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Modeling Disease with Human Inducible Pluripotent Stem Cells

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hiPSCs, reprogramming, liver, disease modeling, differentiation

Abstract

Understanding the physiopathology of disease remains an essential step in developing novel therapeutics. Although animal models have certainly contributed to advancing this enterprise, their limitation in modeling all the aspects of complex human disorders is one of the major challenges faced by the biomedical research field. Human induced pluripotent stem cells (hiPSCs) derived from patients represent a great opportunity to overcome this deficiency because these cells cover the genetic diversity needed to fully model human diseases. Here, we provide an overview of the history of hiPSC technology and discuss common challenges and approaches that we and others have faced when using hiPSCs to model disease. Our emphasis is on liver disease, and consequently, we review the progress made using this technology to produce functional liver cells in vitro and how these systems are being used to recapitulate a diversity of developmental, metabolic, genetic, and infectious liver disorders.

INTRODUCTION

Genetics has a key role in most common disorders. While it is estimated that there are more than 10,000 different monogenic diseases affecting millions of people worldwide (1), most complex disorders involve genetic variants in multiple genes, which either trigger or increase susceptibility to disease in specific environments (2–4). Importantly, this genetic variation is known to determine whether, when, and to what extent a disease varies from person to person. This individual variability results in inconsistent and inefficient treatment strategies (5). Thus, understanding the molecular mechanisms beyond disease penetrance induced by genetic diversity is becoming essential for developing personalized therapeutics. Nevertheless, the lack of suitable human experimental models has limited the study of these mechanisms. Indeed, animal models, although valuable, do not always recapitulate human physiopathology and, therefore, cannot be used to elucidate the detailed molecular mechanisms underlying human illness (5), especially when genetics is implicated. Therefore, generating human disease models that recapitulate pathological events observed in patients is a priority.

Human induced pluripotent stem cells (hiPSCs) (6) provide new tools for disease modeling. Similar to their in vivo counterparts (human embryonic stem cells, or hESCs) (6), hiPSCs possess the ability to self-renew almost endlessly in vitro while maintaining the capacity to differentiate into virtually any cell of the human body. However, unlike hESCs, hiPSCs can be rapidly generated from patients' biopsies, thus providing researchers with a limitless source of patient-specific material that can be used for producing the specific cell types targeted by a disease (**Figure 1**).

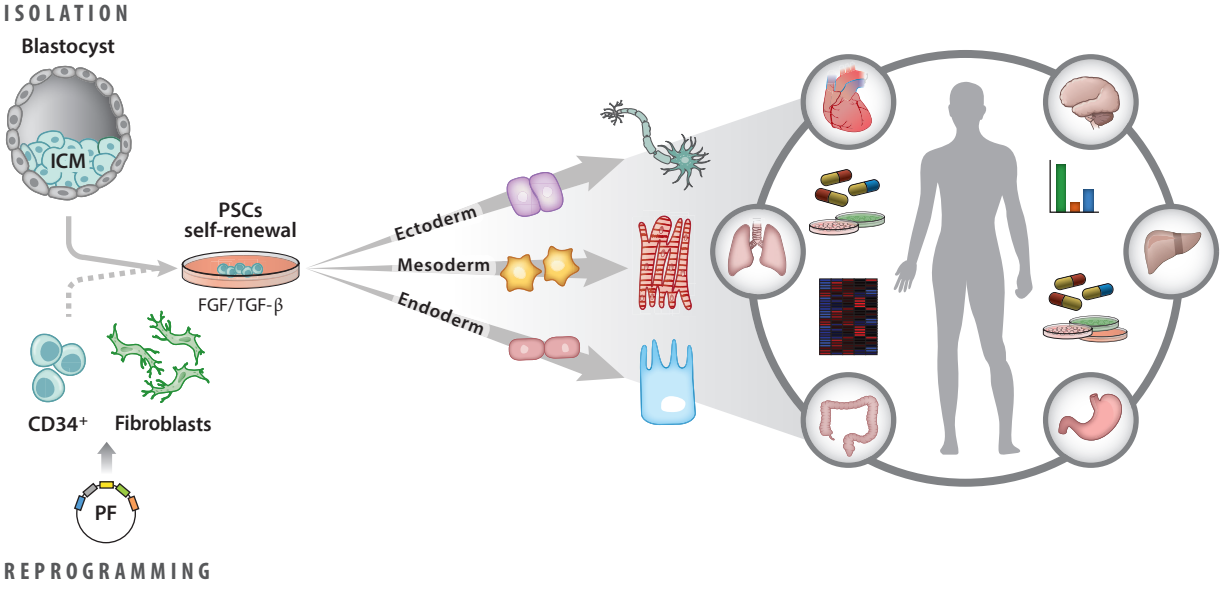

Figure 1

Schematic of the potential use of PSCs in biomedicine. PSCs can be generated either by isolating them from the ICM of human embryos or by reprogramming somatic cells through the overexpression of a small set of transcription factors. Using cocktails of cytokines and growth factors that mimic the natural paths of development, the derivatives of the three primary germ layers—ectoderm, mesoderm, and endoderm—are generated and subsequently differentiated into a diverse number of somatic cells. By choosing the right controls, PSCs can be utilized to study phenotypes associated with disease and to perform drug-screening assays. Abbreviations: FGF, fibroblast growth factor; ICM, inner cell mass; PF, programming factor; PSCs, pluripotent stem cells; TGF, transforming growth factor.

Furthermore, hiPSCs also overcome the ethical drawbacks associated with generating hESCs from human embryos while offering an amenable system model for investigating the role of particular mutations and genes on cellular phenotypes. Finally, hiPSCs can allow for an assessment of the influence of individual genetic backgrounds on the severity of a cellular phenotype related to disease (7–9). Thus, hiPSCs offer an invaluable window into the complex genetic interplay underlying a pathological state, especially that related to congenital or developmental disorders (10–15). In addition, single cell analysis (16), next-generation sequencing analysis (17), live imaging (18, 19), loss- and gain-of-function experiments (20, 21), and large-scale genetic (22, 23) and chemical screening (24–27) have enhanced our ability to study a large number of molecular mechanisms that are difficult to approach using model organisms. Finally, hiPSCs offer the possibility of reasonably fast in vitro recapitulation of pathological phenotypes, which in vivo may need up to several decades to manifest (for example, Alzheimer's disease and diabetes).

It is now clear that the unique characteristics of hiPSCs not only enable a diversity of disorders to be modeled but also allow them to be used to define the role of genetic variants in disease onset and penetrance. This knowledge will be extremely useful in predicting disease risk at an individual level and designing personalized therapeutics to prevent the development of pathological conditions. While these are the ultimate goals, additional technological challenges must be addressed before the clinical promises of hiPSCs can be fulfilled. In this review, we discuss the most relevant advances made in the field of disease modeling with hiPSCs, with particular emphasis on liver diseases, and also the future technological developments necessary to fully recapitulate human disorder in a dish.

HISTORICAL VIEW: DISCOVERY AND DEVELOPMENT OF hiPSCs

The Train of Thought

Seminal studies carried out by Sir John Gurdon (28, 29) at the beginning of the 1960s represent the first examples of successful cellular reprogramming. By developing the technique of somatic cell nuclear transfer, Gurdon and colleagues demonstrated that the information contained in the nucleus of differentiated cells could be reprogrammed after they were transplanted into enucleated oocytes. This new hybrid cell was pluripotent and capable of progressing normally throughout embryonic development, contributing to the formation of all tissue types. These observations established that the nucleus of a differentiated cell is not genetically different from that of its embryonic counterpart and also suggested that molecular factors present in the embryonic environment are sufficient to reprogram somatic cells into an embryonic pluripotent state. Approximately four decades later, James Thomson and colleagues (30) established a method for isolating and expanding in vitro pluripotent cells from human blastocysts. hESCs—with their capability to self-renew almost indefinitely and their potential to differentiate into virtually every cell of the body—revolutionized the fields of biomedicine and developmental biology. Despite these unique characteristics, ethical concerns about the utilization of human embryos during the derivation of hESCs have limited the clinical applications of these stem cells (31). The federal funding ban implemented by the US government during the early 2000s to limit the generation of new hESC lines is one example of a legal limitation motivated by ethical concerns associated with the production of hESCs (32, 33). Consequently, the possibility of generating pluripotent stem cells that are equivalent to hESCs from somatic cells using nuclear reprogramming has always been an attractive option. The proof of concept for such an approach was provided by forcing the fusion of somatic cells with ESCs (34, 35). These studies confirmed that factors present in pluripotent cells were sufficient to reset somatic identity to the pluripotent state. It was not long

after these discoveries that professors Shinya Yamanaka and Kazutoshi Takahashi reported for the first time the reprogramming of mouse fibroblasts into cells with characteristics of ESCs. These iPSCs were generated by resetting the transcriptional and epigenetic programs of somatic cells by overexpressing a set of transcription factors previously known for their role in pluripotency (36). Although the initial attempts required the overexpression of 24 transcription factors, Takahashi & Yamanaka (36) quickly narrowed this list to four factors: Oct3/4 (octamer binding transcription factor 3/4, or Pou5F1) and Sox2 (sex determining region Y box 2), two critical components of the core pluripotency transcriptional network (37, 38); KLF4 (Krüppel-like factor 4), recognized for its potential to inhibit p53-dependent repression of Nanog during differentiation and, later, for its capability to induce LIF-independent self-renewal of mouse ESCs (39); and last, the transcription factor cMyc, a well-known enhancer of cell proliferation and transformation (40). Among these factors, Oct3/4, Sox2, and Klf4 were found to be necessary for the reprogramming process, while cMyc only improved its efficiency (41).

Mouse iPSCs are indistinguishable from mouse ESCs in terms of morphology, gene expression, capacity for teratoma formation, proliferation, and the ability to differentiate into the cells of the three germ layers (36). This extraordinary discovery prompted Yamanaka and colleagues (41) and others (42) to reproduce these findings in human cells, and only 1 year later, both Yamanaka's and Thomson's groups reported almost simultaneously the generation of the first hiPSC lines. Interestingly, Yamanaka's team accomplished this by using the same cocktail of transcription factors that had been used for reprogramming mouse cells, while Thomson and colleagues generated hiPSCs by overexpressing a partially different set of factors (OCT4, SOX2, NANOG, and LIN28) (41, 42). Thomson and colleagues (42) showed that NANOG and LIN28 were beneficial for the reprogramming process of human cells because they increased the survival of nascent hiPSCs, although LIN28 was not essential. More importantly, they demonstrated that hiPSCs could be generated without overexpressing cMyc. This observation proved to be valuable because cMyc can induce malignant transformation (43). The Yamanaka factors remain widely used, although the original cocktail of factors has been modified multiple times to increase the efficacy of reprogramming, although success has been inconsistent (44). In addition, many modifications have been introduced to improve the delivery of the reprogramming factors, such as the use of integration-free methods (44). Episomal vectors, Sendai viruses, and synthetic mRNAs are among the most commonly used methods of generating hiPSCs (45–47) without modifying the host genome, which could interfere with disease modeling or experimental outcomes.

hiPSCs as an Alternative to Human Embryonic Stem Cells for Disease Modeling

hiPSCs and hESCs display telomerase activity; specific cell surface antigens, such as SSEA-3, SSEA-4, Tra-1-60, and Tra-1-81; and similar patterns of DNA methylation on gene promoters; and they can differentiate into derivatives of all three primary germ layers (41, 42, 48). Furthermore, recent analyses of gene expression patterns at the single cell level confirmed previous observations that hiPSCs and hESCs are closely related to the PSCs of the postimplantation epiblast (49–51). Nonetheless, hiPSCs have unique advantages over hESCs. Not only do they circumvent the use of embryos, but they also allow researchers to generate patient-specific pluripotent cells that can be utilized for the in vitro study of a range of biological phenomena. These include the study of developmental disorders, the role of genetic background on cell differentiation and disease, and the derivation of patient-specific platforms for drug screening (5, 44, 52–54).

This extraordinary potential obligates constant scrutiny of the hiPSC technology, and elements such as genomic instability and the potential for differentiation are continually being evaluated (55). Indeed, as with hESCs, there has been an active debate regarding the preference for some

hiPSC lines to differentiate into specific lineages. Particular attention has been paid to the reprogramming process ever since initial observations revealed that specific epigenetic signatures from donor cells could resist the reprogramming process, thus contributing to the formation of limited transcriptional and epigenetic aberrations in hiPSC lines (56–60). It was suggested that this aberrant epigenetic profile might act as an epigenetic memory that could increase the capacity of specific hiPSC lines to differentiate toward lineages close to their parental cells (6, 57–59, 61). Although this hypothesis has not been completely ruled out, the fact that the so-called epigenetic memory tends to disappear in cells cultured for extended periods indicates that this phenomenon may be transient and probably depends on the method of reprogramming and culture conditions used (56, 59). Interestingly, recent findings obtained by comparing genetically matched hESC and hiPSC lines revealed that hiPSCs are also similar in terms of transcriptome, DNA methylome, and capacity to differentiate into the cells of the three germ layers (48). This and other studies have reinforced the view that rather than epigenetic memory, differences in genetic background are the primary source of variability in differentiation potential (48, 61–63). Accordingly, it has been recently shown by mapping expression quantitative trait loci and changes in copy number variation that the divergent gene expression profiles and differentiation efficiency observed among hiPSC lines are strongly associated with changes in their genetic background (7, 22, 63). Of note, a report published in 2017 showed that very few (3 out of 64) hiPSC lines derived from different donors displayed chromosomal abnormalities (64), suggesting that genetic instability is unlikely to affect the capacity of hiPSCs to differentiate in vitro. Overall, the data support the view that hiPSCs are similar to hESCs and that most of the variations in gene expression and epigenetic variations are the consequence of genetic differences among donors.

These findings are highly relevant to disease modeling. Although it is unclear whether genetic background outweighs environmental factors, it is possible that hiPSC lines from different genetic backgrounds might respond differently to extracellular cues. Accordingly, it is anticipated that improvements in current protocols for differentiation will increase differentiation efficiency and the homogeneity of hiPSCs from different genetic backgrounds. In that context, hiPSCs represent an excellent opportunity for assessing the effect of genetic background on cellular phenotype during cell differentiation and disease. Nonetheless, observations made with hiPSCs need to consider the impact of divergent genetic backgrounds on self-renewal and differentiation.

MODELING DISEASES WITH hiPSCs

In vitro disease modeling relies on the availability of specific cell types that are targeted by the disease and display disease phenotypes. Primary cells represent the ideal solution as they are directly representative of the original tissues and organs. However, primary cells are often difficult to obtain, especially from diseased tissues, and they cannot be grown in vitro without losing their functional characteristics (65). Thus, the development of disease models based on human primary cells remains a difficult enterprise despite a broad number of efforts.

hiPSCs have provided a unique opportunity to fill this gap because they can be used to produce an almost infinite quantity of primary-like cells in vitro from a diversity of patients. However, the key challenge has been to generate functional mature cells from hiPSCs. Accordingly, intense research has been carried out during the past decade to advance differentiation protocols and improve the functionality of hiPSC-derived cells so they will resemble primary cells (32, 44, 66). The different approaches used include combining, concentrating, and altering the timing of the use of cytokines and growth factors (41–48), as well as changing the extracellular substrates used and the spatial organization of the cells, and coculturing them with other cell types (67–71). However, in their current state of development, cell types derived from hiPSCs in vitro still exhibit immature

or fetal phenotypes (70, 71). Therefore, biological responses obtained from these cells need to be carefully considered in view of their fetal nature. Nonetheless, the interest in using hiPSC-derived cells for modeling adult disease has been clearly demonstrated for a number of tissues, thereby establishing that these cells can be used to characterize disease phenotypes, especially in the context of monogenic diseases, and the molecular mechanisms of responses to drugs (72). Of note, an increasing number of disease-specific hiPSCs are generated daily from patients afflicted with a variety of genetically inherited and complex disorders affecting virtually every organ (73). Extensive coverage has been given to those hiPSCs related to neurological and cardiac diseases (8, 52, 74); therefore, they will not be described here. Instead, in this review we discuss some of the most significant findings regarding the use of hiPSCs to model liver diseases, which inflict a significant burden on health-care systems, and urgently call for effective therapies.

Modeling Liver Disease

The liver is a multifunctional organ that plays a crucial part in human physiology. It works as a storage site for vitamins, minerals, and glycogen; detoxifies alcohol and drugs; synthesizes plasma proteins, such as albumin and clotting factors; and produces the bile necessary for digesting lipids, among other functions (75). The main functional cell type in the liver is the hepatocyte, which accounts for 70–80% of the organ's mass. Polarization and interaction with nonparenchymal cells are essential for the proper functioning of hepatocytes. Accordingly, hepatocytes interact directly with sinusoidal endothelial cells through their basolateral surface, which facilitates communication between the parenchyma and the bloodstream. At the apical surface, the formation of tight junctions between hepatocytes is required for canaliculus creation and bile acid transport (76). The liver can be attacked by a diversity of disease or injuries that can result in acute organ failure or chronic damage. In this last process, the liver gradually loses its natural organization due to inflammation and fibrosis, which ultimately lead to cirrhosis (65). Liver disease constitutes a leading cause of death worldwide, and liver transplantation remains the only therapy for end-stage liver failure (77). Hence, understanding the pathogenesis of the disease is critical not only for developing new therapies but also for improving diagnosis and prognosis.

Hepatocytes rapidly lose their metabolic activity in vitro (76), and they can be obtained only through invasive methods, which are risky and often not well tolerated by patients. These considerations and their phenotypic instability drastically limit the use of primary hepatocytes to model liver disease in a patient-specific way. Consequently, hiPSC-derived liver cells represent an opportunity to overcome these limitations. Although producing hiPSC-derived liver cells that can recapitulate the physiological responses observed in vivo has proved challenging (12, 70, 78, 79), hiPSC-derived liver cells have already been used to model diverse aspects of liver physiopathology, and this review discusses some of these applications.

Current Approaches to Generating Hepatocyte Cells from hiPSCs

To be useful for disease modeling, hiPSC-derived hepatocytes or hepatocyte-like cells (HLCs) need to recapitulate the functional activities of their native counterparts, including expressing hepatic markers, acquiring specialized structures, and displaying specific enzymatic activity. Several groups have established protocols for directing the differentiation of hiPSCs toward HLCs, and the most successful of these follow the fundamental stages of embryonic development, such as the formation of definitive endoderm, foregut, hepatic endoderm, bipotential hepatoblasts, and HLCs (76, 80). Accordingly, our lab established a protocol for the direct generation of HLCs that mimics liver development in vitro (81). To derive definitive endoderm cells, this approach

uses a chemically defined medium, Activin A, FGF2 (fibroblast growth factor 2), BMP4 (bone morphogenetic protein 4), and a phosphoinositide 3-kinase (PI3K) inhibitor, as well as transient stimulation of the Wnt pathway. Subsequently, the newly formed endoderm cells are grown in the presence of Activin A and B27 medium to induce hepatic progenitor cells. Finally, maturation of the hepatic progenitors into HLCs is stimulated with hepatocyte growth factor and Oncostatin M (**Figure 2**) (81). After 25 days of differentiation, the resulting HLCs share several characteristics with primary hepatocytes: They display occasional binucleated cells, glycogen storage, apical microprotrusions, and a prominent Golgi body (78, 81, 82). Furthermore, these cells express specific hepatocyte markers, such as albumin, CK18 (cytokeratin 18), cytochrome P450 enzymes, ASGPR1 (Asialoglycoprotein receptor 1), C/EBPa (CCAAT/enhancer binding

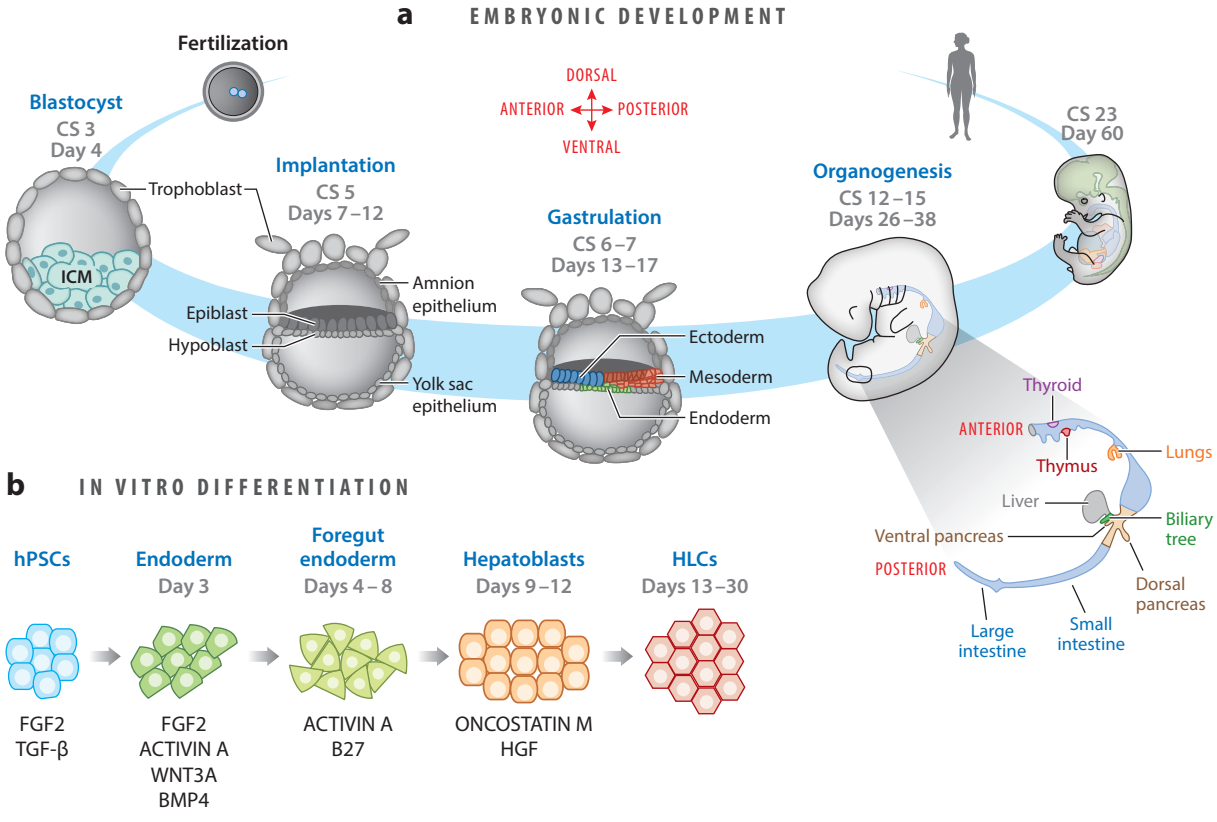

Figure 2

(*a*) Timeline of the differentiation of HLCs and their relationship to early human embryonic development. Days represent the age postfertilization. PSCs are equivalent to the pluripotent cells from the epiblast (Carnegie stage 5, days 7–12 postfertilization). DE cells are efficiently generated upon induction of PSC differentiation by using a defined medium containing ACTIVIN A, BMP4, FGF2, and WNT3A. This process involves a series of morphogenetic changes that resembles DE formation during gastrulation. Specification of foregut and hepatoblast cells is favored by the stimulation of DE cells with high levels of ACTIVIN A on B27 medium. The formation and maturation of HLCs is stimulated by ONCOSTATIN M and HGF. (*b*) Overview of the gut endoderm formed during early mouse embryonic development. Days represent days of in vitro differentiation. Abbreviations: CS, Carnegie stage; DE, definitive endoderm; HGF, hepatocyte growth factor; HLCs, hepatocyte-like cells; ICM, inner cell mass; hPSCs, human pluripotent stem cells. The embryonic stages in the figure are based on data from the Carnegie Collection (**https://embryology.med.unsw.edu.au/embryology/index.php/Carnegie_Collection**), which collects data from the Human Developmental Anatomy Center (USA) and the Carnegie Institute (USA). Data were also derived from References 113 and 114.

Table 1 Functional properties of hepatocyte-like cells (HLCs) derived from human induced pluripotent stem cells

Functional feature	Adult hepatocytes	HLCs	References
Binucleation	Yes	Yes	78, 81, 83, 84, 85, 89, 91, 100
Albumin expression or secretion	Yes	Yes	70, 78, 81, 83, 84, 85, 87–91, 100
A1AT expression or secretion	Yes	Yes	78, 81, 83, 85, 87, 88
AFP expression or secretion	No	Yes	70, 78, 81, 83–85, 87, 88
Urea production	Yes	Yes	83, 100
Glycogen storage	Yes	Yes	78, 83, 85, 87, 88, 91, 100
Indocyanine green uptake	Yes	Yes	81, 100
LDL uptake	Yes	Yes	78, 100
Bile canaliculi formation	Yes	Yes	90, 91
CYP3A4 expression or activity	Yes	No	78, 81, 83, 84, 87–89, 91, 100
CYP3A7 expression or activity	No	Yes	70, 81, 91, 100
EPCAM expression	No	Yes	89
CK18 expression	Yes	Yes	70, 78, 83, 89
HNF4α expression	Yes	Yes	70, 84, 85, 88–91, 100
Susceptible to viral infection	Yes	Yes	85, 86

Abbreviations: A1AT, α-1 antitrypsin; AFP, α-fetoprotein; EPCAM, epithelial cell adhesion molecule; HNF4α, hepatocyte nuclear factor 4α; LDL, low-density lipoprotein.

protein), and PROX1(Prospero homeobox 1) (80). Although these observations reveal that HLCs recapitulate the key features of their in vivo counterparts, the current consensus is that these cells are closer to fetal rather than adult hepatocytes (**Table 1**) (80). In this regard, Rashid et al. (78) proposed that from a developmental point of view, HLCs are likely to be located somewhere between the end of the first trimester of fetal embryonic development and adult hepatocytes. Consequently, HLCs exhibit the expression of the fetal markers α-fetoprotein (AFP) and CYP3A7, while the activity of adult cytochromes, such as CYP3A4, lags behind by several orders of magnitude in comparison with mature primary hepatocytes (52, 80, 82). Interestingly, despite this lack of complete maturation, HLCs are still able to execute hepatocyte-specific functions, including storing glycogen, transporting bile, uptake of low-density lipoprotein (LDL), synthesizing urea, secreting albumin (**Figure 3**) and apolipoprotein B100 (APOB100) to the extracellular medium, metabolizing a low level of drugs, responding to glucagon, and being susceptible to viral infection (76, 78, 82–84). Therefore, HLCs could be used for modeling diseases, especially those of viral infections, lipid metabolism, and hormonal responses. However, it is important to acknowledge that in their current state, HLCs are not compatible with modeling drug toxicology or injury.

Hence, improving current protocols for HLC differentiation is important, and the current strategies include coculturing HLCs with other hepatic cell types, using media supplements to provide cell signaling and to compensate for metabolic changes occurring during differentiation, and employing specialized extracellular matrix. Regarding this last point, defined matrices containing collagen, fibronectin, or vitronectin have already been used to generate HLCs effectively (80). Moreover, David Hay's group (85) recently reported that hiPSCs growing on laminins could be differentiated into HLCs with very high efficiency [~90% of the cells expressed HNF4α (hepatocyte nuclear factor 4α)]. Interestingly, these cells demonstrated higher cytochrome P450 (CYP3A) activity than commercial frozen human primary hepatocytes or HLCs cultured on matrigel (85, 86). While this observation is of great interest, the performance of the HLCs generated using this approach remains limited when compared with freshly isolated primary hepatocytes (85, 86).

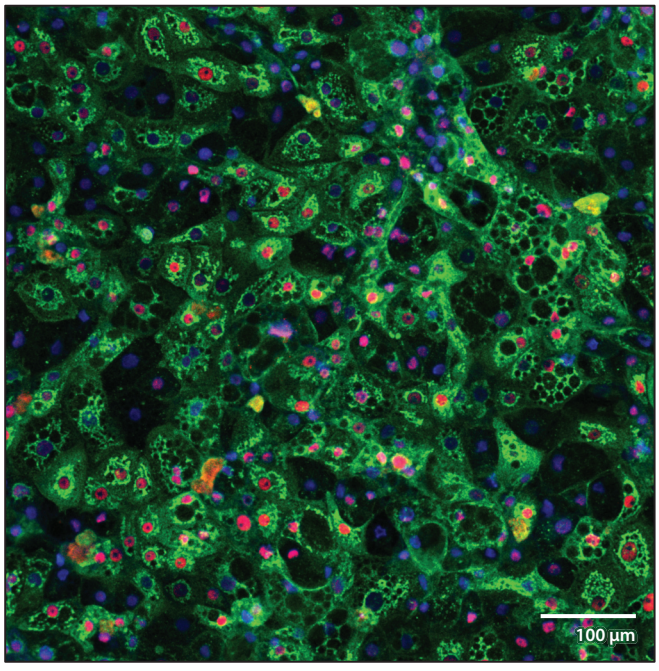

Figure 3

Characterization of human induced pluripotent stem cell–derived hepatocyte-like cells. Immunofluorescence microscopy depicting the presence and localization of albumin (*green*) and HNF4α (hepatocyte nuclear factor 4α) (85) in 30-day differentiated hepatocyte-like cells from human induced pluripotent stem cells using the protocol described in Hannan et al. (81). Nuclei were counterstained with DAPI (*blue*).

Additional efforts to improve the functional maturation of HLCs have focused on reproducing the liver microenvironment more accurately (**Figure 4**). In one approach, researchers successfully established a three-dimensional (3D) protein-based scaffold that allowed the derivation of HLCs in the presence of endothelial cells and human mesenchymal stem cells. Although it was reported that this method generates hepatoblast-like cells with a specific spatial organization, single-cell transcriptomic analyses revealed that these cells retained their fetal status (70, 71, 79). Using a different approach, Gieseck et al. (92) reported a method of culturing HLCs using a 3D collagen-based scaffold that increased the maturation of the HLCs significantly. Accordingly, these HLCs showed improved functionality when compared with HLCs grown in 2D. Specifically, 3D HLCs displayed higher rates of drug metabolism that were associated with increased cell polarization and bile canaliculi formation. Interestingly, these cells remained functional in vitro for more than 75 days, which may be related to the improved disposal of toxins by the polarized cells (87).

Although both of these systems represent steps forward in 3D models of liver development and disease, further work is required to generate fully matured hepatocytes. It remains to be verified whether the inclusion of other nonparenchymal cells within the organoids improves the levels of maturation, functionality, and organization of the HLCs within the organoids.

Cholangiocytes could represent the missing cell type because these biliary cells have tight, functional interactions with hepatocytes. Indeed, these cells regulate bile homeostasis (12, 88), modulate inflammatory responses, and may have an essential function in liver regeneration (89, 90). Furthermore, cholangiocytes originate from the same bipotent embryonic progenitor cells that give rise to hepatocytes (89), which suggests strong coordination during the formation of

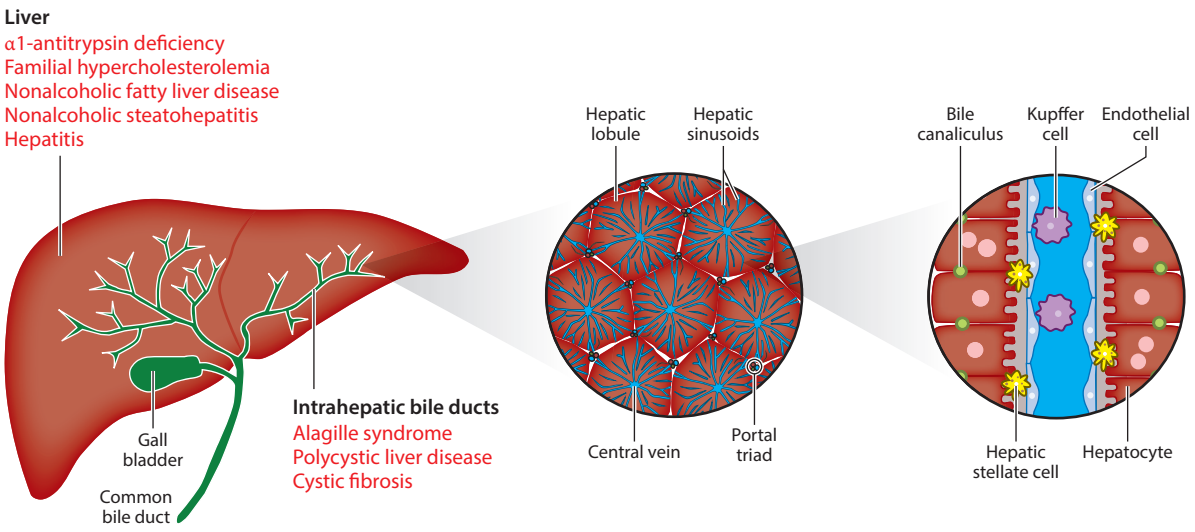

Figure 4

Schematic of the adult liver, with (*left*) the gallbladder, common bile duct, and network of intrahepatic bile ducts (*green*). Red type highlights examples of diseases affecting either the liver or the bile duct network. (*Middle*) The liver consists of several hepatic lobules, which are formed by sheets of hepatocytes surrounded by a network of sinusoids (*blue*) that are lined by endothelial cells. Oxygenated and nutrient-rich blood flows through the sinusoids from the portal triad (composed of the portal vein, hepatic artery, and biliary duct) toward the central vein of the lobule, allowing for the exchange of metabolites between the blood and the hepatocytes. Hepatocytes produce bile; bile is secreted into bile canaliculi (*green*) and transported through the bile ducts, which are lined by cholangiocytes, into the gallbladder. In addition, hepatic stellate cells reside in the space of Disse, between the hepatocytes and the sinusoids; the Kupffer cells—the liver's resident macrophages—reside in the sinusoids.

these two cell types in the embryonic liver. Interestingly, the spatial organization of the hepatoblast during development is critical. Indeed, when the hepatoblasts localize near and around the portal vein, they create a monolayer of immature cholangiocyte progenitor cells called the ductal plate (91). After a series of morphogenetic rearrangements, these cells differentiate into tubular structures that ultimately form the bile ducts (92). The main functions of cholangiocytes are associated with absorption and secretion processes (91, 93). Accordingly, the polarization of these cells during differentiation is critical for reaching their maximum functionality (91). This polarization is difficult to reproduce accurately with cells organized in a monolayer. Consequently, protocols that allow for the differentiation of hepatoblasts in 3D systems are the most effective methods for generating mature cholangiocyte-like cells (CLCs) (88, 91). Interestingly, Sampaziotis et al. (93) recently reported a highly efficient method for generating hiPSC-derived CLCs by using a protocol that mimics biliary development, including a final step of CLC differentiation and functional maturation in 3D culture conditions. Importantly, these CLCs display functions specific to native cholangiocytes, including the capacity to form branching tubular structures, the presence of primary cilia, and the expression of biliary markers—such as CK7 (KRT7), CK19, HNF1B, GGT1, JAG1, NOTCH2, CFTR, SCR, SSTR2, AQP1, and AE2—at levels comparable to those found in primary cholangiocytes (88, 93). Furthermore, CLCs have the capacity to respond to acetylcholine and ATP stimuli, the capacity to respond to GGT and alkaline phosphatase activities, and the ability to transport bile acids. Finally, patient-specific CLCs can be used to model some aspects of polycystic and cystic fibrosis liver diseases, which are known to affect the biliary epithelium. Altogether, these observations show that CLCs derived from hiPSCs could be used as surrogates for primary cholangiocytes. However, as with HLCs, hiPSC-derived CLCs have

not reached a fully adult phenotype, and they still express the fetal biliary marker SOX9 (93). These results raise the question of whether CLCs in vitro also need to be surrounded by other liver cells in order to become fully mature. Hence, current efforts aim to reconstruct the cellular complexity of the liver using organoid technology. To fully mimic the liver microenvironment, the resulting structure should include not only CLCs and HLCs but also Kupffer cells, stellate cells, and endothelial cells.

Considered together, these studies demonstrate that the derivation of new and better 3D coculture systems is feasible and could improve understanding of the physiopathology of a complex organ such as the liver. Furthermore, to obtain in vitro–generated liver cells (hepatocytes and cholangiocytes) with maturation levels beyond the fetal stage, it will be important that new protocols for differentiation incorporate additional elements that would normally be encountered by native hepatocytes during the postnatal period. These include signals associated with the shift from placental to enteral nutrition and the gut microbiota content. This last aspect is of great importance because bacterially derived secondary bile salts are involved in regulating the expression of cytochrome P450 isoforms, such as CYP2C9 and CYP3A4 (80).

hiPSC-Derived Hepatocytes for Modeling Infectious Diseases of the Liver

Diverse pathogens are known to attack the liver and especially to target hepatocytes. Among these pathogens, hepatitis B virus (HBV), hepatitis C virus (HCV), and the parasites that cause malaria are the most prevalent infectious agents, and combined, they account for more than 520 million cases of chronic liver disease worldwide (83). Primary hepatocytes represent the gold standard for studying the physiopathology of liver infection since transformed cell lines, such as HEPG2, do not support their life cycle. Thus, HLCs could provide a complementary platform for studying mechanisms of infection and the life cycle of a virus or parasite, as well as a means for finding novel, effective drugs. In this regard, HLCs have proven to be a successful in vitro system for modeling hepatitis virus infection and virus–host interactions. Indeed, hiPSCs acquire the expression of genes involved in hepatitis infection only after passing the definitive endoderm stage, that is, as they differentiated toward HLCs. Notably, these HLCs are not only able to produce viral RNAs and proteins following infection but they can also support the entire life cycle of the virus (83, 94). Furthermore, it seems that HLCs are capable of producing an appropriate antiviral response, including interferon production, after inoculation with HCV (83, 94). Interestingly, HLCs can survive in vitro for up to 1 week after inoculation with HCV (83). This offers an opportunity not only to examine the effects of relatively long-term infections on hepatocyte function but also to study in more detail the mechanisms that control permissiveness to viral infection, such as the upregulation of microRNA-122 and the suppression of the antiviral gene interferon-induced transmembrane protein 1 (84).

Altogether these observations demonstrate that HLCs provide a promising platform for analyzing hepatocyte responses to viral infection. However, some shortcomings must be addressed before major conclusions can be made. For example, Sakurai et al. (88) reported that virus titers detected in culture supernatants of HBV-infected HLCs were much lower than those of primary human hepatocytes. They attributed this result to the lack of functional maturation of the HLCs derived using current protocols for differentiation. Furthermore, it also will be essential to increase the diversity of the hiPSC lines used in these analyses to assess the impact of the genetic background of host cells on the cellular response and efficiency of infection.

Inherited Metabolic Disorders

Almost 70 inherited metabolic disorders (IMDs) affecting the liver have been described. Although the incidence of IMDs is relatively rare, together they affect 1 in 1,000 individuals and remain a

major cause of liver transplantation, particularly in children (10, 75, 77). One of the most common IMDs is α1-antitrypsin (A1AT) deficiency, an autosomal recessive disorder that affects 1 in 2,000 individuals of northern European descent. It results from a single point mutation in the *SERPINA1* gene (the Z allele; Glu342Lys) that causes protein aggregation specifically within hepatocytes. The accumulation of protein polymers induces hepatocyte cell death, which ultimately causes cirrhosis (95). Although researchers have been trying to model this disease for a long time, efforts to use hiPSCs are relatively recent. Accordingly, a seminal study published in 2010 by Rashid et al. (78) reported the generation of hiPSCs from patients with A1AT deficiency. Interestingly, Rashid et al. showed that these hiPSC lines were able to differentiate into HLCs that displayed key features of the cellular pathology, including the accumulation of mutant A1AT polymers in their endoplasmic reticulum (78). Importantly, in a more recent study, Tafaleng et al. (13) used detailed microscopic and ultramicroscopic analyses to demonstrate that this model system recapitulates not only some of the biochemical features of the disease but also the morphological manifestations observed in patients. These included a delayed degradation and an abnormal accumulation of partially glycosylated A1AT protein in pre-Golgi compartments and the presence of dilated rough endoplasmic reticulum and globular inclusions partially covered with ribosomes. Interestingly, these abnormalities were not observed in HLCs derived from either wild-type donors or patients who presented only with lung disease (13). These findings reaffirmed the notion that HLCs can be used to predict susceptibility to and progression of the disease.

Glycogen storage disease type 1α (GSD1α) and familial hypercholesterolemia (FH) represent two other IMDs that have been modelled using hiPSCs (77, 96). GSD1α regroups autosomal recessive metabolic disorders caused by a deficiency of glucose-6-phosphatase activity, which catalyzes the hydrolysis of glucose-6-phosphate to glucose and phosphate, the final products in gluconeogenesis and glycogenolysis. This pathology affects 1 in 100,000 individuals and is associated with an inability to maintain glucose homeostasis that results in growth retardation, hepatomegaly, lactic acidemia, and hyperlipidemia (97). Interestingly, compared with hepatocytes generated from hiPSCs derived from healthy individuals, GSD1α hiPSC-derived hepatocytes displayed higher levels of intracellular glycogen and lipids concomitant with elevated production of lactic acid. Notably, these cells were also able to respond transcriptionally to glucagon stimulation, demonstrating that the hepatocytes generated from the GSD1α-derived hiPSC lines display some functionality related to lipid and glycogen metabolism and can respond to a key hormone of intermediary metabolism (78).

FH is an autosomal dominant dyslipidemia caused by mutations in the LDL receptor gene that result in elevated levels of LDL cholesterol in the plasma and premature cardiovascular disease. Because hepatocytes are the principal cells that control cholesterol flux in the body, FH patients can be successfully treated with liver transplantation (11). Accordingly, some of the pathological manifestations of FH can be readily recapitulated in vitro by using patient-specific hiPSC-derived HLCs. Indeed, hiPSCs obtained from patients with FH could be differentiated into HLCs that are incapable of incorporating LDL even though they displayed levels of differentiation and functionality comparable to wild-type HLCs (78). Furthermore, in a more recent report, Cayo et al. (11) showed that hiPSCs derived from a patient with cardiovascular disease were also able to produce HLCs. However, in addition to their inability to take up LDL, these HLCs were unable to respond to statin treatment, and they displayed an approximately eightfold increase in the level of secreted APOB100 compared with HLCs derived from genetically independent control PSC lines (11). Together, these observations demonstrate that FH-derived hiPSCs can be used effectively to model diseases affecting lipid uptake and storage in the liver.

The next step for the field is to demonstrate that complex liver diseases that are not defined by single genes with Mendelian penetrance can be modeled in vitro using hiPSCs. Of particular

interest, hiPSC-derived HLCs potentially could be used to better understand the molecular mechanisms by which genetic variation influences quantitative phenotypic traits related to liver function in humans. Accordingly, a recent report showed that population-based cohorts of hiPSC-derived HLCs could be used to perform genome-wide mapping and validation of functional variants or genes, or both, involved in metabolic functions related to the liver (8). These results confirm that hiPSCs can indeed be useful for modeling complex liver diseases. Nonetheless, further studies are necessary to demonstrate that the results obtained are relevant in vivo and, in this context, animal models remain necessary to provide complementary validation.

Hepatic Organoids for Modeling Developmental Disorders Affecting the Liver

When modeling diseases affecting liver development, it must be considered that hepatocytes are not generated in isolation during embryonic life. In this regard, the generation of complex hepatic organoids from hiPSCs could represent a valuable tool for studying developmental disorders affecting the liver. Accordingly, a recent report showed how complex hepatic organoids could be generated through a process meant to recapitulate the natural path of liver development, including development of the endoderm and foregut, and hepatoblast differentiation (12). Importantly, the patterns of mRNA and protein expression observed during the formation of the hepatic organoids are consistent with the patterns observed during liver development in vivo. As an example, on day 3, the majority of the endoderm cells expressed SOX17 and CXCR4. By day 6, the posterior foregut-like structures expressed FOXA3; and by day 9, the developing hepatic organoids expressed multiple markers of the hepatoblast stage, such as TBX3 and AFP. Finally, the organoids expressed the hepatocyte marker CK18 and formed luminal structures that resembled bile ducts, which were surrounded by cells expressing the cholangiocyte marker CK7. Overall, these data suggest that hepatic organoids are capable of recapitulating liver development. Consequently, these organoids displayed many features that are typically observed in the liver in vivo, including expressing the tight junction protein ZO-1, which is important for the formation of bile canaliculi, and primary cilia, commonly observed in primary cholangiocytes in vivo. Moreover, the organoids displayed biosynthetic and drug biotransformation properties characteristic of the human liver, with some level of CYP34A-dependent activity. Additionally, the organoids contained cells that accumulated glycogen and also were capable of secreting albumin and several types of bile acids into the supernatant. Importantly, the organoids displayed some capacity for self-renewal and, thus, could be expanded in vitro (12). Considering all of these properties, it was proposed that hepatic organoids could be used to study the impact of genetic mutations on human liver development. As a proof of principle, hepatic organoids were used to characterize the effect of different mutations in the *JAG1* gene on biliary tract development. Mutations in the *JAG1* gene cause the majority (~97%) of cases of Alagille syndrome (ALGS), an autosomal dominant genetic disorder in which the Notch signaling pathway is severely impeded, causing defects in the intrahepatic biliary tree (12, 98–103). Consequently, hiPSCs derived from ALGS patients formed hepatic organoids that developed normally through the hepatoblast stage, including forming HLCs. However, they did not contain cholangiocytes or bile ductular structures, nor were they capable of regenerating secondary organoids. Furthermore, the organoids showed signs of intrahepatic cholestasis and fibrosis, both of which could be explained by the reduced levels of RNA expression from several Notch signaling components (JAG1, NOTCH2, HEY1) and cholangiocyte markers (CK7, CFTR). These results showed that mutations in *JAG1* influence not only the efficiency of hepatic organoid formation but also the organoids' ability to form duct-like structures containing cholangiocytes, as well as their capability to transport bile and their capacity for regeneration (12).

Interestingly, not all mutations in the *JAG1* gene affected the formation of normal hepatic organoids. Indeed, hiPSCs generated from individuals carrying the Gly274Asp *JAG1* mutation, which is present in patients with cardiovascular defects but without any liver or bile duct abnormalities, were able to produce hepatic organoids with the same morphology and efficiency as control hiPSCs. Taken together, these findings indicate that hepatic organoids can recapitulate with fidelity the phenotypes dictated by different types of *JAG1* mutations in vivo (12). It is worth mentioning that, so far, there is not a strong correlation between the type and location of the *JAG1* mutation and the severity of disease. Accordingly, it has been suggested that additional genomic modifiers may be responsible for the highly variable clinical manifestations that characterize ALGS (98–103).

The preeminent thesis about ALGS posits that it is caused by the haploinsufficiency of *JAG1* because individuals with whole gene deletions can have phenotypes identical to those with intragenic mutations (99–104). However, it has also been reported that at least in vitro, mutant JAG1 proteins can act through a dominant negative mechanism to inhibit Notch signaling (98–103). Hence, it remains to be determined whether ALGS is a consequence of haploinsufficiency or a dominant negative effect of the mutated *JAG1* gene. In this regard, Guan et al. (12) also showed that unlike hiPSCs with a heterozygous *JAG1* mutation (ALGS hiPSCs), hiPSC lines engineered to carry a heterozygous *JAG1* knockout were able to efficiently form intact hepatic organoids that presented all the features displayed by control hepatic organoids. Interestingly, when ALGS hiPSCs were converted to haploinsufficient knockouts, these new hiPSC lines were able to form functional hepatic organoids. These data strongly suggest that ALGS liver abnormalities are caused predominantly by a dominant negative effect of the *JAG1* gene mutation (12).

Overall, hepatic organoids seem to reproduce critical events of liver development and, thus, could be useful for uncovering new insights into disorders of human development, such ALGS. This system could overcome some of the limitations of previous in vitro methods. Increasing the complexity of the organoids by incorporating other nonparenchymal cells could also further increase the spectrum of liver disease that can be modeled in vitro.

Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis

The first manifestation of nonalcoholic fatty liver disease (NAFLD) is the accumulation of lipids in hepatocytes (105, 106). The persistence of this condition triggers a plethora of metabolic alterations—including mitochondrial dysfunction, endoplasmic reticulum stress, hepatic insulin resistance, and an inflammatory response—through which NAFLD evolves toward nonalcoholic steatohepatitis (NASH) (106–109). Altogether, 29% of patients with NASH will progress to cirrhosis, and one-third of these will develop cancer (110). Consistent with this statistic, NAFLD and NASH are the second most common causes of liver transplantation (107). However, predicting the evolution of the disease is impossible because diagnosis involves invasive methods, such as liver biopsy, which are not well tolerated by patients (106, 107). Modeling NAFLD and NASH by using HLCs could facilitate the identification and functional validation of biomarkers for prognosis, stratification, and drug development. Accordingly, a first attempt to model NAFLD in vitro has been recently reported by Graffmann and colleagues (111, 112) who showed that HLCs could be used to model intracellular lipid accumulation. Furthermore, this study showed that the resulting HLCs displayed the biochemical alterations associated with steatosis, including upregulation of the lipid droplet–coating protein Perilipin 2, as well as numerous genes of the peroxisome proliferator–activated receptor pathway (112). Thus, this HLC model can recapitulate some of the metabolic features of NAFLD. Although these results represent a step forward in modeling NAFLD and NASH, further development is required to recapitulate in full the physiopathology

in humans. Indeed, disease progression is linked with lipotoxicity and not only with lipid accumulation. Furthermore, it involves a complex inflammatory response that can be produced only by immune cells, such as macrophages. Hence, developing coculture systems in which hepatocytes interact with other nonparenchymal cells will improve the utility of HLCs in recapitulating the features of NAFLD and NASH.

CONCLUSIONS

Animal models have provided a vast amount of knowledge concerning diseases and have been useful in developing most of the drugs available. However, they clearly have limitations for modeling complex human disorders, especially when genetic diversity is involved. Consequently, hiPSCs derived from patients could deliver a novel tool for modeling human diseases and their genetic mechanisms. Accordingly, hiPSCs are already used to generate cells from virtually every organ of the body, and the resulting cells have been used to model a diversity of diseases. However, only a handful of studies have used hiPSC-derived disease models to actually uncover new mechanisms of disease or to identify new drugs. The technical requirements of such work and the lack of standardization in cell culture systems partly explain this relatively slow progress. Furthermore, data generated from an in vitro model need to be validated, ideally using clinical information from individual patients. These comparative studies take time and consume resources. Finally, a lack of functional maturation may also limit the direct relevance of cells generated from hiPSCs.

However, protocols are progressing rapidly, and new approaches are constantly being developed to improve the functional maturation levels of hepatocytes, differentiation consistency, reproducibility, and conditions for scaling up the production of hepatocytes. In parallel, increasingly complex models of human tissues and organs are being engineered. The hope is that using tissue organoids containing more than one cell type and that are embedded into 3D conditions mimicking organ architecture will improve the function of hiPSC-derived cells. The careful selection of control cell lines and the development of hiPSC line cohorts derived from multiple genetic backgrounds will also increase the reproducibility and interpretation of data obtained from hiPSCs. Ultimately, these improvements will not only transform our capacity to study the impact of genetics on disease onset but also allow the development of truly personalized medicine.

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