Drug Discovery: Recent Progress and the Future

Review
Pharmaceutical Research for Inherited Metabolic Disorders of the Liver Using Human Induced Pluripotent Stem Cell and Genome Editing Technologies

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Orthotopic liver transplantation, rather than drug therapy, is the major curative approach for various inherited metabolic disorders of the liver. However, the scarcity of donated livers is a serious problem. To resolve this, there is an urgent need for novel drugs to treat inherited metabolic disorders of the liver. This requirement, in turn, necessitates the establishment of suitable disease models for many inherited metabolic disorders of the liver that currently lack such models for drug development. Recent studies have shown that human induced pluripotent stem (iPS) cells generated from patients with inherited metabolic disorders of the liver are an ideal cell source for models that faithfully recapitulate the pathophysiology of inherited metabolic disorders of the liver. By using patient iPS cell-derived hepatocyte-like cells, drug efficacy evaluation and drug screening can be performed. In addition, genome editing technology has enabled us to generate functionally recovered patient iPS cell-derived hepatocyte-like cells in vitro. It is also possible to identify the genetic mutations responsible for undiagnosed liver diseases using iPS cell and genome editing technologies. Finally, a combination of exhaustive analysis, iPS cells, and genome editing technologies would be a powerful approach to accelerate the identification of novel genetic mutations responsible for undiagnosed liver diseases. In this review, we will discuss the usefulness of iPS cell and genome editing technologies in the field of inherited metabolic disorders of the liver, such as alpha-1 antitrypsin deficiency and familial hypercholesterolemia.

Key words genome editing; alpha-1 antitrypsin; familial hypercholesterolemia; human induced pluripotent stem cell; inherited metabolic disorder of the liver

1. INTRODUCTION
The liver plays central roles in maintaining metabolic homeostasis. Therefore, inherited metabolic disorders of the liver (IMDs) can be life-threatening. Many IMD patients require liver transplantation for treatment of their severe metabolic disorders, but not all patients indicated for transplantation receive it due to a shortage of donated livers. As an alternative to liver transplantation, a drug or drugs effective for the treatment of IMDs are highly anticipated. To facilitate the pharmaceutical research, a disease model that faithfully recapitulates the pathophysiology of IMD patients will be indispensable. For many IMDs, however, no such disease model exists, and it can be difficult to obtain biological samples from IMD patients, especially those with rare IMDs. Human induced pluripotent stem (iPS) cell technology is expected to solve these problems.1 Human iPS cells can be easily established from somatic cells such as fibroblasts of IMD patients and proliferate indefinitely. Accordingly, by establishing IMD patient-derived iPS cells (IMD-iPS cells), it would be possible to obtain abundant patient samples for pharmaceutical research. Many groups including ours have developed an efficient hepatocyte differentiation protocol by adopting genetic manipulation,2–6) three-dimensional culture,7–9) co-culture,10–12) extra-cellular matrix,13–15) or small-molecule compounds.16–18) Thus, it is possible to generate human iPS cell-derived hepatocyte-like cells (HLCs) which recapitulate the hepatic functionality in vivo.19–22) Therefore, it is suggested that HLCs derived from IMD-iPS cells would be a useful cell model in terms of drug development.

Among various IMDs, alpha-1 antitrypsin (AAT) deficiency and familial hypercholesterolemia (FH) have been the most actively studied. AAT deficiency (AATD) is a disease caused by mutations in the AAT gene. AATD can cause severe liver diseases, including liver cirrhosis and hepatocellular carcinoma.23) The incidence of AATD is 1 in 1800 to 2000 live births. The PiZ variant (Z-AAT) is the most common of the medically significant null variants, and it is caused by a (G > A) point mutation at codon 342 (Glu342Lys) in exon 5 of the AAT gene.24) The mutation promotes spontaneous Z-AAT polymerization and retention of the Z-AAT polymers in the endoplasmic reticulum (ER) of hepatocytes, resulting in pro-
tein overload that causes the liver diseases. FH is an autosomal dominant hypercholesterolemia caused by mutations in the low-density lipoprotein receptor (LDLR) gene or LDLR-related genes. FH is characterized by an elevation of serum low density lipoprotein (LDL)-cholesterol which lead to xanthoma formation and premature cardiovascular disease. The incidence of compound heterozygous or homozygous mutations in the LDLR gene or LDLR-related genes (referred to collectively as HoFH) is 1 in 150000 to 300000. The phenotype of HoFH is much severer than those of heterozygous FH (HeFH). It is also reported that statins are ineffective in HoFH, despite their effectiveness in HeFH. In this report, we will overview recent progress in the pharmaceutical research for IMDs, such as AATD and FH, using human iPS cell and genome editing technologies.

2. PHARMACEUTICAL RESEARCH USING PATIENT IPS CELLS

In 2010, to the best of our knowledge, two groups were the first to succeed in establishing human iPS cells from patients with IMDs, including Crigler–Najjar Syndrome, AATD, and FH. Thereafter, human iPS cells were established from various patients with IMDs (Table 1), and the IMD-iPS cells have contributed to the disease modeling and drug screening for IMDs.

Rashid et al. were the first to establish human iPS cells from a patient with AATD (AATD-iPS cells), and they differentiated these iPS cells into HLCs (AATD-iPS-HLCs). The AATD-iPS-HLCs could recapitulate the key pathological features of AATD, namely retention of the Z-AAT polymers in the ER. Choi et al. conducted drug screening using the AATD-iPS-HLCs. Among 3131 clinical drugs (including 2800 drugs that have been approved by U.S. Food and Drug Administration (FDA) or have entered phase II clinical trials), 5 drugs could reduce Z-AAT accumulation in the AATD-iPS-HLCs. Carbamazepine, one of the final 5 hit drugs, was consistently shown to decrease Z-AAT accumulation in AATD model mice. It is well known that there is a wide variability in the severity of AATD-mediated liver injury. Some AATD patients suffer from severe liver disease (SLD) that necessitates liver transplantation, while others with the same genetic defect suffer from no liver disease (NLD). To elucidate the cause of this variation, Tafaleng et al. established human iPS cells from both groups of AATD patients (SLD-iPS cells and NLD-iPS cells). By means of a pulse-chase labeling assay, they then demonstrated a significant difference in the fate of Z-AAT between the two types of cells. That is, the intracellular Z-AAT disappeared more slowly in SLD-iPS-HLCs (half-time 3.6 ± 0.1 h) than in NLD-iPS-HLCs (half-time 2.2 ± 0.3 h). Moreover, transmission electron microscopy examination demonstrated that globular inclusions, large vesicular structures enveloping Z-AAT, were observed in SLD-iPS-HLCs but not in NLD-iPS-HLCs. These results suggest that the degradative response in AATD patients with NLD might be sufficient to prevent Z-AAT accumulation. In addition, this study provided diagnostic criteria for predicting individual differences in AATD-mediated liver injury. Wilson et al. performed microarray analyses to examine the differences between AATD-iPS-HLCs and healthy donor-derived iPS-HLCs. They identified the AATD-specific transcriptomic signature, namely, expression of 135 genes diverged from controls. Additionally, they found that carbamazepine treatment increased the protein expression levels of autophagosome

| Year | Journal | Authors | Disease | Ref. |
|------|---------|---------|---------|------|
| 2010 | Stem Cell Reviews and Reports | Ghodsizadeh et al. | Tyrosinemia type 1 | 29 |
| | | | Glycogen storage type lb | |
| | | | Progressive familial hereditary cholestasis | |
| | | | Crigler–Najjar syndrome | |
| 2010 | The Journal of Clinical Investigation | Rashid et al. | Alpha-1 antitrypsin deficiency | 30 |
| | | | Glycogen storage disease type 1a | |
| | | | Familial hypercholesterolemia | |
| | | | Crigler–Najjar syndrome | |
| | | | Hereditary tyrosinemia type 1 | |
| 2012 | Hepatology | Cayo et al. | Familial hypercholesterolemia | 31 |
| 2013 | Hepatology | Choi et al. | Alpha-1 antitrypsin deficiency | 32 |
| 2015 | Hepatology | Li et al. | Alpers–Hutenlocher syndrome | 33 |
| 2015 | Hepatology | Tafaleng et al. | Alpha-1 antitrypsin deficiency | 34 |
| 2015 | Stem Cell Reports | Wilson et al. | Alpha-1 antitrypsin deficiency | 35 |
| 2015 | Stem Cells | Soga et al. | Niemann–Pick disease type C | 36 |
| 2017 | EBioMedicine | Bi et al. | Tangier disease | 37 |
| 2017 | Cell Stem Cell | Cayo et al. | Familial hypercholesterolemia | 38 |
| 2017 | Scientific Reports | Imagawa et al. | BSEP-deficiency (progressive familial intrahepatic cholestasis type 2) | 39 |
| 2017 | Biochemical and Biophysical Research Communications | Yoshitoshi-Uebayashi et al. | Citrullinemia type 1 | 40 |
LDL.42) This compound heterozygous mutations in the region at residue 807 in exon 17, which is unable to internalize apoB-100.38) Consistent with a previous report that statins are play a marked elevation in the secretion of apolipoprotein B et al. have demonstrated that FH-JD-iPS cell-derived HLCs gene causes the loss of LDLR-mediated LDL-C uptake. Cayo candidates for FH-JD. 38) The screen was performed using 2320 per et al. performed a drug screen to identify novel drug can- could be predicted using FH-iPS-HLCs. Furthermore, CayoLovastatin treatment. This result suggests that the drug efficacy uptakes capacity in FH-JD-iPS-HLCs was not recovered by for AATD. 41) Moreover, it is considered that the discovery of novel drug-target molecules will be promoted by performing comprehen- 3. PHARMACEUTICAL RESEARCH USING A COMBINATION OF IPS CELL AND GENOME EDITING TECHNOLOGIES

By introducing genome editing technology into patient iPS cells, the detailed study of inherited genetic disorders has been greatly accelerated. Genome editing technology is based on the use of engineered nucleases including zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins 9 (CRISPR/Cas9) systems. These chimeric nucleases enable efficient and precise genetic modifications by inducing targeted DNA double-strand breaks that stimulate the cellular DNA repair mechanisms, including nonhomologous end joining (NHEJ) and homology-directed repair (HDR). Until recently, for example, the homologous recombination efficiency in human ES/iPS cells was quite low, but it has greatly improved by using these nucleases. In addition, several genes and chemical compounds with the potential to enhance the genome editing efficiency of human ES/iPS cells have been discovered, making the genome editing of human ES/iPS cells easier than ever. As a result, many researchers have performed genome editing experiment in IMD-iPS cells (Table 2).

To the best of our knowledge, targeted gene correction of AATD-iPS cells was first reported by Yusa et al. By using ZFN, they corrected a point mutation (Glu342Lys) in the AAT locus that is responsible for Z-AAT production. Enzyme-linked immunosorbent assay (ELISA) analysis revealed the absence of mutant polymeric Z-AAT and efficient secretion of normal monomeric AAT in the culture supernatant of corrected AATD-iPS-HLCs. Choi et al. later performed a similar gene correction experiment using TALEN instead of ZFN at higher efficiency (the efficiencies were 4 and 25–33% in Yusa et al. and Choi et al., respectively). Additionally, CRISPR/
Cas9 system-mediated gene correction of AATD-iPS cells has also been reported.\(^{59}\) Recently, Segeritz et al. conducted functional and "omics" comparisons between AATD-iPS-HLCs and genetically corrected AATD-iPS-HLCs to identify new molecular markers and disease signatures.\(^{65}\) They showed that Z-AAT polymer processing was associated with disrupted mitochondrial structure, presence of the oncogenic protein AKR1B10 and two upregulated molecular clusters centered on members of the inflammatory and unfolded protein response pathways. These approaches would be useful for identification of new therapeutic targets for the treatment of AATD.

In addition to correction of AATD-iPS cells, genome editing technology contributes to the functional correction of FH-iPS-HLCs. Omer et al. used a CRISPR/Cas9 system to perform gene correction of iPS cells derived from an HoFH patient (HoFH-iPS cells)\(^{61}\) with a 3-base pair homozygous deletion in exon 4 of the \(LDLR\) gene, which results in <5% \(LDLR\) activity.\(^{65}\) They adopted a double-nicking strategy using Cas9 nickase (Cas9n). The double-nicking strategy is a novel approach that combines a Cas9n with paired guide RNAs to introduce targeted double-strand breaks.\(^{67}\) This strategy has been proven effective for reducing off-target mutations.\(^{68}\) As expected, sequencing of gene-corrected HoFH-iPS cells compared to non-corrected cells revealed no changes in the 6 selected regions of the genome that had the highest potential for off-targeting.\(^{61}\) Thereafter, they demonstrated that CRISPR-mediated genetic correction could be successfully used to recover the function of cholesterol metabolism in HoFH-iPS cell-derived HLCs. Finally, in 2017, Pashos et al. used iPS cell and genome editing technologies to examine the function of lipid-associated genetic mutations that were discovered by a genome-wide association study (GWAS) and were potentially responsible for FH.\(^{69}\)

Collectively, the above findings clearly demonstrate that functional correction of patient iPS-HLCs can be achieved by using genome editing technology (Fig. 2). In addition, a comparison between gene-corrected cells and non-corrected cells could lead to the discovery of new therapeutic target molecules. Moreover, empirical analysis based on patient iPS cells and genome editing technology would accelerate the identification of disease-related genetic mutations.

### Table 2. Genome Editing Investigations Using iPS Cell Lines from Patients with Inherited Metabolic Disorders of the Liver

| Year | Journal | Authors | Disease | Ref. |
|------|---------|---------|---------|------|
| 2011 | Nature  | Zhang et al. | Alpha-1 antitrypsin deficiency | 55 |
| 2011 | Human Molecular Genetics | Fattahi et al. | Familial hypercholesterolemia | 56 |
| 2013 | Molecular Biotechnology | Choi et al. | Alpha-1 antitrypsin deficiency | 32 |
| 2014 | Stem Cell Reports | Maetzel et al. | Niemann-Pick disease type C | 57 |
| 2014 | Proceedings of the National Academy of Sciences of the United States of America | Park et al. | Hemophilia A | 58 |
| 2015 | Molecular Therapy | Smith et al. | Alpha-1 antitrypsin deficiency | 59 |
| 2016 | Molecular Therapy–Nucleic Acids | Lee et al. | Hyperargininemia | 60 |
| 2017 | Hepatology Communications | Omer et al. | Familial hypercholesterolemia | 61 |
| 2017 | Cell Reports | Liu et al. | Abetalipoproteinemia | 62 |
| 2018 | Stem Cell Research & Therapy | Lyu et al. | Hemophilia B | 63 |
| 2018 | Cell Reports | Ramaswamy et al. | Hemophilia B | 64 |
| 2018 | Journal of Hepatology | Segeritz et al. | Alpha-1 antitrypsin deficiency | 65 |

Fig. 2. Pharmaceutical Research Using a Combination of Human iPS Cell and Genome Editing Technologies

(Colors figure can be accessed in the online version.)

4. CONCLUSION

Although we mainly discussed IMDs which show hepatocyte dysfunction, there are also some IMDs caused by dysfunction in liver nonparenchymal cells. It was recently reported that hepatic satellite cells can be generated from human iPS cells.\(^{70,71}\) This technology would be useful for modeling the hepatic fibrosis in IMDs such as congenital hepatic fibrosis (CHF). Several IMDs exert symptoms not only in the liver, but also in other organs. For example, Niemann-Pick disease type C patients suffer from splenomegaly and neurologic disorders in addition to liver disorder. For modeling of such IMDs, characterization of multiple cell types will be necessary.

The disease modeling and drug screening for IMDs can be successfully performed by using patient iPS cells. In addition, functional correction and comparison studies have been progressing steadily by introducing genome editing technology into IMD-iPS cells. We hope that these technologies will reveal new therapeutic drugs for IMDs that cannot be treated using existing drugs.
Conflict of Interest  The authors declare no conflict of interest.

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