beam or visible light in SLA via digital light projection.[44,45] Some light sensitive hydrogels, such as polyethylene glycol-diacrylate (PEGDA) and gelatin-methylacryloyl (GelMA), are commonly used in vat polymerization. Although such techniques allow for high-resolution printing, they can lead to cell damage because of UV light exposure; furthermore, the cross-linking efficiency and toxicity of the photoinitiator must be considered. Inkjet bioprinting has also been used for regenerative medicine[42]; it allows fast and precisely controlled printing to generate small molecules, growth factors, and cell-incorporated droplets, but the drawbacks of this technique

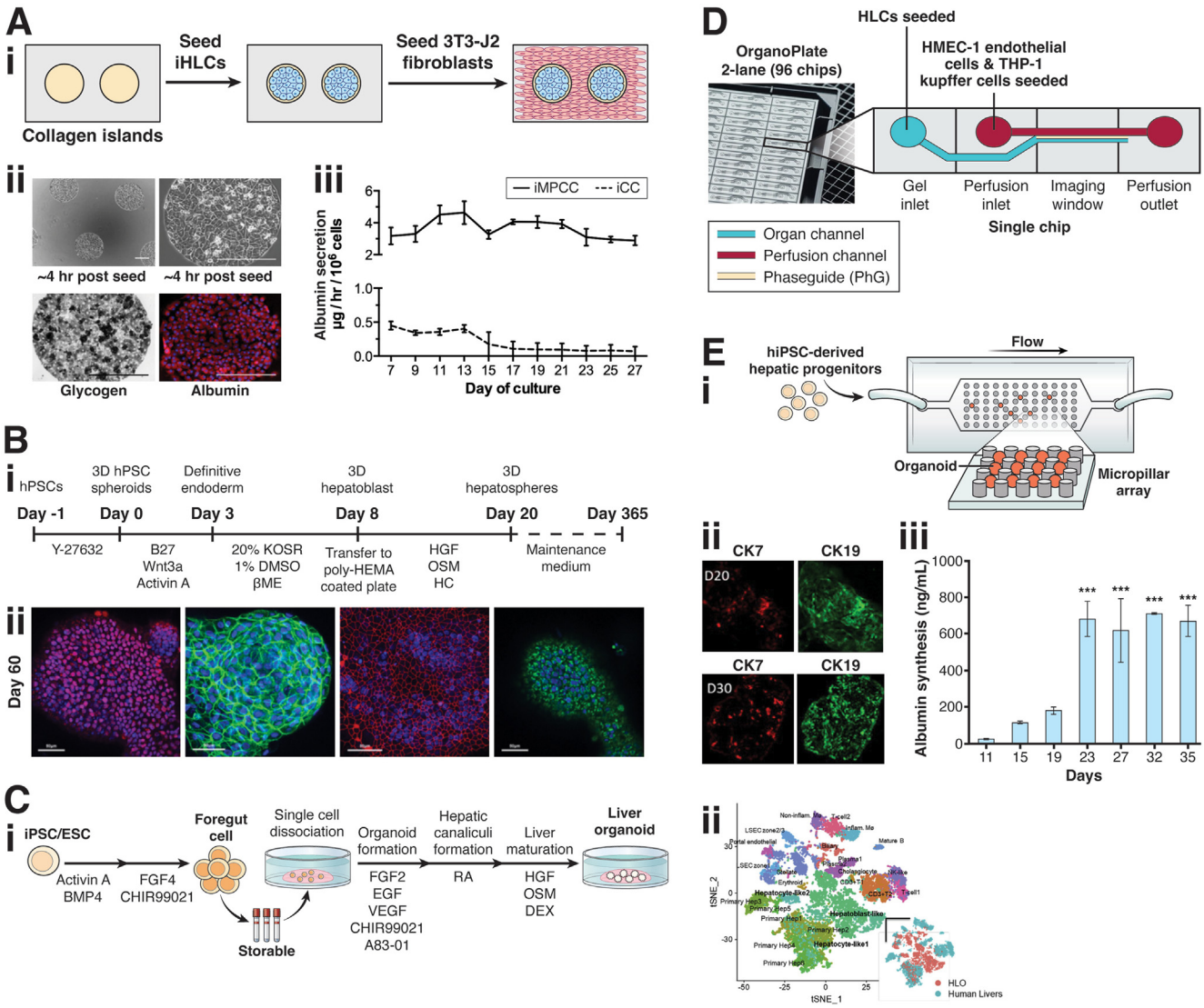

Figure 2. Advanced culture platforms for HLC differentiation/maturation. (*A*) Schematic of iHLC and 3T3-J2 micropatterned cocultures or iMPCCs (*top*-). Phase contrast, glycogenstained, and albumin-stained images of micropatterned iHLCs (*bottom left*). The iHLCs in iMPCCs display significantly higher maturation than iHLCs in conventional confluent monocultures or iCC (*bottom right*).[27] (*B*) A long-term culture protocol for generating 3D liver tissue (*top*). (*Bottom, left to right*) Confocal microscopy images of 3D liver tissue with the expression of HNF4A, ECAD, ZO1 (*bottom right second*), and ALB.[32] Scale bar: 50 μm. (*C*) Overview of human liver organoids (HLOs) generation from storable PSC/ESC-derived foregut cells (*left*). Single cell RNA sequencing (scRNA-seq) profiling of 5177 HLOs cells and 8439 primary human liver cells (*right*).[33] Each point on the integrated t-distributed stochastic neighbor embedding (tSNE) map indicates a single cell. Different color indicates different cell source. (*D*) Schematic of the OrganoPlate with one perfusion channel and one organ channel. HLCs were seeded in the organ channel, while human microvascular endothelial cells (HMEC-1) and THP-1 macrophages were seeded in the perfusion channel, which was separated by a Phaseguide from the organ channel.[34] (*E*) Generation of a human liver organoids-on-a-chip platform. Schematic of liver organoids-on-a-chip (*top*). Immunohistochemical staining images of CK7 and CK19 in organoids on day 20 and day 30 (*bottom left*). The expression of CK7 and CK19 indicated cholangiocyte formation in the organoids. Continuous albumin expression was tested from day 11 to day 35 (*bottom right*).[35]

include the limited materials used for bioinks, bioprinting-induced cell damage, and the low resolution of the bioprinted constructs.

Stereolithography and microvalve-based printing techniques have been used to print iPSCs, hESCs, hepatic progenitor cells, and HLCs. To evaluate if the printing process is gentle enough to maintain the pluripotency of stem cells, a valve-based printing technique was applied to print iPSCs and hESCs.[41] The printed cells showed no differences in the expression of hepatic markers (eg, HNF4A, albumin), cell polarity, and the presence of hepatic morphology as compared with nonprinted control subjects. This was the first demonstration that the bioprinting process did not adversely affect stem cell viability, pluripotency, and further differentiation. The non-toxic, biocompatible bioink used were GelMA, glycidol methacrylate-hyaluronic acid (GMHA),

and Geltrex®. Ma et al[44] used digital light projection to generate a 3D hydrogel-based triculture model with a microscale hexagonal architecture containing hiPSC-HPCs, HUVECs, and adipose-derived stem cells; this printed construct showed higher hepatic functions (eg, albumin, urea, CYP3A4, and CYP2C9) than conventional monolayers. Several natural (eg, alginate, cellulose, and decellularized ECM [dECM]) and synthetic (eg, polylactide-co-glycolide, polyethylene glycol, and poly-L-lactic acid [PLLA]) materials can be used to generate bioprinted constructs. Wang et al[46] examined HLC maturation in 3D bioprinted PLLA-collagen scaffold and a rat liver dECM scaffold. The iPSC-derived hepatocyte-like cells (iHLCs) on both scaffolds displayed polarized phenotype and formed bile canaliculi-like structures. The iHLCs cultured on the dECM scaffold showed significantly higher CYP2C9, 3A4, and 1A2 enzyme activities and lower expression of fetal hepatic genes (eg, α-fetal protein [AFP] and CYP3A7) as compared with either PLLA-collagen scaffold and conventional 2D monocultures.

Microfluidic (Liver-on-a-Chip) Devices

Microfluidic devices allow for the automated exchange of culture medium and exposure of certain cell types to fluid shear stress as in vivo. MIMETAS launched a commercial microfluidic cell culture platform for iPSC-derived hepatocytes, OrganoPlate LiverTox, comprised of two microfluidic channels, one for the 3D cultivation of iPSC-derived hepatocytes and another for the perfusion of endothelial cells and THP-1 monocytes (Figure 2*D*).[34] With diffusive flow from the perfusion channel into the hepatic channel, HLCs displayed stable albumin and urea secretions, increased CYP3A4 activity, and decreased AFP secretion for 15 days of culture. Haque et al[47] reported the development of a microfluidic device to mimic the liver progenitor cell (LPC) niche. This microfluidic platform is composed of two parallel microscale cell culture chambers interconnected by microgrooves. Each chamber was used for the cultivation of PHHs and HLCs, respectively.[47] The authors showed that when the HLCs were cocultured with healthy PHHs, the paracrine interactions of PHH and HLCs facilitated the differentiation LPCs toward the hepatic lineage. In contrast, when HLCs were cultured with alcohol-induced inflammatory PHHs secreting more TGF-β1, LPCs differentiated into cholangiocyte-like cells. Lastly, Rashidi et al[48] demonstrated that HLCs cultured under fluid shear stress showed increased CYP1A2 and CYP2D6 activities.

The liver tissue exhibits metabolic and functional changes across the liver sinusoid, a phenomenon called zonation, which is brought about by the gradients of oxygen, hormones, nutrients, and other signaling factors.[49] Tonon et al[50] developed a microfluidic device to differentiate hESCs into HLCs by controlling the O_2 gradient; this device includes linear channels for cell culture along with a 95% N_2/5% CO_2 gas mixture continuous flow. A computational analysis was performed to produce a stable O_2 gradient range from 160 mm Hg (21%) to 15 mm Hg (2%). The cells were first differentiated for 8 days under ambient O_2 and then differentiated for another 9 days under the O_2 gradient. The HLCs displayed lower CYP3A4 activity and glycogen synthesis under high pO_2, which is consistent with the metabolic changes found in the different zones of the hepatic lobule.

Organ-organ crosstalk is important in physiology and disease, including for drug metabolism and disposition. Thus, microfluidic devices are now being designed with chambers for multiple tissue types connected via flowing culture medium. For instance, in one study mouse embryonic fibroblast (MEF)-derived HLCs were cultured within a 3D porcine liver dECM hydrogel to promote cell-cell and cell-ECM interactions; HLCs displayed higher albumin expression on the dECM-coated substrate as compared with collagen.[51] To generate a vascularized liver organoid-like tissue structure, HLCs were cocultured with HUVECs under gravity-driven medium flow, which facilitated the formation of HUVEC vascular networks. Coculture with medium perfusion significantly increased HLC maturation as assessed by higher albumin and urea secretions and CYP3A4 activity. Additionally, the liver tissue was cultured with stem cell–derived intestinal and stomach organoids. Treatment of the multitissue device with primary bile acid chenodeoxycholic acid decreased CYP7A1 expression in the liver compartment, likely caused by fibroblast growth factor 15 (FGF15) expression in the intestine,[52] suggesting paracrine crosstalk between the two organoid types.

Synthetic Biology Approaches to Mature HLCs

HLCs and stem cell–derived liver organoids tend to have a more fetal-like phenotype as compared with adult human tissue.[33,53,14] To address this limitation, key transcription factors in hepatocyte differentiation and liver organogenesis have been identified and targeted for directed differentiation (Figure 1*B* and *C*). Hepatocyte nuclear factor HNF4A and GATA6 have been identified as key regulators of hepatic development in mouse studies.[54–56] GATA6 is highly expressed in the endoderm stage of development and modulates HNF4A expression.[53] HNF4A is highly expressed in the hepatic progenitor stage and regulates 40% of the actively transcribed hepatic genes (eg, HNF1B, CEBPA, FOXA2, and GATA6), which are vital to hepatic functions including the production of serum factors, proteins, and apolipoproteins.[55,56] Thus, Guye et al[53] used heterozygous lentiviral transfection of a doxycycline inducible GATA6 gene to create multilineage fetal liver organoids (FeLO) from human iPSCs; cells with high GATA6 expression produced endoderm lineage cells, low GATA6 expression produced mesoderm lineage cells, whereas no GATA6 expression produced ectoderm lineage cells. In a follow-up study with the FeLOs, PROX1, and ATF5 were identified via RNA sequencing as potential targets based on their low level of expression in the FeLO as compared with the adult livers.[15] ATF5 is a transcription factor associated with the proliferation and differentiation of several tissues and causes the

activation of cytochrome P-450 (CYP450) enzymes in hepatocytes,[57] whereas PROX1 is a key transcription factor for the metabolic maturation of hepatocytes. Overexpression of ATF5 and PROX1 via lentivirus transduction channeled FeLO toward a more liver-like identity as confirmed by RNA-sequencing and ATAC-sequencing analysis; however, the CYP3A4 activity was still low as compared with freshly isolated PHHs.[15] To improve this, the authors stimulated CYP3A4 activity with clustered regularly interspaced short palindromic repeat (CRISPR)-based transcriptional activation (CRISPRa). CRISPRa uses a deactivated cas9 protein to place a promoter near the gene of interest without cutting the DNA. The engineered liver organoids with PROX1, ATF5, and CYP3A4 activation/upregulation had improved albumin, α-1 antitrypsin (AAT), angiopoietin like 3, and complement 3 expressions over the baseline FeLOs and had similar levels as PHHs. Another study also showed that overexpression of ATF5, CEBPA, and PROX1 in HLCs with an adenovirus construct increased hepatocyte-related mRNA and protein expression including CYP450 enzymes, AAT, and HNF4A.[58]

In addition to improving differentiation/maturation, synthetic biology techniques can be used to induce the proliferation of stem cell–derived hepatic progenitors. For instance, one study performed a high throughput screen of various fibrotic proteins to test their influence on the proliferation of hESC-derived hepatoblasts. A combination of fibrotic niche proteins that outperformed industry standard Matrigel in terms of stimulating proliferation was identified from this screen and characterized with proteomics. The fibrotic niche proteins deemed essential for the proliferation of hepatic progenitors included collagen III, IV, IL17, IL18, and M-CSF.[59]

Despite all of the significant advances described previously in using synthetic biology techniques to improve HLC differentiation, there are many unexplored potential future applications for synthetic biology with HLCs and liver organoids. For example, many organoids are self-organizing, which can lead to high variability and an architecture that does not recapitulate in vivo structures. This issue could be addressed by designing cell-cell interactions through a synthetic notch pathway that controls cadherin expression and thus cell migration and organization in 3D spheroids.[60,61] In addition to spatial control, precise temporal control of signaling pathways plays an important role in hepatocyte differentiation. For example, BMP signaling is upregulated or downregulated depending on the stage in differentiation.[62] Serial CRISPR-Cas9 activation schemes could be designed to achieve this activation and inhibition in a time dependent manner.[63]

Applications of HLCs in Disease Modeling

HBV and HCV Infection

Xia et al[64] investigated HBV infection in HLC 2D monocultures, which expressed relevant host factors for HBV, such as HNF4A and sodium taurocholate cotransporting polypeptide (NTCP). HLCs supported efficient HBV infection for 4 weeks, and the spread of HBV infection in the HLCs could be inhibited via treatment with viral entry inhibitor Myrcludex B. Nie et al[65] evaluated HBV infection in an iPSC derived liver organoid (hiPSC-LO) that incorporated iPSC-derived endoderm, mesenchymal stem cells and HUVECs with a ratio of 10:1:7 (Figure 3*Ai*). The hiPSC-LO supported long-term HBV infection showing HBcAg, pregenomic (pgRNA), and HBV DNA expression, and the collected infected supernatant was able to infect human hepatocytes, suggesting the generation of live virus particles by the organoids (Figure 3*Aii*).

Schwartz et al[66] infected iPSC-derived HLC monocultures with HCV and demonstrated persistent TNFα secretion for 2 weeks following infection. HLCs also displayed upregulated CXCL10 and IL28B expression suggesting the potential to study immune response to HCV as compared with the cancerous Huh7 cell line (Figure 3*C*).[69] A genome-wide association study showed that polymorphisms in the IL28B gene are highly correlated to HCV clearance and response to IFN-based treatment.[70] HLCs express IL28B in response to viral infection 2 days after infection, suggesting the utility of HLCs for modeling the effects of IL28B polymorphisms in HCV infection, clearance, and antiviral drug response.[66,70]

Sa-ngiamsuntorn et al[67] generated a protocol to reprogram mesenchymal stem cells into iPSCs, which were further differentiated into HLCs. These HLCs expressed four HCV receptors (Claudin-1. Occludin. Scavenger receptor class B member 1, and CD81) on their basolateral membrane and apolipoproteins and LDL-receptors on the apical membrane. As compared with Huh7 cells, HLCs displayed better cell polarity and viral uptake; furthermore, HLCs, unlike Huh7 cells, could be infected with HCV genotypes 1a, 1b, 3a, 3b, 6f, and 6n (Figure 3*D*).

iPSCs and definitive endoderm cells are not permissive for HCV infection, whereas iPSC-derived hepatic progenitor cells can be persistently infected.[71] Yoshida et al[72] found that iPSC express CD81 and Occludin but they lacked two key receptors (SR-B1, CLDN1) and miR-122 related to HCV replication. Wu et al[71] did a comparison of gene expression profiles at the transition points from resistance to susceptibility to HCV infection during iPSC differentiation. The investigators demonstrate that the activation of certain host factors (eg, miR122, EGFR/EphA2, PI4KIIIa) and the downregulation of antiviral host factors (eg, IFITM1) were correlated with HCV permissiveness/infection.

Malaria

More than 200 million cases of malaria occurred globally from 2010 to 2019, with more than 400,000 deaths worldwide.[73] Malaria is caused by protozoan parasites, including *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malaria*, and *Plasmodium knowlesi*. Controlling plasmodium parasite replication at the liver stage is an attractive target for developing antimalarial drugs and vaccines. Primaquine treatment in patients with glucose-6-phosphate dehydrogenase deficiency disease leads to dangerous hemolysis.[74] Resistance to malaria

treatments (eg, artemisinins and chloroquine) has been detected in many countries.[75] In addition, it is important to test for highly polymorphic genetic variants related to drug metabolism because they can influence the efficacy of antimalarial drugs. HLCs can serve as a useful model to study a specific patient's response to malaria infection and antimalaria drug efficacy in the context of host genetics. Toward that end, Ng et al[76] investigated the feasibility of infection of iPSC-derived HLCs with *Plasmodium burgher*, *Plasmodium yoelii*, *P falciparum*, and *P vivax*. They found that the differentiated cells at the hepatoblast stage were permissive to malaria infection; further maturation of HLCs via treatment with small molecule FPH1 increased HLC sensitivity to antimalarial drug efficacy.

Nonalcoholic Fatty Liver Disease

NAFLD is one of the most common chronic liver diseases worldwide and affects an estimated 30%–40% of the US population.[77] It is characterized by the accumulation of intracellular lipid droplets due to factors such as high-fat diets, sedentary lifestyle, and metabolic disorders including type 2 diabetes and obesity.[77–79] Certain genetic risk factors, such as gene polymorphisms in PNPLA3, MBOAT, and TM6SF2, have also been associated with an increased risk of developing NAFLD.[78,80] Approximately 20% of patients diagnosed with NAFLD develop nonalcoholic steatohepatitis (NASH), which is characterized by hepatocyte ballooning, necrosis, inflammation, and fibrosis; NASH predisposes patients to cirrhosis and liver cancer.[79] There are currently no FDA-approved pharmaceutical treatments for NAFLD/NASH. Thus, iPSC cell lines derived from patients diagnosed with NASH/NAFLD can provide valuable insights into the genetic markers of disease progression, and enable drug development to include these patient types during compound screening. For instance, Gurevich et al[79] published a differentiation process for generating iPSC cell lines from healthy and NASH-diagnosed donors. Although functional data was comparable between healthy and diseased groups, the NASH donor-derived HLCs displayed spontaneous lipidosis (a characteristic feature of NAFLD/NASH) without fatty acid (FA) treatment and retained higher levels of lipid accumulation than the healthy control. Graffman et al[81] used ESC- and iPSC-derived HLCs from healthy donors to investigate gene-level changes in hepatocyte-like cells after inducing steatosis. Through this study, the authors were able to observe upregulations in Peripilin 2 (PLIN2), a lipid droplet coating protein, and several genes in the peroxisome proliferator activated receptor (PPAR) pathway, which is involved in regulating lipid metabolism. Having demonstrated that gene-level changes caused by lipid accumulation can be identified in HLCs, the authors went on to create four iPSC lines from donors with varying degrees of steatosis and then studied the responses of HLCs to lipid accumulation and treatment with the adiponectin-like synthetic small molecule AdipoRon, which has been shown to improve insulin sensitivity and to reduce fasting blood sugar levels in obese mice; however, responses to both the FA treatment and AdipoRon treatment varied between the iPSC lines.[82] For example, the healthy control donor line accumulated the largest lipid droplets during FA treatment, which increased in size after incubation with AdipoRon. Furthermore, none of the steatotic cell lines responded to AdipoRon treatment despite containing the enzymes involved in the signaling pathways targeted by Adiporon. In another study, Parafati et al[78] investigated fatty acid–induced and endoplasmic reticulum (ER) stress-induced steatosis in HLCs using high content imaging in the monolayers.

Building on their 2D monolayer, Gurevich et al[79] demonstrated that their iPSC-derived HLCs could be cultured within 3D organoids containing isogenic iPSC-derived mesenchymal stem cells, macrophages, and endothelial cells from normal and NASH sources. Ouchi et al[83] developed both diseased and healthy multicellular liver organoids derived from ESC and iPSC cell lines that recapitulated steatosis, inflammation, and fibrosis under FA treatment. These organoids, unlike those used by Gurevich et al[79] where multiple cell types were mixed and allowed to aggregate, involved differentiating multiple cell lineages directly from iPSCs into 3D organoids. The authors also demonstrated a novel method of measuring organoid fibrosis through atomic force microscopy.[83] In a follow-up study by the same group, Kimura et al[84] treated 24 HLC-based organoids from different patients with fatty acids to first induce a relative insulin insensitive state and then probe the genetic factors that exacerbated a NASH-like phenotype in the liver organoids; retroactive analysis of human clinical trial data coupled with findings from the organoids revealed a critical interplay between genetic predisposition and metabolic status that can dramatically alter drug responses.

Using microfluidic technology, Wang et al[35] developed an organoid-on-a-chip model that allowed for the differentiation and formation of iPSC-derived liver organoids that developed a NAFLD-like phenotype under exposure to FAs. The immediate benefits of this model are the ability to develop functional organoids in a perfused system for NAFLD disease modeling and analysis. The chip was demonstrated to form iPSC-derived organoids consisting of cholangiocytes and HLCs with the expression of cholangiocyte markers (eg, CK7 and CK19) and hepatic markers (eg, HNF4A, ALB) that were amenable to long-term culture up to a month (Figure 2*E*). After steatosis induction, liver organoids displayed lipid droplet accumulation, enhanced expression of lipid metabolic-related genes, increased inflammatory cytokines secretion (eg, IL8, IL17, TNF α), and intracellular reactive oxygen species production (Figure 3*B*). Besides organoids, other 3D models involving the coculture of multiple cell lineages have been used to model NAFLD. For example, Kumar et al[85] developed a polyethylene glycol (PEG) hydrogel, functionalized with ECM components and cell-adhesion molecule peptides, at an optimal stiffness to support the maturation of iPSCs and ESCs to HLCs. This hydrogel, named "Hepmat," supported the coculture of HLCs and predifferentiated PSC-derived hepatic stellate, endothelial and macrophage-like cells up to 40 days. The authors then demonstrated that these cocultures could support

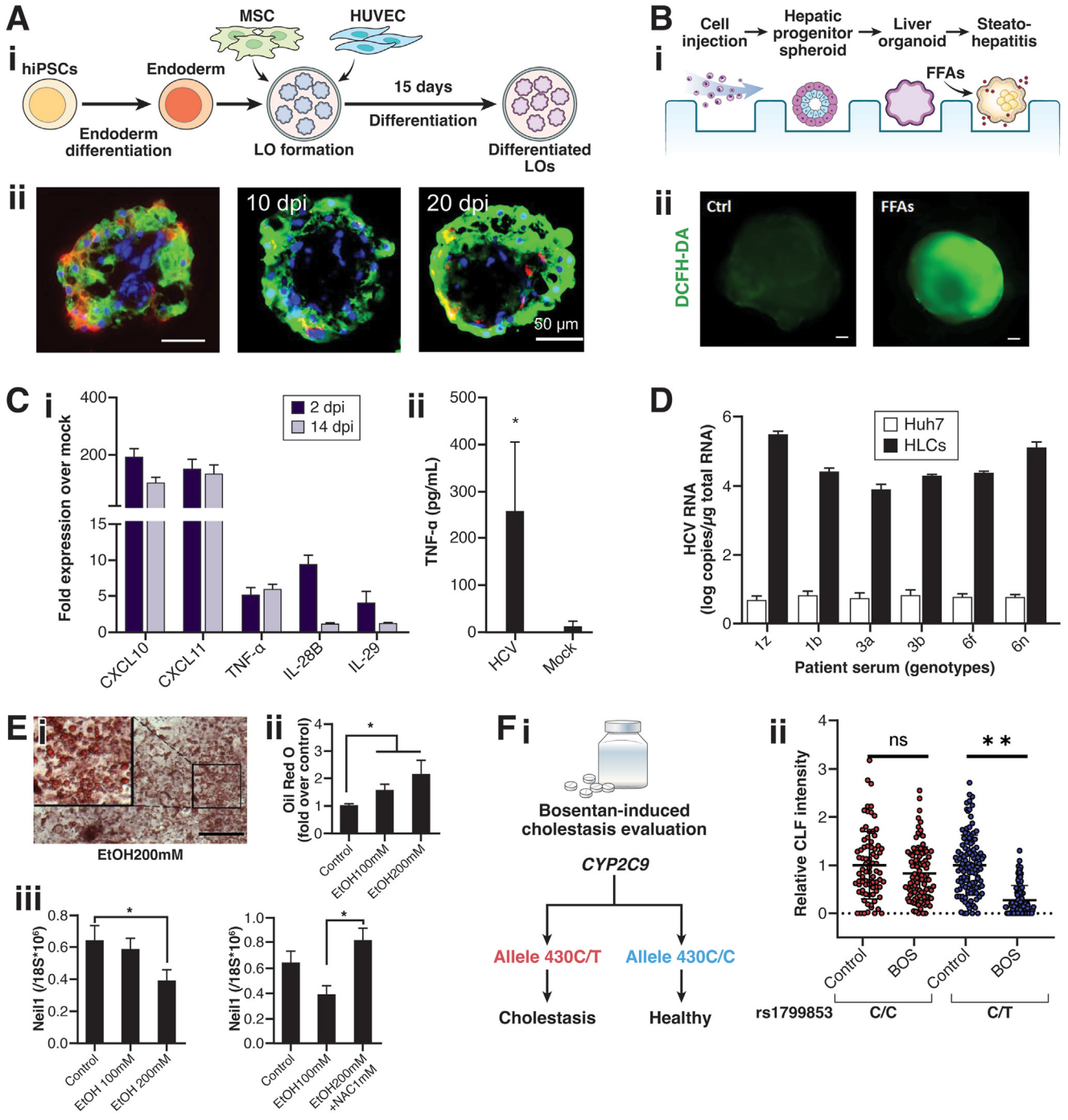

Figure 3. Disease modeling with HLCs. (*A*) Schematic overview of human liver organoid (HLO) formation from iPSCs (*top*). Immunofluorescence staining image of ALB (*green*) and NTCP (*red*) in HLOs (*bottom left*). Immunofluorescence staining of ALB (*green*) and HBV core antigen (*red*) in infected HLOs at 10- and 20-days postinfection (dpi).[65] Scale bar: 50 μm. (*B*) Schematic representation of the generation of liver organoids-on-a-chip and free fatty acids (FFAs)-induced steatohepatitis (*top*). Staining for reactive oxygen species (ROS) generation (*green*) via use of the DCFH-DA dye.[35] Scale bar: 100 μm. (*C*) Inflammatory response of iHLCs to HCV infection. mRNA expression of each cytokine in infected group relative to the mock condition (*left*). TNF-α secretion was measured post infection day 14 (*right*).[66] (*D*) HCV RNA expression in Huh7 and HLCs. Different HCV genotypes were investigated.[67] (*E*) Alcohol increased lipid accumulation and oxidative mitochondrial injury in iHLCs. Lipid was stained by Oil Red O (*top left*) and stain was then quantified (*top right*). The expression of Neil1 before and after *N*-acetyl-L-cysteine (NAC) treatment was measured at day 25 in iHLCs that were exposed to alcohol at 200 mM (*bottom*).[68] (*F*) Schematic design for testing bosentan DILI on liver organoids derived from patients with (C/T) and without (C/C) a susceptibility gene (*left*). Cholylysyl-fluorescein (CLF) intensity after bosentan treatment of organoids from patients with (C/T) and without (C/C) a DILI susceptibility gene 34; rs1799853, a single nucleotide variant in the CYP2C9 gene.

TGFβ-induced fibrosis and inflammation induced by FA treatment better than representative cellular monocultures.[85] A benefit of this approach to 3D culture as compared with differentiated organoids is that the ratios of each cell population can be better controlled to reflect in vivo liver populations.

Alcoholic Liver Disease

Alcohol liver disease (ALD) is comprised of alcoholic fatty liver disease (AFLD) and advanced alcoholic hepatitis, cirrhosis, and cirrhosis complications. It is a leading cause of mortality in the United States. From 2015 to 2016, AFLD affected 4.7% of adults in the United States.[86] About 1.7% of ALD patients in combination with stage 2 or greater fibrosis can progress to severe liver diseases, such as cirrhosis and liver cancer.[86] To advance the study of ALD pathogenesis, several iPSC-derived liver models have been generated. Tian et al[68] evaluated the effect of ALD on each stage of HLC differentiation and found that alcohol did not affect definitive endoderm formation as assessed by SOX17 and CXCR4 expression. At the early stage of HLCs maturation, alcohol significantly decreased cell proliferation but did not influence cell differentiation because the expression of hepatic progenitor markers, such as AFP, CK19, CD133, and EpCAM, were not altered by alcohol treatment. In the mature stage, alcohol did not significantly alter hepatic markers (eg, ALB, CYP3A4, CK18) expression, but induced disease phenotypes with higher lipid droplet accumulation and tumor biomarker (eg, GPC3, FLNB) expression and oxidative mitochondrial damage; treatment with *N*-acetyl-L-cysteine restored the mitochondrial repair enzyme Neil-1 activity and improved markers associated with HCC and steatosis (Figure 3*E*).

Wang et al[87] generated hESC-derived liver organoids that could be expanded to 10^{18} cells after 5 months of culture with a normal karyotype even after at least 4 months. Fetal liver mesenchymal cells (hFLMCs) were cocultured with the organoids at a ratio of 2:1. After ethanol treatment for 7 days, the organoids displayed increased CYP2E1 activity and upregulated secretion of ALT, AST, lactate dehydrogenase (LDH), and fibrogenic markers (eg, LOXL2, COL1A1, COL3A1) expression. Ethanol treatment also led to enhanced oxidative stress, mitochondrial membrane potential depolarization, and proinflammatory responses showing IL1 signaling activation and IL17 expression.

Monogenic Diseases

iPSC is a powerful model system for studying monogenic diseases because they maintain the genetics of the patient donor. Classical α-antitrypsin deficiency (ATD) is a genetic metabolic disease characterized by the intracellular accumulation of misfolded α-antitrypsin Z (ATZ) protein in hepatocytes. HLCs made from patients with ATD showed an accumulation of the misfolded α-antitrypsin protein.[88] In another study, HLCs were generated from wild-type control patients, genetic mutant patients with no overt ATD-associated disease, and those with severe ATD-associated disease.[89] The genetic mutant lines did have an accumulation of ATZ regardless of liver disease status, whereas the control lines did not. In addition, HLCs derived from patients with more severe liver disease had a delay in ATZ degradation as compared with the HLCs from patients with no overt disease.

Familial hypercholesterolemia IIA (FH) is caused by mutations in the low-density lipoprotein receptor (LDLR), which leads to high levels of low-density lipoprotein cholesterol. The iPSCs from patients with FH were generated and had their gene mutation corrected.[88,90–92] The HLCs derived from FH patient iPSCs showed abnormal LDLR protein expression and lower low-density lipoprotein uptake consistent with the disease phenotype. The gene-corrected HLCs showed some recovery from the disease phenotype.

Glycogen storage disease type 1a (GSD1a) is characterized by mutant glucose-6-phophatase, which is an important enzyme in gluconeogenesis and glycogenolysis. HLCs created from a patient with GSD1a showed increased glycogen, lipid, and lactate accumulation as compared with a healthy control subject, which is in line with the disease phenotype.[88]

Wilson disease is characterized by a mutation in a gene that encodes for a transporter protein responsible for copper export into the bile and blood. One group created HLCs from iPSCs derived from patients with Wilson disease and saw abnormal localization of the transporter protein and defective copper transport; the phenotype could be reversed by correcting the mutation and treating with curcumin.[93] Another group showed that gene-corrected HLCs from patients with Wilson disease could slow the manifestation of Wilson disease in a mouse model following implantation.[94]

Genetics can also play a role in patient susceptibility to drug-induced liver injury (DILI). One group made liver organoids from iPSC lines from patients with or without a bosentan-induced liver injury susceptibility gene.[33] When treated with bosentan, the organoids with the susceptibility gene had a significant impairment of bile acid excretion shown by lower cholyl-lysyl-fluorescein intensity, but the organoids without the susceptibility gene did not (Figure 3*F*).

Conclusions and Future Outlook

HLCs, as an alternative cell source to PHHs, hold great promise in multiple research areas, such as disease modeling, precision drug development, and cell-based therapies. Although in vitro generated HLCs have improved in maturation with several of the approaches discussed here, much work needs to be done to achieve adult functions at similar levels as PHHs. Allowing the cells to progress through full maturation via cell autonomous pathways will likely be too costly and inefficient. Therefore, we anticipate that combinations of gene activation and suppression coupled with microenvironmental engineering (eg, micropatterning, microfluidics, bioprinting, and so forth) may be most suited to achieve sufficiently mature

cells for downstream applications. Furthermore, for regenerative medicine, implantation of the in vitro differentiated HLCs into the host may allow accelerated maturation because of the presence of complex blood factors and organ-organ crosstalk.

It is clear from the studies discussed here that HLCs can be further matured in response to homotypic and heterotypic cell-cell interactions, soluble factor gradients, ECM proteins, and medium perfusion. Although these factors have been tested individually and in some limited combinations in a few studies, their combination into an integrated platform that can also retain its cost-effectiveness and throughput for screening applications has yet to be realized and will likely be an important goal to achieve as a collaborative effort between clinician scientists, developmental biologists, and biomedical engineers. Furthermore, the ability to culture many iPSC lines into such a platform with reproducible results will be important for routine use in pharmaceutical practice and to elucidate the mechanisms of liver development.

Although some of the liver organoids generated so far contain liver-like nonparenchymal cells (NPCs), protocols for generating functionally mature stem cell–derived liver NPCs need to be further improved to enable liver organoids that more faithfully recapitulate liver functions. Organoids with the full complement of liver cells will be particularly useful to model liver diseases, such as HBV and NAFLD in which cellular crosstalk is critical for disease progression. Although there has been some progress in showcasing hepatic-NPC interactions in organoids, much work needs to be done to model progressive disease with molecular changes that mimic the in vivo situation.

Although 3D organoids hold the promise to be more physiologic in their architecture, cellular complexity, and phenotypic responses, the ability to monitor their phenotype using advanced imaging techniques and embedded biosensors for metabolite and protein production will be important to ensure the scalability of organoids to high-throughput drug screening and ultimately for cell-based therapies.

In conclusion, significant progress has been made in advanced approaches to generate HLCs and use them to model several liver diseases. We anticipate that continued progress in this space will enable the use of HLC-based liver organoids for several applications and eventually significantly reduce the use of animal models for biomedical research and pharmaceutical development.

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Conflicts of interest
The authors disclose no conflicts.

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