# Human Induced Pluripotent Stem Cell-Derived Liver Models

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The liver is the largest organ of the human body and is composed of parenchymal cells, namely hepatocytes and cholangiocytes, and numerous other cells, also called non-parenchymal cells, namely hepatic stellate cells (HSCs), liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs, the resident liver macrophages) and various immune cells. The pharmaceutical industry is in high need of efficient and relevant in vitro liver models, which can be incorporated in their drug discovery pipelines to identify potential drugs and their toxicity profiles. Current liver models often rely on cancer cell lines or primary cells, which both have major limitations. However, the development of human induced pluripotent stem cells (hiPSCs) has created a new opportunity for liver disease modeling, drug discovery and liver toxicity research. HiPSCs can be differentiated to any cell of interest, which makes them good candidates for disease modeling and drug discovery. Moreover, hiPSCs, unlike primary cells, can be easily genome-edited, allowing the creation of reporter lines or isogenic controls for patient-derived hiPSCs.

liver disease modeling human induced pluripotent stem cells (hiPSCs)

## 1. 2D Culture Systems

The 2D monolayer cell culture is a traditional in vitro model for studying the response of hepatocytes to drugs. This model has the advantages of easy and low-cost operation. Multiple studies have been carried out wherein 2D cultured hPSC–hepatocyte-like cells (HLCs) were used for drug toxicity testing and modelling liver disease. Even if hPSC–HLCs have lower drug biotransformation capabilities than short-term cultured PHHs, correct identification of toxic drugs and chemicals appears superior over that of immortalized cell lines, commonly used in pharmaceutical studies [1][2]. Moreover, like for PHHs, hPSC–HLCs can also be used for the identification of drug-mediated steatosis, apoptosis and cholestasis [3]. Due to the relatively easy genome engineering of iPSC, mode of action (MOA) of drugs can be evaluated in real time using HLCs derived from PSCs wherein stress pathway reporter genes have been incorporated, which cannot be achieved using PHHs [4]. 2D hPSC–HLC culture systems exploiting patient-derived iPSC, including genetically corrected isogenic cells, can also be used to model genetic liver diseases and identify new drug candidates for diseases, such as familial hypercholesterolemia, glycogen storage disease, Wilson's disease, Alpers syndrome, α1AT deficiency, Crigler-Najjar Type 1, defective mitochondrial respiratory chain complex disorder and hereditary tyrosinemia [5][6][7][8][3][9][10][11][12][13][14]. In addition, 2D hPSC–HLC models also allow studying infectious liver diseases caused by Hepatitis B, C and E (reviewed in [15]) or malaria [16]. Finally, such 2D differentiation cultures could be upscaled and automated such that screening of toxicity or efficacy of drugs can be conducted at a medium throughput level (100–3000 compounds) and have been

used for drug testing and liver toxicity screenings by different research groups, but are to the best of knowledge not yet implemented in industrial testing platforms [2][3][17][18][19].

Nevertheless, even though 2D PSC–HLC cultures are an easy and stable model to perform drug screens, they remain immature, express fetal markers, as well as some 'de-differentiation' characteristics also observed in long-time plated PHHs [20][21]. HLCs express low levels of a number of phase I and phase II drug metabolization genes and enzyme activity [22][23][24][25]; consequently, for drugs that are metabolized to toxic metabolites by certain *CYP450* genes, toxicity will likely not be detected and this may lead to false-negative or false-positive results. In addition, as not all transmembrane transport proteins responsible for drug uptake are expressed at levels seen in PHHs, this may also affect drug toxicity and efficacy evaluation [23][24]. As with PHH mono-cultures, 2D HLC mono-cultures fail to reflect the complexity of the multicellular liver.

## 2. 3D Culture Systems

To maintain PHH maturity and/or enhance and maintain hPSC–HLC maturity, 3D cultures have been developed. Terminology used for such cultures ranges from aggregates or spheroids to organoids. Although in more recent years, these different terms have been associated with specific approaches to create 3D liver (and other tissue) models, many studies use the terms interchangeably. Aggregate or spheroid cultures consist of pre-differentiated lineage specific cells/precursors that are allowed to aggregate and then be maintained in 3D culture. This can be carried out using a single cell type or by incorporating multiple different cell types that can be derived from either one or multiple germ layers. Organoids by contrast, are in general derived from a single cell, whereby spontaneous differentiation and patterning occurs, yielding multicellular cultures that do or do not display a central cavity. Although many organoids contain differentiated progeny from only a single germ layer (e.g., hepatocytes and biliary cells), some reports have suggested that liver organoids may also contain cells of a second mesodermal germ layer, important to recreate the multi-germ layer origin of complex liver tissue.

Spheroid/aggregate cultures or organoid cultures can be generated scaffold-free, or by incorporation in a scaffold. Scaffolds for liver model creation usually are porous, soft hydrogels, that enable free transmission of liquid, gasses and molecules. These scaffolds can be generated from natural polymers (e.g., gelatin, collagen, laminin or mixed ECM components as in Matrigel) or synthetic polymers (such as polyethylene glycol (PEG), polyacrylic and self-assembling peptides), whereby mechanical, physiochemical and biological characteristics can be tailored to support the maintenance of the specific (liver) cells within spheroids and organoids. These hydrogel solutions can also be used as so-called bio-inks when 3D liver models are created using bioprinting.

Using a 3D structure including a number of (non-)parenchymal cell types in the liver allows to better recapitulate the in vivo liver by improving the liver-specific metabolism, as well as partially replicating the organization of the liver, the cell–cell signaling and partial zonation in the liver; these are all factors that can contribute to better prediction of drug toxicity. Unfortunately, the potential for high-throughput screening is more limited due to the labor-intensive setup, which limits the use of these models. Nevertheless, they represent a more functionally relevant model for, perhaps, a secondary toxicological study of candidate compounds.

## 2.1. 3D Hepatocyte Monocultures

To counteract de-differentiation of PHHs when cultured in 2D in classical culture dishes, PHHs can be cultured as aggregates/spheroids, which then allows preservation of the PHH function for up to 2–3 weeks [26]. These studies were replicated to hPSC-derived HLCs by multiple research groups. For example, Ogawa et al. demonstrated that PSC–HLCs, harvested on day 26 from 2D cultures, could form aggregates. When these aggregates were cultured in the presence of 8 bromo-cAMP, HLCs attained greater maturity, demonstrated by drug metabolization, albumin secretion and transcriptome analysis [27]. Similar results were also described by Takayama et al., who demonstrated that HLC spheroids created from day 11 2D PSC–HLC progeny displayed greater CYP450 activity compared with HLCs maintained in 2D, and that the HLC spheroids could more accurately detect hepatotoxic drugs compared to 3D HepG2 spheroids [28]. Researchers' group recently created HLC aggregate cultures by embedding day 8 PSC-HLCs in a tailor-made PEG based hydrogel, identified by screening >250 combinations of hydrogels with different stiffness, degradation ability and ECM/cell adhesion molecule (CAM) peptide functionalization (which was termed hepatocyte maturation or HepMat hydrogel) [29]. PSC-HLCs in HepMat hydrogels could be maintained for >30–40 days and attained greater CYP3A4 activity compared with 2D cultures. In contrast to the spheroids created without scaffolds, HLCs embedded in HepMat hydrogels created smaller spheroids, and some cells remained as smaller clusters or single cells. As also documented in all HLC spheroid/aggregate culture studies described above, the HepMat-cultured HLCs retained fetal features (including expression of AFP) and CYP450 activity remained lower than that of freshly isolated PHHs. Another example is the study by Ng et al., who developed inverted colloidal crystal PEG scaffolds to embed PSC-derived hepatic progenitors [30]. Improved HLC functionality over 2D cultured HLCs was shown, and following implantation of the spheroid containing hydrogels in murine liver, the porous structure of the crystal PEG-based hydrogel allowed for vascularization.

Thus, although culture of HLCs derived from PSCs in 3D spheroids/aggregates with or without scaffolds improves HLC functionality, the cells remain immature. CYP enzyme activities may be improved compared to 2D cultures but still do not fully reach levels observed in PHHs, which has to be taken into account when performing drug toxicity screens.

## 2.2. 3D Complex Cultures with Multiple Liver Cell Types

An alternative is to create cell models encompassing not only hepatocytes but also additional epithelial cells (e.g., cholangiocytes) as well as non-parenchymal cells, such as endothelial cells, mesenchymal stellate cells and macrophage/Kupffer cells. It has been hypothesized that in such co-cultures the hepatocyte niche might be better recreated, and this should allow for further maturation of hepatocytes, as is seen during liver development and in postnatal liver. Furthermore, the inclusion of multiple liver cell types should enable to more closely recapitulate liver diseases and especially enable improved assessment of drug toxicity as liver diseases or toxicity is seldomly caused by damage to hepatocytes only. Therefore, these complex models are of great clinical interest. Several approaches have been used, including the creation of aggregates/spheroids from pre-differentiated cells and organoid formation from PSCs, either scaffold-free or in scaffolds.

Among the former studies are, for instance, studies whereby aggregates/spheroids are derived from a mixture of PSC-derived progeny and non-PSC-derived cells, for example, Coll et al., cocultured PSC–HSCs with the hepatoma cell line HepaRG [31]. The function of the hepatoma cells was improved, and coculturing of iPSC–HSCs in these spheroids induced a more quiescent HSC phenotype compared with 2D cultured PSC–HSCs. Treatment of these spheroids with fibrogenic and hepatotoxic compounds resulted in a fibrogenic response and ECM secretion. Takebe et al. created aggregates of PSC-derived immature HLCs, combined with human umbilical cord endothelial cells (HUVECs) and mesenchymal stromal cells (MSCs), in so-called liver buds [32]. Engraftment of the liver bud in the brain of SCID mice supported further maturation of the HLCs. Specifically, hepatic cord-like structures and inter-HLC tight junctions were formed, along with functional HLC maturation. In another example, Ramli et al. committed PSCs to endoderm, created aggregates of these foregut cells and induced maturation to a mixture of hepatocytes and cholangiocytes [33]. These spheroids had functional bile canaliculi and troglitazone, known to cause cholestasis, could disrupt bile flow and cause apoptosis. Although culture with excess free fatty acids resulted in structural changes associated with nonalcoholic steatohepatitis (NASH), lack of non-parenchymal cells, such as KCs and HSCs, prevented detection of inflammation and fibrogenesis.

Others created hepatobiliary cultures by directed differentiation from PSC-aggregates, creating so called hepatic organoids. Wu et al. created PSC aggregates that were then subjected to different sequential cocktails of growth factors and signaling molecules to an HLC and cholangiocyte fate (called hepatobiliary organoids, HBOs) [34]. When supplemented with extra cholesterol, these organoids containing central cysts, filled with bile and could be maintained for more than 45 days. HLC maturation was also observed in longer term-maintained HBOs. Guan et al. used a similar approach, but also used Matrigel as a scaffold to create mixed hepatic and biliary organoids [35]. They formed organoids (so-called hepatic organoids, HOs) consisting either of mainly HLCs, mainly cholangiocyte-like cells or a mix of both HLCs and cholangiocyte-like cells. As for the study by Wu et al., HLC functionality improved over time in culture, although the characterization was relatively limited. The authors could successfully use the HO culture system created from a number of patient-derived iPSCs to study mutations in the *JAG1* gene, essential in NOTCH signaling, which clinically causes Tetralogy of Fallot and Alagille Syndrome.

To further increase the complexity of the models, the liver mimics should encompass not only epithelial cells, but also mesodermal non-parenchymal cells, such as HSCs, LSECs, KCs or even lymphoid cells. Several models have been described. In one type of model, PSCs are allowed to differentiate as organoids into both mesodermal and endodermal progeny. Rashidi et al. created aggregates of undifferentiated PSCs and induced hepatic differentiation in 3D [36]. They demonstrated that these 3D PSC-cultures consisted of a central mesodermal core and were surrounded by multiple layers of hepatocytes. These organoids could be maintained for up to 1 year in vitro, and this was associated with progressive loss of AFP secretion but maintained CYP3A activity. They further demonstrated that the organoids could support liver recovery following partial hepatectomy in rodents. A different example is the model created by Ouchi et al., who induced iPSC aggregates to a foregut fate, followed by embedding in Matrigel and further differentiation towards liver cells [37]. Single cell RNAseq (scRNAseq) analysis revealed that the resulting human liver organoids (HLOs) consisted mainly of hepatocytes (~60%); 30% of the cells had a mesenchymal phenotype, while a small number of biliary cells and Kupffer cells were also present. This and a subsequent study also demonstrated that the transcriptome of a fraction of HLO-derived hepatocytes resembles

that of primary liver derived hepatocytes [37][38]. The organoids displayed inducible CYP3A4 activity, the mesenchymal compartment displayed vitamin A storage after retinol treatment, and organoids secreted inflammatory cytokines following LPS stimulation. In response to free fatty acid treatment, signs of steatohepatitis were detectable, which could be reversed by treatment with FGF19. They further demonstrated that the HLOs can also be used to study drug toxicity using a medium throughput screen for, e.g., cholestatic and mitochondrial toxic drugs [38]. Furthermore, Guan et al., created a multi-lineage hepatic organoid that expresses the causative mutation for Autosomal Recessive Polycystic Kidney Disease (ARPKD) and showed that organoids develop abnormal bile ducts and fibrosis, a key feature of ARPKD liver pathology. The ARPKD organoids showed increased collagen formation and activation of the PDGFRB pathway. Use of the PDGFR inhibitor, Imatinib and Crenolanib, could successfully decrease collagen accumulation, indicating that this model could be used to test anti-fibrotic drugs [39]. As a final example, researcher's group created similar complex liver spheroids, but by embedding PSC-progeny committed separately to hepatoblasts, endothelial cells, stellate-like cells and macrophages, in the HepMat hydrogel described above [29]. Instead of scRNAseq, multiple iterative labeling for antibody neodeposition (MILAN) was used to define the composition of these co-cultures. As was observed in the multicellular organoid cultures above, among the hepatocyte progeny in the HepMat-based multicellular liver cultures contained $AFP^{-}/ALB^{+}/CYP3A4^{+}$ mature hepatocytes, $AFP^{+}/ALB^{+}/CYP3A4^{-}$ intermediate hepatocytes, $AFP^{+}/KRT19^{+}$ hepatic progenitors, $AFP^{-}/KRT19^{-}$ cells with a cholangiocyte phenotype, some of them forming bile ducts, $CD68^{+}$ macrophages, $VIM^{+}$ mesenchymal cells and $CD31^{+}$ endothelial cells. As for the HLOs, the multicellular HepMat spheroid cultures displayed higher CYP3A4 functionality compared to hydrogel cultures containing only HLCs, and the co-culture system enabled modeling steatohepatitis caused by oleic acid, which could be reversed/blocked by the candidate anti-NASH drugs, obeticholic acid and elafibranor.

Thus, 3D complex cultures with different liver cell types with/without scaffolds has improved HLC functionality substantially compared to 2D cultures or even 3D monocultures, even if not all studies compared 3D HLC monocultures vs. 3D multicellular co-cultures. Although some HLCs in the 3D multicellular co-cultures start to resemble the phenotype of PHHs, a significant fraction of HLCs remain immature. In addition, how closely the NPCs, present in these multicellular co-cultures, resemble primary freshly isolated NPCs is also not yet fully established.

## 2.3. Bioprinting hPSC-Derived Liver Models

As discussed earlier, the hydrogels used to create scaffold-based organoids/spheroids can also be used in combination with bioprinting, which might enable the creation of anatomically more correct liver mimics. Different methods of bioprinting, such as bio-extrusion [40], inkjet bioprinting [41], valve-based bioprinting [42][43] and digital light processing-based bioprinting [44], have already been used to recreate 3D tissue structures from stem cells. Faulkner-Jones et al. was the first to print liver mimics from PSC-derived HLCs [43]. Using microvalve-based printing, they created a 40-layer HLC-containing alginate hydrogel construct and demonstrated that the printed cells express HNF4α and secrete ALB to similar levels of 2D controls. However, no additional tests were performed to demonstrate the maturation degree of the HLCs. In contrast, Goulart et al. printed iPSC-derived HLC spheroids, iPSC-derived endothelial cells and iPSC-derived mesenchymal cells in an alginate/pluronic-based hydrogel at a

ratio of 75–20–5%, respectively [45]. They demonstrated that printing HLC spheroids resulted in prolonged metabolic functionality and a reduced EMT transition, compared to printing HLCs in a single cell suspension. However, no comparison with PHHs was performed. Furthermore, Ma et al. created 3D hydrogel-based tricultures consisting of PSC-derived hepatic progenitor cells with endothelial and mesenchymal supporting cells [44]. The three different cell types were printed via the digital light processing based bioprinting, using a gelatin methacrylate bioink, to recreate the hepatic lobular architecture. The gene expression of hepatic markers, such as *HNF4α, TTRI* and *ALB*, were increased compared to 2D and 3D hepatic progenitor mono-cultures, and ALB and urea production were significantly increased.

These studies have shown that bioprinting liver cells is indeed possible; however, more studies need to be performed to address if recreation of the hepatic lobular structure significantly enhances the functionality of HLCs as well as the NPC compartment. Nevertheless, the use of bioprinting may permit higher-throughput fabrication of the 3D in vitro co-culture models, and may offer better reproducibility and precision compared to conventional methods of fabrication of 3D models. This in itself may constitute a major advantage for drug discovery purposes.

## 3. Microphysiological Systems

Even though the generation of 3D static cultures has improved the maturation and functionality of the hPSC-derived liver cells, a perfusion system, which facilitates the delivery of nutrients and oxygen to the tissue and re-circulation of endogenous factors, may further aid maturation and specification of the cells in the microtissue and also recreate the typical liver zonation. Microphysiological systems (MPS), also known as ‘organ-on-a-chip’ (OoC), are microfluidic platforms that recreate properties of tissue microenvironments in a dynamic condition. Biomimetic human liver OoCs have advanced from simpler 2D cell models to spheroids or organoids to address the growing need to understand mechanisms of complex diseases, the response to drug exposure and its effect on the tissue [46]. The technology provides spatiotemporal control of the environment and an efficient method for removal of cellular waste products, leading to improved tissue homeostasis. The systems can also allow for the integration of sensors for continuous monitoring. Although OoCs have been developed for culturing primary cells or hepatic cell lines, this technology has also been implemented in the field of PSC-hepatic progeny.

Schepers et al. incorporated day 8 PSC progeny (i.e., hepatoblast) aggregates and inserted these in a microfluidic system. Maintenance of albumin secretion and CYP3A4 activity was demonstrated for up to 3 weeks [47]. Similarly, Wang et al. incorporated PSC-derived embryoid bodies (EBs) in micropillar microfluidic devices and performed in situ differentiation to the hepatic lineage. Resulting organoids contained both HLCs and cholangiocyte-like cells [48]. Perfusion enhanced mature hepatic marker expression (*ALB* and *CYP3A4)* and HLC functionality, including hepatotoxic response to acetaminophen (APAP) in a dose- and time-dependent manner. Similarly, Giobbe et al. differentiated hPSCs directly to hepatocytes and cardiomyocytes in a OoC system and demonstrated a significantly increased functionality as compared with their unperfused control cells [49]. Leclerc et al. demonstrated that perfusion of immature HLCs in microfluidic biochips triggered upregulation of several pathways related to cellular reorganization, stress response and drug metabolism [50].

Hollow fiber bioreactor systems have also been used to differentiate hiPSCs to HLCs [51][52][53]. Miki et al. and Sivertsson et al. differentiated definitive endodermal (DE) derived cells or hepatoblasts towards HLCs in perfused 3D bioreactors [53][54]. This demonstrated that perfusion allowed for improved HLC differentiation compared to conventional 2D cultures, including enhanced CYP expression, albumin and urea synthesis [53][54]. Meier et al. demonstrated that when HLC spheroids were maintained in a hollow fiber perfusion bioreactor, ALB and α1AT secretion improved as well as the functionality of some CYP450 enzymes compared to that of spheroids maintained in static cultures [51]. Furthermore, Freyer et al. reported the formation of bile duct-like structures and improved albumin and urea production in 3D perfusion systems [52]. Recently, Grebenyuk et al. created a microfluidic device that, uniquely enabled by a 3D-printable 2-photon-polymerizable hydrogel formulation, allowed a precise microvessel printing at scales below the diffusion limit of living tissues. This microfluidic system allows long-term perfusion of liver tissue and showed improvement in maturation compared with 2D cultures [55][56]. However, as no comparisons were performed between 3D static and 3D perfused cultures, it remains unclear whether perfusion of 3D cultures enhanced maturation.

In a further increased complex culture model, Lee-Montiel et al. incorporated PSC hepatic progeny and PSC-cardiac progeny derived from the same iPSC in a functionally coupled OoC system. This enabled the characterization of drug-drug interactions between cisapride and ketoconazole, as the authors could demonstrate that ketoconazole inhibited the CYP3A4 induced conversion of cisapride to norcisapride, leading to cardiac arrhythmia [57].

The use of perfused microphysiological systems, unlike static cultures, in theory should further improve differentiation and maturation of liver cells form hPSCs. However, currently it is unclear if improved maturation is achieved in perfused vs. non-perfused 3D culture systems, and full maturity has not yet been reached. One of the major benefits of this model is its ability to circumvent fluctuations in parameters, such as pH, oxygen, nutrients and metabolites, which is of interest for drug toxicity testing as these parameters may influence the pharmacokinetics of a tested drug [58]. However, although its complexity could lead to major improvements and advantages for drug discovery, this complexity also makes the model less suitable for high-throughput drug screenings.

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