

# Modeling Liver Diseases Using Induced Pluripotent Stem Cell (Ipsc)-Derived Hepatocytes

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## Abstract

The induced pluripotent stem cells (iPSCs) are reprogrammed somatic cells in a stem state. The iPSCs can give rise to cells of all three germ layers and provide an unlimited supply of tissue-specific differentiated cell types for disease modeling and cell therapy. The generation of patient-specific iPSC lines and studying disease phenotype in a dish using differentiated hepatocytes open up new avenue towards personalized medicine. There has been active investigation on generating homogenous functional human hepatocytes from iPSCs. Liver carries out secretory and metabolic functions. Recent studies showed that iPSC derived-human hepatocytes are useful for *in vitro* investigation of genetic liver disorders, drug screening and metabolism, hepatitis C viral infection and assessing efficacy of cell therapy. Inherited metabolic disorders, including  $\alpha$ 1-antitrypsin deficiency (A1AD), familial hypercholesterolemia, glycogen storage disease type 1a and Wilson's disease have been modeled using disease-specific iPSC lines. The iPSC hepatocytes derived from patients with A1AD were used for drug screening. Advancement made in precise genetic engineering technology using designer nucleases provides a new tool for gene correction, and reverse genetic engineering of disease causing genotype in pluripotent stem cells. Moreover, iPSC-hepatocytes from various genetic backgrounds are valuable resource for evaluating drug interactions and drug metabolism. In this review, we summarize the recent developments on the various applications of iPSC-derived human hepatocytes for disease modeling.

**Keywords:** Induced pluripotent stem cell; iPSC; Hepatocytes; Disease modeling; Inherited metabolic disorders; Hepatitis C virus; Drug development

## Introduction

Liver is a vital organ that carries out a multitude of functions including: plasma protein secretion, bile production, and metabolism of carbohydrates, lipids, proteins, xenobiotics and hormones. Hepatocytes are the functional unit of the liver and are arranged as hepatic plates among sinusoidal spaces in liver lobules. The lateral faces of adjacent hepatocytes contain bile canaliculi, through which bile is excreted. Bile canaliculi merge to form intrahepatic bile ducts that are lined with biliary epithelial cells. The non-parenchymal cells in the liver are sinusoidal endothelial cells, Kupffer cells (liver macrophages), and hepatic stellate cells. During liver development, hepatocytes and biliary epithelial cells (cholangiocytes) originate from the embryonic endoderm layer; whereas, non-parenchymal cells emerge from mesoderm (middle layer).

Liver disease is a major human health problem. According to Centers for Disease Control and Prevention (CDC), an estimated 500 million people worldwide are affected with viral hepatitis which comprises of both hepatitis B and C and out of this approximately 1 million people die annually [1]. Besides viral hepatitis, 35% of heavy alcohol drinkers develop alcoholic hepatitis. Viral and alcoholic mediated liver damage leads to chronic inflammation and fibrous scar tissue formation. Fibrotic formation and the continuous liver regenerative and degenerative processes provide a fertile niche for pathogenesis of hepatocellular carcinoma (HCC).

Liver is also affected by inherited metabolic diseases, such as alpha1-antitrypsin (A1AT) deficiency, hereditary hemochromatosis (HH), Wilson's disease among others. The A1AT, a protease inhibitor (PI), deficiency is predominantly caused by a single amino acid substitution (glutamic acid to lysine) at position 342 in the Z allele

[2]. This autosomal dominant mutation causes improper folding and retention of polymerized A1AT protein in the endoplasmic reticulum of hepatocytes which results in a low serum level of A1AT. Deficiency of A1AT causes uninhibited protease activities of trypsin, elastase, cathepsin G and other proteinases in the lung leading to destruction of air sacs, and emphysema. Moreover, chronic accumulation of polymerized A1AT in the liver proceeds to chronic hepatitis, fibrosis, cirrhosis and hepatocellular carcinoma [3-6]. Hereditary hemochromatosis is an autosomal recessive condition of iron overload in the liver, heart, and pancreas [7,8]. Type I HH is caused by a point mutation at amino acid position 282 (cysteine to tyrosine) of gene HFE (hemochromatosis) [9,10]. Increased iron absorption and subsequent storage-associated tissue injury are the outcomes of HFE mutation (C282Y). Wilson's disease (hepato-lenticular degeneration) is a genetic condition of copper overload in liver and brain [11,12]. This condition is inherited as an autosomal recessive genetic disorder. Various mutations in copper transport gene ATP7B are associated with the development of Wilson's disease [13,14]. Missense mutations at ATP7B protein positions 778 (R778L) and 1069 (H1069Q) are the major contributors of the disease phenotype [15,16]. Animal and cell culture models have been used for understanding the molecular and cellular basis of liver diseases [17-26].

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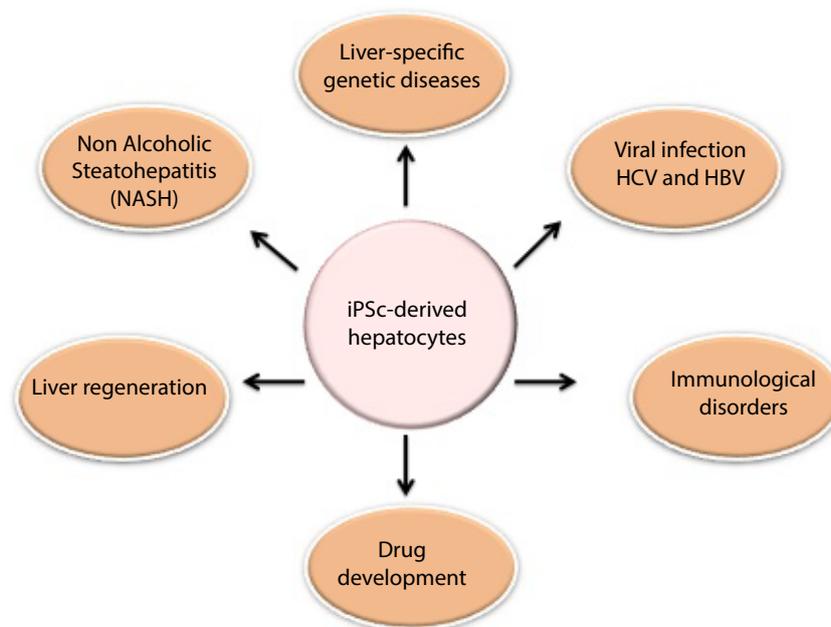
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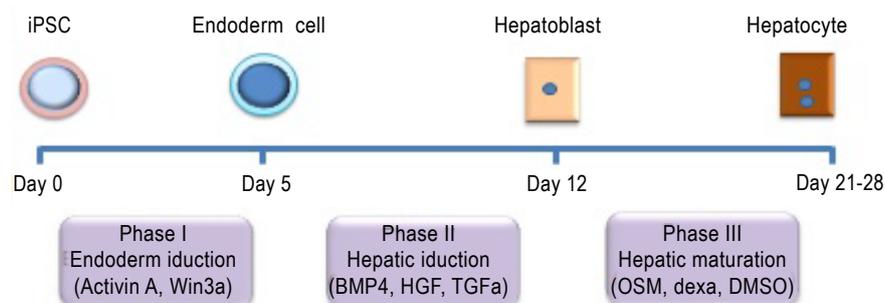
Reprogramming terminally differentiated somatic cells into induced pluripotent stem cells (iPSCs) has revolutionized the field of stem cell biology [27-32]. Recent advances in iPSC technology provides renewable source of various types of functional cells. Disease-specific iPSC lines are valuable tool for understanding disease processes. Human somatic cells can be reprogrammed to iPS cells by using the four factors, *OCT4*, *SOX2*, *KLF4*, and *L-MYC* and efficiency of reprogramming is improved by additional factors *LIN28* and *p53* shRNA [29,33,34]. Non-integrating episomal plasmid-based expression vectors are useful for generating transgene integration-free iPSC lines [30]. In general, the generated iPSC lines are tested for expression of pluripotency markers (SSEA4, TRA-1-81, Oct3/4, Sox2) and genomic stability. Furthermore, pluripotent state of iPSCs is assessed by *in vivo* teratoma assay to observe the formation of tissues from three embryonic layers (tissues of ectodermal, endodermal and mesodermal origins). These iPSCs allow for derivation of various cell types that can be used for disease modeling and repair and regeneration of tissues. Figure 1 shows the various utilities of iPSC-hepatocytes.

Generating functional hepatocytes from iPSCs and human

embryonic pluripotent stem cells (hESCs) is critical for modeling liver diseases. In recent years, the conditions required for differentiating pluripotent stem cells into functional hepatocytes *in vitro* have been under intensive study [35-50]. A three stage differentiation program is commonly used to differentiate pluripotent stem cells to mature hepatic lineage cells within a 3 to 4 week period (Figure 2). Hepatic differentiation consists of a first phase of definitive endoderm formation, a second phase of hepatic lineage specification and a final third phase of hepatic maturation [36,42,49,50]. Activin A and Wnt3a are the factors used for derivation of endoderm cells. For hepatic specification and maturation, hepatocyte growth factor (HGF), bone morphogenetic protein 4 (BMP4), transforming growth factor- $\alpha$  (TGF- $\alpha$ ), dexamethasone, dimethyl sulfoxide (DMSO), oncostatin M (OSM) and additional factors are used. Table 1 provides a list of methods used for derivation of hepatic lineage cells from pluripotent stem cells. The iPSC-derived functional hepatocytes provides a platform for studying genetic disorders, viral infection, drug metabolism and toxicology analysis as well as cell therapy applications in regenerative medicine. The following sections describe current progress of iPSC applications specifically in liver biology.



**Figure 1:** Utilities of iPSC-derived functional hepatocytes for disease modeling and other applications.



**Figure 2:** Schematic showing the experimental outline of differentiation of human iPS cells into functional hepatocytes. The differentiation protocol consists of three phases of varying durations and each differentiation phase requires different cocktails of cytokines, growth factors and chemicals. Hepatoblasts are bipotential liver progenitor cells that give rise to both hepatocytes and cholangiocytes. Dexa: dexamethasone.

Cell type	Protocol Duration	Phase I - Endoderm induction factors	Phase II - Hepatic induction factors	Phase III - Hepatic maturation factors	Reference
iPSCs	12 days	Day 1-3; activin A (100 ng/mL), Wnt 3a (50 ng/mL), HGF (10 ng/mL).	Day 4-7; L-glutamine (1 mM), NAA (1%), 2-Mercaptoethanol (0.1 mM), DMSO (1%).	Day 8-12; OSM (20 ng/mL), Dexamethasone (0.5 μM), ITS premix (50 mg/mL).	Chen et al. [49]
iPSCs	25 days	Day 1-3; CHIR99021 (3 μM), Ly294002 (10 μM), Activin (100 ng/ml), FGF2 (40 ng/ml), BMP4 (10 ng/ml).	Day 4-8; Activin A (100 ng/ml), FGF2 (40 ng/ml), and B27.	Day 9-25; HGF (20 μg/ml), OSM (10 μg/ml).	Rashid et al. [36]
hESCs	20 days	Day 1-5; Activin (100 ng/mL), FGF2 (20 ng/mL), BMP4 (10 ng/mL), LY294003 (10 μM).	Day 6-10; FGF10 (50 ng/mL), Retinoic acid (10 <sup>-7</sup> M), SB431542 (10 μM).	Day 11-20; FGF4 30 ng/ml, HGF 50 ng/mL, EGF 50 ng/ml.	Touboul et al. [46]
hESCs	18 days	Day 1-3; Activin A (100 ng/ml), ITS (1%).	Day 4-8; FGF4 (30 ng/ml), BMP2 (20 ng/ml).	Day 9-18; HGF (20 ng/ml) for 5 days, subsequently OSM (10 ng/ml), dexamethasone (0.1 μM).	Cai et al. [41]
hESCs	14 days	Day 1-3; Activin A (100 ng/mL), FGF-2 (100 ng/ml).	Day 4-11; 10% FBS or KSR, NAA (1 mM) L-glutamine, 1% DMSO, HGF (100 ng/mL).	Day 11-14; 10% FBS or KSR, NAA (1 mM), L-glutamine, dexamethasone (10 <sup>-7</sup> M).	Basma et al. [42]
hESCs or iPSCs	21 days	Day 1-4; Activin-A (100 ng/ml), b-FGF (8 ng/ml), Wnt-3A (25 ng/ml).	Day 5- 10; FGF-10 (50 ng/ml), Retinoic acid (0.1 mM), SB431542 (1 mM).	Day 11-21; FGF-4 (30 ng/ml), EGF (50 ng/ml), HGF (50 ng/ml).	Wu et al. [75]

HGF: hepatocyte growth factor; NAA: non-essential amino acids; DMSO: dimethyl sulfoxide; OSM: Oncostatin M; ITS: insulin-transferrin-selenium; FGF: fibroblast growth factor; BMP: bone morphogenetic protein; EGF: epidermal growth factor; FBS: fetal bovine serum; KSR: knockout serum

**Table 1:** List of various methods to differentiate pluripotent stem cells to mature hepatic lineage cells.

## Modeling Inherited Genetic Disorders of Liver

Liver is the largest metabolic organ in the body and is involved in the metabolism of carbohydrates, fat and protein. These various metabolic processes are executed by the collective efforts of cellular receptors, signaling messengers, transcription factors, enzymes and co-factors. Loss-of-function mutations in key proteins in metabolic pathways can severely perturb the cellular homeostasis. The iPSC lines derived from patients with genetic liver diseases can be used for studying the disease pathobiology at the cellular level utilizing *in vitro* simple cell culture conditions. Table 2 presents the list of liver genetic disorders studied using iPSCs. Rashid and colleagues used disease-specific iPSCs to model α1-antitrypsin deficiency (A1ATD), familial hypercholesterolemia (FH), glycogen storage disease type 1a (GSD1a) in a dish [36]. The generated iPSCs retained the ability to differentiate to mature hepatocytes and demonstrated their unique metabolic defects during differentiation. They found that A1ATD iPSC-hepatocytes had a higher level of polymerized A1AT protein in the cytoplasm. Impairment of LDL uptake was observed in the FH iPSC-hepatocytes. Hepatocytes derived from GSD1a-specific iPSC exhibited excessive accumulation of cytoplasmic glycogen. Crigler-Najjar syndrome, hereditary tyrosinemia type 1 and progressive familial hereditary cholestasis disease-specific iPSC lines were generated as well [36,51]. These disease-specific iPSC lines were differentiated into hepatocytes; however, the disease phenotype was not assessed. The iPSC line generated from a patient with Wilson's disease having a Arg778Leu substitution in the ATP7B gene produced functional hepatocytes that had defect in copper transport [52]. Exogenous expression of wild-type ATP7B gene through integrating lentiviral vector rescued the disease phenotype demonstrating a potential cell therapy application. The modeling of other inherited diseases such as hemochromatosis and hepatobiliary cystic fibrosis can provide additional mechanistic insights into respective disease processes.

## Gene Correction and Genetic Engineering Approach for Liver Disease Modeling

In addition to disease modeling, the *ex vivo* gene corrected patient-specific iPSC lines can be used for autologous transplantation as a therapeutic option. Recent advancement in the development of designer nucleases such as zinc finger nucleases (ZFNs), transcription activator-like effector nuclease (TALEN), and RNA-guided Cas9 nuclease from CRISPR (clustered regularly interspaced short palindromic repeats) system paved the way for precise gene editing in mammalian cells, including iPSCs [53-60]. Yusa and colleagues showed the application of ZFNs and *piggyBac* technology for the correction of a point mutation (Lysine residue at position 342 corrected to Glutamic acid) in the α1-antitrypsin (A1AT) Z allele that is responsible for α1-antitrypsin deficiency disorder [61]. This was achieved using a two-step genetic engineering approach. First, a gene targeting vector having the correct DNA sequence and puromycin antibiotic selection marker was recombined with mutant iPSC line A1AT locus Z allele. Subsequently, the puromycin selection marker sequence was seamlessly excised by *piggyBac* transposase enzyme. A concern in use of *piggyback* transposons for gene targeting is that the excised transposon can re-integrate in random genomic locations and cause unintended mutagenesis. Oligonucleotide-based site-directed mutagenesis following precise double-strand break by designer nucleases can allow for gene editing [62,63].

Modeling diseases through reverse genetic engineering (RGE) of iPSCs and hESCs can allow for the generation of homozygous and heterozygous mutant isogenic cell lines without the need of generating disease-specific iPSCs from patients. This RGE approach also provides the flexibility of knocking out any gene or genetic element in human cells and allowing for disease phenotype screens. Researchers described the function of human *SORT1* gene by creating homozygous mutant hESC lines [60]. The hepatocytes derived from *SORT1*<sup>-/-</sup> cells exhibited several fold increase in apolipoprotein B (apoB), indicating that sortilin

	IMD	Genotype	Functional analysis of iPSC hepatocytes and comments	Reference
1	α1-antitrypsin deficiency (A1ATD)	Homozygous Glu342Lys mutation in Z allele	Polymerized AAT protein demonstrated in the mutant iPSC-hepatocytes.	Rashid et al. [36]
	Glycogen storage disease type 1 a (GSD1a)	Deficiency of hepatic glucose-6-phosphatase enzyme	Increased accumulation of glycogen in the cytoplasm of differentiated hepatocytes.	
	Familial hypercholesterolemia (FH)	Defective LDL receptor (Autosomal-dominant mutation)	Demonstrated defect in LDL uptake by mutant iPSC-hepatocytes.	
	Crigler-Najjar syndrome	Homozygous for 13-bp deletion in exon 2 of UGT1A1 gene	Disease phenotype not characterized.	
	Hereditary tyrosinemia type 1	Val166Gly codon substitution in one of fumarylacetoacetate hydrolase (FAH) alleles	Disease phenotype not characterized.	
2	Hereditary tyrosinemia type 1	FAH protein codon substitution Gln64His	Disease phenotype not characterized.	Ghodsizadeh et al. [51]
	Glycogen storage disease type 1 a (GSD1a)	SLC37A gene mutation 1124- 2A>G	Disease phenotype not characterized.	
	Progressive familial hereditary cholestasis	Multifactorial	Disease phenotype not characterized.	
	Crigler-Najjar syndrome	UGT1A1 protein missense mutation Leu413Pro	Disease phenotype not characterized.	
3	Wilson's disease (WD)	Arg778Leu substitution in ATP7B	Mislocalization of mutant ATP7B protein and lack of copper transport function in iPSC-hepatocytes.	Zhang et al. [52]

**Table 2:** Generation and characterization of iPSC lines derived from patients with inherited metabolic disorders (IMD).

protein coded by *SORT1* reduces apoB secretion and blood LDL-cholesterol level. Additionally by RGE, the function of human *AKT2* gene coding for RAC-β serine/threonine protein kinase was defined. Missense mutation in *AKT2* protein resulting in glutamic acid to lysine codon substitution at position 17 (Glu17Lys) led to hypoinsulinemia, hypoglycemia, and increased deposition of body fat in affected patients [64]. The hESC lines engineered to have *AKT2<sup>-/-</sup>* and *AKT2<sup>E17K</sup>* genotypes reproduced the disease phenotype in where the differentiated *AKT2<sup>-/-</sup>* hepatocytes had significantly high levels of glucose in the media and *AKT2<sup>E17K</sup>* hepatocytes with low media glucose compared to that of normal iPSC-hepatocytes [60]. Reverse genetic engineering approach can be a powerful tool for dissecting the effects of individual gene or single nucleotide polymorphisms (SNPs) of a multifactorial disorder using iPSC lines generated from individuals of various genotypes.

In addition to precise genome targeting approach, integrating lentiviral vector is a useful tool for transgene expression or gene knockdown in iPSCs. A recent study demonstrated that disease causing PiZ variant of A1AT could be efficiently knockdown by lentiviral vector expressing specific short-hairpin RNA (shRNA) in disease-specific iPSC-derived hepatocytes [65].

### (iPSC)-Derived Hepatocytes for Modeling Viral Infection

Hepatitis C virus (HCV) and hepatitis B virus are liver specific pathogens. Over 170 million people are infected with HCV globally [66]. The persistent nature of the infection leads to the development of chronic hepatitis, steatosis, fibrosis, cirrhosis, and primary liver cancer. Currently, there is no HCV vaccine available. HCV is a single-stranded, positive-sense RNA virus belonging to family *Flaviviridae*. Identification of a cell culture replication competent genotype 2a HCV strain JFH-1 (Japanese Fulminant Hepatitis- 1) greatly accelerated the field of HCV research [67-69]. Intra and inter genotypic HCV chimeric

strains based on JFH-1 isolate have been useful for characterization of viral coded functional domains [70-73]. Along with this discovery, a human hepatoma (Huh) cell line and its derivatives are commonly used for generating infectious HCV particles for functional and genetic studies. Robust viral production is observed in sub-clones of Huh-7 cell line that lack the innate immune sensor RIG-I [74].

Primary human hepatocytes and iPSC-derived hepatocytes are useful for evaluating host-HCV pathogen interactions, specifically the innate immune aspect [75,76]. Functionally, mature hepatocytes are required for HCV replication. Schwartz et al. demonstrated that the iPSC-derived hepatocyte-like cells (iHLC) expressed HCV permissive factors miR-122 and cell entry receptors CD81, CLDN1, occludin and SRBI [76]. The iHLCs supported HCV infection and treatment of HCV infected cells with NS5B polymerase inhibitor (2'CMA) and NS3/4A protease inhibitor (VX-950) resulted in complete viral inhibition. Furthermore, the infected cells upregulated inflammatory genes such as CXCL10, CXCL11, TNF-α, IL28B and IL29 in response to HCV infection. Another study reported that the HCV infection of differentiating hepatic cells correlated with the induction kinetics of miR-122, an essential permissive factor [75]. ShRNA mediated knockdown of cyclophilin A (CyPA), another HCV replication permissive factor, hindered viral infection. Ability of the hepatocytes derived from pluripotent stem cells to support HCV replication can be a hallmark assay for assessing the maturation status of hepatocytes. Hepatic progenitors defined based on the expression of KDR surface protein cultured in two-dimensional or three-dimensional conditions exhibited differential permissivity to HCV infection [77]. Combining reverse genetic engineering technology and HCV infection study can provide novel insights into the mechanism of hepatitis C pathobiology.

### (iPSC)-Hepatocytes for Drug Screening and Modeling Drug Metabolism

Hepatocytes carry out both anabolic and catabolic activities of

nutrients, metabolites, hormones, drugs and other compounds for storage and excretory purposes. The liver cytochrome p450 (CYP) enzyme system and membrane transporters are critical for drug metabolism and clearance [78-82]. To validate the maturation and functionality of hepatocytes-derived from pluripotent stem cells, induction of CYP enzymes upon drug treatment (example: rifampicin and phenobarbital), characterization of drug metabolites generated by CYP system and transporter activities have been investigated [36,51,83]. CYP system comprises of phase I and phase II enzymes that involve in oxidation-reduction and conjugation reactions of xenobiotics. Regarding phase I enzymes, immature hepatocytes express CYP1A1 and CYP3A7, and mature hepatocytes produces CYP3A4, CYP2B6, CYP2C19, CYP2C9, CYP1A2 and CYP2D6 [83-85]. For identification of new therapeutic compounds, the hepatocytes of iPSC lines derived from patients with  $\alpha$ 1-antitrypsin deficiency were used. Through high throughput screening approach, five clinical drugs were identified which were shown to reduce the defective A1AT protein accumulation in the cytoplasm [86]. This landmark study demonstrated the utilization of iPSC-hepatocytes for drug development towards inherited metabolic disorders. There is a huge potential for modeling drug interactions and pharmacokinetic properties using library of iPSC-hepatocytes generated from various genetic background. Also the pluripotent nature of iPSC allows for generation of various cell types such as liver, heart, neurons, lung and other cells. This opens up the possibility of evaluating drug toxicity on each cell type derived from patient-specific iPSC, during clinical practice as a component of personalized medicine.

## Discussion

Using iPSC lines for modeling liver disease is an emerging field. Disease-specific iPSC lines have been established from individuals affected with various monogenic defects. Mutant phenotype of disease-specific iPSC lines has been demonstrated at the cell culture level, which provides a screening platform for identifying novel therapeutic compounds. iPSC and hESC -derived hepatocytes have been useful for investigating hepatitis C virus replication and host-pathogen interaction.

Though the opportunities of using iPSC-hepatocytes for disease modeling are enormous, the limitations of the system need to be evaluated. Pathogenesis of liver disease is the outcome of complex interactions between disease causing agents, liver parenchymal cells (hepatocytes, biliary epithelium and hepatic progenitor cells), non-parenchymal cells (stellate cells, sinusoidal endothelium and Kupffer cells), and inflammatory and immune factors. Moreover, pathogenesis of fibrosis and cirrhosis due to viral, alcoholic and metabolic causes can take years to several decades. Thus, modeling complex diseases require reconstitution of various cellular and molecular components in the right proportion in an appropriate anatomical niche. Three dimensional culture techniques and liver organoids can address some of the drawbacks. Furthermore, iPSC-based cell culture model can be used as a complement to animal models for investigating liver disease.

There is a great potential for use of iPSCs to study the genetic and molecular basis of nonalcoholic steatohepatitis (NASH) and alcoholic fatty liver disease. Metabolomics, genomics, and proteomics analysis of iPSC-hepatocytes that are exposed to nutritional, organic and inorganic compounds can shed light on the pathobiology of NASH. The iPSC-hepatocytes can play key role in drug development process. Drugs and xenobiotics are metabolized by liver cytochrome p450 enzyme system and are subsequently excreted by hepatic transporter proteins. Polymorphisms in genes coding for CYP system account

for variations observed in the pharmacokinetic properties of drugs among different individuals. Preclinical toxicological and metabolic evaluation of investigational drugs in iPSC-hepatocytes generated from individuals of diverse genetic backgrounds would provide early evidence for potential side effects and safety concerns before expensive human clinical trials. Gene corrected autologous iPSC lines and differentiated target cells can be used as cell source for the treatment of inherited liver disorders, especially in pediatric patients.

Continuous passaging of iPSC lines could potentially introduce genetic mutations which could influence the disease phenotype that is being studied; thus, periodic evaluation of genomic integrity is necessary during *in vitro* expansion of these cells. One major concern when using iPSCs is the formation of teratomas. Pluripotent stem cells have the ability to form teratomas which can limit the use of iPSCs as such in the clinical setting; however, fully differentiated cells derived from iPSCs could possibly be used following extensive safety analysis.

Human hepatocytes derived directly from fibroblast can be an alternative approach to disease modeling without the use of iPSCs. Recently, several groups have demonstrated that human fibroblast can be directly reprogrammed to functional hepatocytes using a defined cocktail of chemical and transcription factors [84,85,87]. One study has shown that fibroblast can be induced to enter the endodermal phase, which can be further differentiated into functional hepatocytes [84]. Additional groups have used hepatocyte-specific transcription factors such as a combination of HNF1A, HNF4A, and HNF6 or FOXA3, HNF1A and HNF4A to reprogram human fibroblast into hepatocytes [85,87]. Directed differentiation of fibroblasts to hepatocytes is an exciting option for liver disease modeling. Overall, iPSC technology provides new tool to advance the research in the field of liver biology.

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