Analysis of the mechanism underlying liver diseases using human induced pluripotent stem cells

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\section*{ABSTRACT}
Results of recent studies have shown that disease models using human induced pluripotent stem (iPS) cells have recapitulated the pathophysiology of genetic liver diseases, viral hepatitis and hepatic fibrosis. The utilization of human iPS cells as a model of liver diseases has several substantial advantages compared with primary hepatocytes and cancer cell lines, such as the potential for unlimited expansion and similarity of biological characteristics to normal liver cells. In this review, we have focused on modeling liver diseases using human iPS cells and discussed the experimental evidence that supports the utility of such disease models, including that in our recent studies. Genetically modified or patient-derived human iPS cells can mimic congenital liver disease phenotypes. Human iPS-derived hepatic cells can be infected with the hepatitis viruses. The co-culture of human iPS-derived hepatocytes and mesenchyme partially mimics the process of liver fibrosis. Human iPS cell-derived hepatic cells and the co-culture system of such cells will contribute to the progress of studies on the pathophysiology of genetic and non-genetic liver diseases and development of novel therapeutic strategies for treating liver diseases.

\section*{1. Introduction}
Chronic liver diseases caused by genetic disorders, viral infections, autoimmune diseases and metabolic diseases, including non-alcoholic steatohepatitis, remain a worldwide public health threat placing individuals at risk of developing end-stage liver disease and hepatocellular carcinoma [1]. There is an urgent need to develop novel therapies to treat liver diseases; however, studies on this are hampered by a lack of appropriate disease models that reveal the pathophysiology of chronic liver diseases. Animal models of liver diseases, such as genetically modified mice, have been developed and helped clarify this pathophysiology; however, there are several phenotypic differences between human diseases and animal models.

Stem cells have been identified in a variety of tissues, where they play central roles in the homeostasis of normal tissue and tissue repair [2]. Human induced pluripotent stem (iPS) cells are somatic cells genetically reprogrammed to be pluripotent by the transient expression of genes essential for maintaining the properties of embryonic stem cells [3]. Human iPS and human embryonic stem cells exhibit the potential for differentiation into various cell types, including hepatic lineages [4–8]. Such pluripotent stem cells are candidates for regenerative medicine and a promising tool to evaluate the pathophysiology of genetic or non-genetic diseases. Differentiation methods of various types of somatic stem cells and their progeny, including neurons [9], cardiomyocytes [10], lung [11], intestine [12], hepatocytes [4], cholangiocytes [5] and immunocompetent cells [13], have been reported. Human iPS cell lines derived from patients with genetic diseases are often used for such research models because target cells derived from patients may mimic the pathophysiological abnormalities owing to genetic disorders [9,11,14–17]. However, studies involving the use of iPS cells derived from patients with rare genetic diseases might yield in unreliable data owing to different genetic backgrounds, different multipotency of iPS cell lines, lack of a phenotypic marker, and various mutation patterns in disease. In addition, the cell–cell interaction of epithelia, mesenchymal cells and immunocompetent cells should be reproduced for studying non-genetic and multifactored diseases. Here, we have focused on the modeling of liver diseases using human iPS cells and discussed the experimental evidence supporting the utility of
such disease models, including that in our recent studies.

2. Diseases models of genetic liver diseases

Several reports have shown that human iPS cells differentiate into hepatocyte or cholangiocyte lineages in vitro [4–8]. Hepatocyte-like cells derived from iPS cells (iPS-Heps) exhibit properties of hepatocytes, such as albumin secretion and metabolic functional properties. Several pathophysiological disorders can be reproduced using human iPS-Heps; on the contrary, the phenotypes of iPS-Heps are immature compared with adult mature hepatocytes with respect to albumin production, cytochrome P450 (CYP) activity and metabolic functions [18,19]. Recent reports have shown that patient-derived iPS-Heps have been used to investigate the pathophysiology of genetic disorders, such as α1-antitrypsin deficiency, glycogen storage disease type 1, Crigler–Najjar syndrome, hereditary tyrosinemia type 1, Gaucher disease, Niemann-Pick disease type C and Wilson’s disease [14–16,20,21]. Rashid et al. reported that iPS-Heps derived from patients with α1-antitrypsin deficiency recapitulate key pathological features of the disease, such as the aggregation of misfolded alpha1-antitrypsin in the endoplasmic reticulum [14,22,23]. Glycogen accumulation was observed in iPS-Heps derived from patients with glycogen storage disease type 1a [14].

A molecular signature related to lipid metabolism was impaired in iPS-Heps derived from a patient with Niemann-Pick disease type C in consistent with the phenotype of such diseases [21]. Yi et al. reported that iPS cells derived from patients with Wilson’s disease were differentiated into both hepatic and neural lineages and that a gene expression analysis and cDNA sequencing confirmed the expression of the mutant ATP7B gene in Caucasian patients with Wilson’s disease [16]. Sampaziotis et al. showed that iPS cells derived from patients with cystic fibrosis are differentiated into cholangiocyte-like cells and that the experimental drug of cystic fibrosis rescued the disease phenotype of cholangiopathy in vitro [7]. The studies on genetic liver diseases using patient-derived iPS cells are summarized in Table 1. These reports reveal that patient-derived iPS cells can mimic the phenotype of genetic liver disease; however, the pathophysiology of these genetic diseases has already been clarified in previous studies involving the use of molecular biology techniques and disease animal models, such as knockout mice. These studies showed only some of the phenotypes of liver diseases.

3. Genetically modified iPS cells as a model for liver diseases

While conducting studies involving the use of patient-derived iPS cells to clarify the unknown pathophysiology of rare genetic liver diseases, there might be confusion owing to unreliable data because of different genetic backgrounds of patients, lack of a phenotypic marker, various mutation patterns in the disease and different multipotency of the iPS cell lines. Therefore, genetically engineered human iPS cells derived from healthy individuals are used to study diseases including those involving interferon regulatory factor-8 deficiency [30], Alzheimer’s disease [31], Parkinson’s disease [32] and polycystic liver disease [28]. PRKCSH (protein kinase C substrate 80 K-H) is a responsible gene for polycystic liver disease. Kamiya et al. reported that PRKCSH deficiency promotes the proliferation of iPS-derived cholangiocyte-like cells, which mimic

| Diseases | Source of iPS cells | Mutated genes | Phenotypes of iPS-derived hepatic lineages | References |
|----------|---------------------|---------------|-------------------------------------------|------------|
| α1-antitrypsin deficiency | Patient-derived | α1-antitrypsin (SERPINA1) | Aggregation of misfolded α1-antitrypsin in the endoplasmic reticulum | [14,23,22] |
| Congenital hepatic fibrosis | Genome-edited | PKHD1 | Abnormal proliferation of cholangiocytes due to production of IL-8 and CTGF in an autocrine manner | [24] |
| Crigler–Najjar syndrome | Patient-derived | UGT1A1 | Not shown (elevated indirect bilirubin) | [25,14] |
| Cystic fibrosis-associated cholangiopathy | Patient-derived | CFTR | Impaired chloride/water transportation in cholangiocytes | [7] |
| Gaucher disease | Patient-derived | GBA | Not shown (glucosylceramide accumulation in lysosomes in neurons and macrophages) | [26,27,20] |
| Glycogen storage disease type 1a | Patient-derived | G6PC | Increased glycogen storage and lipid accumulation, excessive production of lactic acid | [25,14] |
| Hereditary tyrosinemia type 1 | Patient-derived | FAH | Not shown (elevated fumarylacetoacetate) | [25,14] |
| Niemann-Pick disease type C | Patient-derived | NPC1 | Abnormal lipid metabolism, impaired autophagy and ATP production | [21] |
| Polycystic liver disease | Genome-edited | PRKCSH | Abnormal differentiation and proliferation of cholangiocytes | [28] |
| Wilson’s disease | Patient-derived | ATP7B | Not shown (lower osteogenesis activity in osteoblasts) | [29,16] |

SERPINA1: serpin family A member 1; PKHD1: polycystic kidney and hepatic disease 1; UGT1A1: UDP glucuronosyltransferase family 1 member A1; CFTR: cystic fibrosis transmembrane conductance regulator; GBA: glucosylceramidase beta; G6PC: glucose 6-phosphatase; FAH: fumarylacetoacetate hydrolase; NPC1: NPC intracellular cholesterol transporter 1; PRKCSH: protein kinase C substrate 80 K-H; ATP7B: ATPase copper transporting beta.
the uncontrolled proliferation of cholangiocytes in polycystic liver diseases (Table 1) [28].

We have recently reported on the pathological molecular mechanism of congenital hepatic fibrosis (CHF), which was clarified using genetically engineered human iPS cells. The pathological mechanism of CHF is quite different from that of liver cirrhosis owing to chronic hepatitis; hepatic fibrosis in CHF patients is prominent with nodular formation, but necroinflammatory changes of hepatocytes and the activation of hepatic stellate cells are not evident in the CHF liver [33]. The gene responsible for CHF is \( PKHD1 \) (polycystic kidney and hepatic disease 1), which encodes the fibrocystin protein localized in the primary cilia of cholangiocytes [34,35]. Animal models of CHF, such as gene-targeted \( Pkhd1 \) mutated mice, have been developed [36–39]; however, there are several phenotypic differences between human CHF and these animal models. Thus, a disease model using human cells is necessary to study CHF pathophysiology. It is difficult to clarify such mechanisms using an iPS cell model derived from CHF patients because of the numerous mutation patterns without specific correlations between genetic and phenotype variations in CHF patients. Furthermore, the complete functional loss of \( PKHD1 \) is lethal in the fetal period [40–42].

Based on the background described above, we examined the pathophysiology of CHF using genetically modified iPS cell lines derived from a healthy individual [24]. Our data provide evidence that the abnormally increased production of IL-8 and CTGF derived from cholangiocytes lacking fibrocystin function is essential for the pathogenesis of CHF; moreover, the findings demonstrate that IL-8 and CTGF are candidate therapeutic molecular targets for CHF (Table 1). This study also indicated that genetically engineered human iPS cells derived from healthy individuals are used as an appropriate tool for the study of disease caused by unknown gene function.

4. Disease models of hepatitis C virus (HCV) and hepatitis E virus (HEV)

The study using human iPS cells has several substantial advantages compared to primary hepatocytes and hepatoma cell lines, such as the potential for unlimited expansion and the similarity of biological characteristics to normal hepatocytes. Moreover, the influence of the maturity of host cells on the life cycle of the hepatitis virus and specific gene functions in the host-to-virus interactions can be evaluated using iPS-Heps. Interferon (IFN)-\( \alpha \)-related innate immune responses are particularly important for the elimination of hepatitis virus from host cells [43]; however, hepatoma cell lines (such as HuH-7 and HepG2) partially lack the ability of innate immune responses. In contrast to hepatoma cell lines, primary human hepatocytes are used as host cells for the productive infection of the hepatitis virus without these problems [43,44]. However, the availability of human primary hepatocytes is limited because long-term culture remains difficult, and the genetic modification of target genes in these cells is also unavailable.

In consistent with the abovementioned concept, several reports have shown the benefit of human iPS-Heps as an in vitro replication system of HCV and HEV. Human iPS-Heps support the entire life cycle of HCV, including immune responses to infection [45–47]. Human iPS-derived definitive endoderm was not permissive for HCV infection, whereas iPS-Heps were persistently infected and secreted infectious particles. Permissiveness to HCV infection was correlated with induction of the liver-specific microRNA-122 [48]. Wu et al. reported that human iPS-Heps are permissive for non-cell culture-adopted HEV in contrast to hepatoma cells. HEV replication induces an antiviral innate immune response in iPS-Heps [49]. These reports indicate that human iPS-Heps can be used as an experimental model to develop antiviral agents against HCV and HEV.

5. Disease models of hepatitis B viruses

The development of therapeutic strategies against hepatitis B virus (HBV) has been a key area of research because nucleos(t)ide analogs cannot eliminate HBV from host cells because of the persistence of HBV covalently closed circular DNA (cccDNA) [50]. Sodium taurocholate cotransporting polypeptide (NTCP) was reported to be an entry receptor for HBV, and the overexpression of NTCP in hepatoma cell lines rendered them susceptible to HBV infection [51]. However, hepatoma cell lines lack several cellular pathways, including innate immune responses, and the utilization of iPS cells as a model of hepatitis B virus infection for drug development has been reported [19,52,53].

Previous studies including our data showed that iPS-Heps can respond to anti-viral IFN-\( \alpha \) stimulation, which is susceptible to HBV [19,52,53]. Immature proliferating hepatic progenitor-like cell lines derived from iPS cells (iPS-HPCs) at a relatively immature stage of hepatic differentiation can be infected with HBV in vitro [19]. We have shown that iPS-HPCs overexpressing NTCP can be used as a model for HBV persistence and that these cells maintain innate immune responses against HBV [19]. These studies demonstrated that iPS-Heps and
iPS-HPCs can be utilized as an experimental model to investigate HBV–host interactions, develop novel therapies and elucidate the association between HBV persistence and cell differentiation.

6. Disease models of drug-induced liver injury and autoimmune hepatic diseases

Human iPS-Hep cells can be utilized to enhance hepatic safety risk assessment to avoid drug-induced liver injury. Hepatic culture systems, such as hepatoma and HepaRG cell line, have limited utility, since they do not fully mimic functional hepatocytes, and do not sufficiently provide the cells with genetic polymorphisms [54]. Human iPS-Hep cells are a promising source overcoming this problem; however, there are several problems in the safety risk assessment of drugs using iPS cells. Human iPS-Hep cells remain immature compared with primary hepatocytes, suggesting that several metabolic function including CYP in such cells is relatively lower than that of primary hepatocytes [55]. Moreover, there are differences in the quality of human iPS cell lines and iPS-Heps, which may result in a lack of reproducibility of assays.

On the other hand, several reports showed that human iPS-Heps can predict hepatotoxicity of drugs. Li et al. showed that iPS-Heps derived from a patient with Alpers-Huttenlocher syndrome are more sensitive to valproic acid-induced apoptosis, which mimic the increased risk of valproic acid-induced liver injury [56]. Choudhury et al. showed the use of patient-specific iPS-Heps for modeling idiosyncratic hepatotoxicity to a tyrosine kinase inhibitor drug (Pazopanib) associated with significant hepatotoxicity of unknown mechanistic basis. Pazopanib caused disruption of iron metabolism and increased oxidative stress in iPS-Heps derived from patients compared with those derived from normal individuals [57]. A recent study showed that a cell sheet composed of human iPS-Heps expresses albumin, CYP3A4 and CYP1A2 at a comparable level to primary human hepatocytes [58]. These studies support the idea that human iPS-Heps will overcome the problems in immaturity and be clinically utilized to predict drug-induced liver injury in future.

There are few reports about the analysis of autoimmune liver diseases using human iPS cells, whereas Joyce et al. showed that murine iPS-derived myeloid-derived suppressor cells regulate immune responsiveness in vivo and have a therapeutic effect against hepatitis in murine autoimmune hepatitis model [59]. Further technical progress will be necessary for the study on autoimmune hepatitis, primary biliary cholangitis, and primary sclerosing cholangitis, which coordinately control both parenchymal cells and immunocompetent cells derived from human iPS cells in a simultaneous culture system.

7. Disease models of liver fibrosis

Human iPS-Hep cells with genetic modifications may be of value for research into various diseases as described above; however, previous studies also showed that the phenotypes of iPS-Hep cells are immature compared with adult hepatocytes with respect to albumin production, CYP activity and metabolic functions [4,5,18,19]. This problem of the immature nature of iPS-Hep cells as hepatocytes must be resolved. Moreover, the interaction between epithelial cells (hepatocytes and cholangiocytes) and mesenchymal cells, such as hepatis stellate cells, Kupffer cells, sinusoidal endothelial cells and immunocompetent cells, is important for the development of chronic hepatitis and liver cirrhosis in vivo [60].

To reveal the pathophysiology of chronic hepatitis and liver fibrosis including non-alcoholic steatohepatitis (NASH), such 'non-parenchymal liver cells' derived from human iPS cells are important in in vitro studies. It is possible that the co-culture of iPS-derived hepatic cells and iPS-derived hepatic stellate cell-like cells (iPS-HSCs) contributes to both modeling liver fibrosis and the hepatic maturation of iPS-derived hepatic cells [61–64].

Supporting this hypothesis, Camp et al. showed that three progenitor cells, hepatic endoderm, endothelium and septum mesenchyme, derived from iPS cells can effectively generate liver buds [61]. The liver buds derived entirely from human iPS cells can functionally rescue against acute liver failure via transplantation [61]. Kou et al. reported that liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells (HSCs) can be generated from human iPS cells [62]. In this study, such iPS cell-derived HSCs and LSECs secreted growth factors and extracellular matrices (ECM) and promoted the proliferation and maturation of iPS-HPCs in vitro.

We have also shown a new strategy for the differentiation of human iPS-HSCs [64]. The phenotype of human iPS-HSCs resembled that of primary HSCs, and the co-culture of iPS-HPCs with iPS-HSCs induced hepatic maturation in iPS-HPCs. Human iPS-HSCs facilitate the maturation of iPS-HPCs in 2D direct co-culture but not in transwell co-culture, indicating that a direct cell–cell interaction between HSCs and HPCs is important for the hepatic maturation of progenitors. Next, we generated iPS-HSCs overexpressing LIM homeobox 2 (LHX2), which suppresses myofibroblastic changes.
in HSCs in mice. The upregulation of LHX2 in iPS-HSCs increased hepatic maturation compared to normal iPS-HSCs. LHX2 upregulation altered the ECM expression profile in the iPS-HSCs, such as laminin and collagen. This study demonstrated that genetically modified iPS-HSCs are a useful tool for improving the hepatic maturation of iPS-derived hepatic cells [19]. Genetically modified iPS-HSCs will be helpful for a validation analysis of potential mechanisms and will be valuable for further screening of novel molecular targets. These strategies may mimic the molecular pathophysiology of chronic hepatitis including cell–cell interactions between epithelial cells and mesenchymal cells [61,62,64].

Coll et al. showed that HSCs are generated from human iPS cells, which are activated into myofibroblasts in vitro [63]. Human iPS-derived HSCs exhibit the phenotype resembling quiescent human HSCs in 3D culture with hepatocytes (the HepaRG cell line), and iPS-derived HSCs were transformed into myofibroblasts via treatment with TGF-β, thioacetamide and acetaminophen in vitro. Activated iPS-derived HSCs secrete pro-collagen [63]. Conversely, recent reports showed that HSCs, portal fibroblasts and mesothelial cells exhibit distinct phenotypes, and both HSCs and portal fibroblasts contribute to liver fibrosis [65]. The phenotype of iPS-HSCs resembles that of immature HSCs [62,64]. Previous reports have shown liver mesenchymal cells can be generated from human iPS cells; however, it is difficult for iPS cells to be differentiated into HSCs alone, but into neither portal fibroblasts nor mesothelial cells. The differentiation method should be improved to generate the specific population of liver mesenchyme. Nevertheless, these studies revealed that the co-culture system of iPS-Heps (or iPS-HPCs) and iPS-derived mesenchymal cells may generate novel in vitro systems mimicking liver fibrosis, called as ‘liver organoids’ (Figure 1). Such strategies are promising and will contribute to the development of novel classes of drugs against chronic hepatitis and liver fibrosis.

8. Conclusion

Models of liver diseases involving the use of human iPS cells have contributed to the elucidation of the pathophysiology of liver diseases with genetic and non-genetic etiology. Recent reports have shown that primary murine hepatocytes derived from adult livers can be maintained for a long time in a 3D organoid culture system [66–68]. Human liver organoids derived from human iPS cells may be established using the technique assisted by the methods for the long-term culture of primary hepatocytes [69]. Human iPS cell-derived organoids composed

![Figure 1. Schema of human iPS-derived liver cells and organoids for the research of liver diseases. Human iPS cells derived from healthy volunteers and patients can be used for the study on various liver diseases. Human iPS cells are differentiated into target cells, including hepatocytes, Kupffer cells, hepatic stellate cells and cholangiocytes, and the resulting cells are used as a model for the liver diseases at present. In the case that cell–cell interaction is important for the study, human iPS-derived liver organoids will be used as a model for the diseases in future. These iPS-derived disease models mimic the molecular mechanisms of liver diseases and will contribute to the drug development in chronic hepatitis and fibrosis.](image-url)
of hepatocytes, cholangiocytes, HSCs, Kupffer cells and immunocompetent cells may completely mimic the molecular mechanisms of liver diseases (Figure 1) and can contribute to the development of novel therapies against chronic hepatitis and fibrosis in the future.

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Disclosure statement

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