Current Topics

Recent Advances in Research on Particulate Formulations such as Lipoproteins, Liposomes, Extracellular Vesicles, and iPS-Derived Cells

Review

Generation of Human Induced Pluripotent Stem Cell-Derived Hepatocyte-Like Cells for Cellular Medicine

Sayaka Deguchi,^a Kazuo Takayama,^{*a,b,c} and Hiroyuki Mizuguchi^{*a,c,d,e}

^aLaboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University; Osaka 565–0871, Japan: ^bPRESTO, Japan Science and Technology Agency; Saitama 332–0012, Japan: ^cLaboratory of Hepatocyte Regulation, National Institutes of Biomedical Innovation, Health and Nutrition; Osaka 567–0085, Japan: ^dGlobal Center for Medical Engineering and Informatics, Osaka University; Osaka 565–0871, Japan: and ^eIntegrated Frontier Research for Medical Science Division, Institute for Open and Transdisciplinary Research Initiatives (OTRI), Osaka University; Osaka 565–0871, Japan.

Received August 31, 2019

Liver transplantation and hepatocyte transplantation are effective treatments for severe liver injuries, but the donor shortage is a serious problem. Therefore, hepatocyte-like cells generated from human induced pluripotent stem (iPS) cells with unlimited proliferative ability are expected to be a promising new transplantation resource. The technology for hepatic differentiation from human iPS cells has made great progress in this decade. The efficiency of hepatic differentiation now exceeds 90%, making it possible to produce nearly homogeneous hepatocyte-like cells from human iPS cells. Because there is little contamination of undifferentiated cells, there is a lower risk of teratoma formation. To date, the transplantation of human iPS cell-derived hepatocyte-like cells has been shown to have therapeutic effects using various liver injury model mice. Currently, studies are underway using model animals larger than mice. The day when human iPS cell-derived hepatocyte-like cells can be used as cellular medicine is surely approaching. In this review, we introduce the forefront of regenerative medicine applications using human iPS cell-derived hepatocyte-like cells.

Key words human induced pluripotent stem cell; organoid; differentiation; liver transplantation; hepatocyte transplantation

1. INTRODUCTION

Liver transplantation is one of the effective treatments for end-stage liver injuries. Although the 5-year survival rate after liver transplantation is high (70% or greater), the lack of donors is a serious problem (Fig. 1, left). In the U.S.A., 13147 people were newly added to the liver transplantation waiting list (<https://optn.transplant.hrsa.gov/>) in 2018, but only 8250 liver transplants were performed. The number of people waiting for liver transplants is increasing. Therefore, hepatocyte transplantation is expected as a “bridge” that could prevent the progression of liver injury in patients while waiting for liver transplantation (Fig. 1, right).

In general, human hepatocytes obtained from patients without liver diseases are transplanted to recipient patients *via* a portal vein. It was reported that cryopreserved human hepatocytes (approximately 7.0×10^7 cells) were transplanted twice to an 11-d-old infant with ornithine transcarbamylase deficiency who was too young to receive liver transplantation.¹⁾ By performing hepatocyte transplantation, the serum ammonia concentration decreased from 1940 to 40 $\mu\text{g/dL}$. In other case, isolated human hepatocytes (8.8×10^8 cells) were transplanted to a 37-year-old woman with fulminant liver failure.²⁾ After the transplantation, the bilirubin level and factor VII coagulation activity normalized.

Although hepatocyte transplantation is effective in treating liver injury, there is an important issue to be solved. It is difficult to stably obtain the large amounts of functional human hepatocytes necessary for hepatocyte transplantation. This is partly because most surplus hepatocytes are obtained from the outer edge of the liver.³⁾ To solve this problem, human induced pluripotent stem cell-derived hepatocyte-like cells (human iPS cell-derived HLCs) are expected to become a new cell source for hepatocyte transplantation.

The supply of human iPS cell-derived HLCs is unlimited, and they are excellent tools with functions similar to those of human hepatocytes, as described in the next section. In this review, we describe ongoing efforts to apply human iPS cell-derived HLCs to regenerative medicines such as hepatocyte transplantation (or liver organoid transplantation).

2. DIFFERENTIATION OF HUMAN iPS CELL-DERIVED HEPATOCYTE-LIKE CELLS AND LIVER ORGANOIDS

Differentiation protocols from human iPS cells into HLCs have been developed in various laboratories (Fig. 2). In general, HLCs are differentiated from human iPS cells *via* definitive endoderm and hepatoblast-like cells by adding humoral factors to mimic liver development.^{4–6)} Si-Tayeb *et al.* succeeded

*To whom correspondence should be addressed. e-mail: takayama@phs.osaka-u.ac.jp; mizuguchi@phs.osaka-u.ac.jp

Fig. 1. Liver Transplantation and Hepatocyte Transplantation

Liver transplantation is an effective treatment for liver injuries. However, the shortage of donor livers is a serious problem. In order to delay the progression of liver injury while waiting for liver transplantation, hepatocyte transplantation is conducted. As a new cell source for hepatocytes transplantation, human iPS cell-derived hepatocytes are expected to be useful because of their capacity for unlimited self-renewal. (This figure was created using Biorender <<https://biorender.com>>.) (Color figure can be accessed in the online version.)

in producing human iPS cell-derived HLCs by stepwise addition of activin A, bone morphogenetic protein 4 (BMP-4), fibroblast growth factor 2 (FGF-2), hepatocyte growth factor (HGF), and oncostatin M.⁷ The percentage of albumin (ALB)-positive cells was greater than 80%, suggesting that nearly homogenous HLCs can be generated using their differentiation protocol. Takayama *et al.* also developed efficient hepatic differentiation protocols by transducing hepatic transcription factors such as *SRY-box transcription factor 17* (SOX17), *hematopoietically expressed homeobox* (HEX), *hepatocyte nuclear factor 1 alpha* (HNF1 α), and HNF4 α .^{8–10} To achieve efficient gene transduction into human iPS cell-derivatives, they used fiber-modified adenovirus vectors. As a result, the percentage of ALB-positive cells increased to 90%. In addition, urea secretion, low density lipoprotein (LDL) uptake capacity, and CYP induction potency were observed. It is known that a three-dimensional cell microenvironment is important for hepatocyte homeostasis. Nagamoto *et al.* reported that the gene expression levels of *ALB* and *CYPs* in human iPS cell-derived HLCs were increased by overlaying a Swiss 3T3 cell sheet.¹¹ Takayama *et al.* also reported that the ALB secretion level and CYPs activities in the human iPS cell-derived HLCs were improved by using a three-dimensional micro patterned culture plate.¹² Those results suggest that the hepatic functions of human iPS cell-derived HLCs could be enhanced by mimicking the three-dimensional cell microenvironment.

Recently, technology for the generation of human organoids has also been developed. Takebe *et al.* reported that human liver organoids can be generated by co-culturing human iPS cell-derived hepatic endoderm cells with human umbilical vein endothelial cells (HUVECs) and human mesenchymal stem cells (MSCs) (Fig. 2). They transplanted human liver organoids into a cranial window of a nonobese diabetic/severe combined immunodeficient (NOD/SCID) mouse, and confirmed the formation of tube structures connecting with host vessels.^{13–15} They succeeded in generating a three-dimensionally vascularized liver.

However, in order to conduct safe and effective transplan-

Fig. 2. Differentiation of Human iPS Cell-Derived Hepatocyte-Like Cells and Liver Organoids

The differentiation methods from human iPS cells into hepatocyte-like cells have been developed in various laboratories. Recently, liver organoid technology has also been developed. (This figure was created using Biorender <<https://biorender.com>>.) (Color figure can be accessed in the online version.)

tation of human iPS cell-derived HLCs in clinical practice, a system for detecting harmful cells among human iPS cell-derived HLCs must be developed.

3. SAFETY EVALUATION OF HUMAN iPS CELL-DERIVED HEPATOCYTE-LIKE CELLS

There are some risks in the transplantation of human iPS

Fig. 3. Safety Evaluation Systems to Transplant Human iPS Cell-Derivatives

There are risks of immune rejection and tumorigenicity in the transplantation of human iPS cell-derivatives. Therefore, safety evaluation systems of human iPS cell-derivatives are required. Approaches to minimize the risks of transplantations are summarized. (This figure was created using Biorender <<https://biorender.com>>.) (Color figure can be accessed in the online version.)

cell-derivatives. Human iPS cells were established from somatic cells using reprogramming factor-expressing viral vectors. However, transgenes are sometimes inserted into the host genome. Reactivation of transgene expression would increase the risk of human iPS cell-derivatives forming a tumor. In addition, immune rejection may also occur in allotransplantation of human iPS cell-derivatives. Moreover, residual undifferentiated cells among the transplanted cells could pose a risk of teratoma formation. Therefore, a safety evaluation system of human iPS cell-derivatives is necessary (Fig. 3).

When human iPS cells were established from human dermal fibroblasts for the first time, transduction of reprogramming factors was performed using a retroviral vector.¹⁶⁾ However, that vector would insert transgenes into the host genome. In order to avoid the risk, an episomal vector is now widely used for establishing human iPS cells. For example, Okita *et al.* adopted oriP/EBNA-1-based plasmid vectors that can express transgenes for a long time without transgene integration.^{17,18)} Gene expression, methylation, and karyotype analyses showed that human iPS cells generated by episomal vectors were similar to human embryonic stem (ES) cells. Thus, it is considered that human iPS cells established using episomal vectors are preferable for application to regenerative medicine.

“HLA homozygous haplobanks” are expected to be an effective approach to avoid immune rejection driven by HLA mismatches between human iPS cell-derivatives and recipients.¹⁹⁾ HLA homozygous iPS cells are stocked so that they can be provided immediately to HLA-compatible recipients. It is estimated that approximately 140 unique HLA homozygous types would be needed to cover 90% of the Japanese population.¹⁷⁾ However, to collect such a large number of

HLA homozygous types, 150000 donors would be needed. Therefore, researchers are pursuing several other approaches to avoid immune rejection. Morizane *et al.* transplanted iPS cell-derived neurons into the brains of cynomolgus macaques under the treatment of immunosuppressant.²⁰⁾ They showed that the administration of tacrolimus suppressed immune rejection to the same extent as major histocompatibility complex (MHC)-matched iPS cell-derivatives. Recently, genome editing technologies, such as clustered regularly interspaced short palindromic repeats/CRISPR associated protein (CRISPR-Cas9) systems have been used to expand the applications of human iPS cells.^{21–23)} By using genome editing technologies, “universal cells” that are invisible to the host immune system have been established.²⁴⁾ For example, Xu *et al.* generated two types of HLA-edited human iPS cells that can escape from the host immune surveillance.²⁵⁾ They showed that HLA-edited iPS cell-derived blood cells could survive after transplantation despite the co-transplantation of allogenic CD8⁺ T cells, while wild type iPS cell-derived blood cells were almost completely eliminated. From the above, it is suggested that immune rejection can be avoided not only by using HLA type matched human iPS cells, but also by using appropriate immunosuppressant and genome editing techniques.

It is also necessary to evaluate the risks of tumorigenicity of residual undifferentiated cells. In order to detect residual undifferentiated cells, flow cytometry, real-time RT-PCR, and *in vivo* tumorigenicity test are performed. The contaminating human iPS cells can be detected up to 0.1% by flow cytometry analysis of TRA-1-60-positive cells, and can also be detected up to 0.001% by droplet digital PCR analysis of *LIN28A* mRNA.^{26,27)} The *in vivo* tumorigenicity test is the most widely performed test to evaluate the risk of teratoma formation after

transplantation of human iPS cell-derivatives. It was reported that 1×10^4 contaminating human ES cells in 5×10^5 human ES cell-derived retinal pigment epithelial cells generated teratoma in immunodeficient mice.²⁸⁾ On the other hand, Kanemura *et al.* showed that 1×10^3 undifferentiated iPS cells could form teratoma in immunodeficient mice.²⁹⁾ Because teratoma formation efficiency largely depends on the type of immunodeficient mice and transplantation method, Kusakawa *et al.* attempted to construct an *in vivo* tumorigenicity test with high sensitivity. They revealed that a combination of NOD/Shi-scid IL2R γ^{null} (NOG) mice and matrigel was highly sensitive for the detection of 1×10^2 tumorigenic cells.³⁰⁾ From the above results, it is clearly important to detect residual undifferentiated cells using a sufficiently sensitive method. In addition, some researchers have eliminated residual undifferentiated cells in human iPS cell-derivatives to avoid the risks of teratoma formation. Mitsui *et al.* developed conditionally replicating adenoviruses that exhibit efficient viral replication and cytotoxicity in undifferentiated cells, but not in differentiated normal cells.³¹⁾ Recently, Kang *et al.* found that YM155, a surviving inhibitor, can eliminate undifferentiated human iPS cells.³²⁾ They demonstrated that residual undifferentiated cells in human iPS cell-derived HLCs completely disappeared when treated with YM155 and teratoma formation was effectively prevented by YM155 pretreatment. Therefore, in order to achieve safe transplantation of human iPS cell-derivatives, an

effective method for detecting and reducing contamination of residual undifferentiated cells is necessary.

At present, the development of human iPS cell-derived HLCs aimed at clinical transplantation has not progressed sufficiently, but it is expected that safe and effective transplantation of human iPS cell-derived HLCs will be achieved in the near future. Because clinical applications would need to transplant large amounts of human iPS cell-derived HLCs, research to develop more sensitive safety tests for human iPS cell-derivatives should be continued.

4. TRANSPLANTATION OF HUMAN iPS CELL-DERIVED HEPATOCYTE-LIKE CELLS FOR LIVER INJURY

To examine whether human iPS cell-derived HLCs would be therapeutically effective in the treatment of liver injury, many researchers have attempted to transplant human iPS cell-derived HLCs into liver injury model mice. The hepatocyte transplantation methods are classified into orthotopic and heterotopic transplantations. In this section, we introduce several studies that succeeded in curing liver injury model mice using human iPS cell-derived HLCs (Table 1).

In order to engraft transplanted hepatocytes in the liver, intravenous injection, intrasplenic transplantation, and sheet transplantation have been conducted. Asgari *et al.* reported

Table 1. Treatment of Liver Injuries with Transplantation of Human iPS Cell-Derived Hepatocyte-Like Cells And Liver Organoids

	Year	Cells	Mice	Transplantation method	Results	Ref.
Orthotopic transplantation	2011	Human iPS cell-derived definitive endoderm cells, hepatoblast-like cells and HLCs	Chronic liver injury was induced by treating for 4 weeks with dimethylnitrosamine	Intravenous injection	Survival rate increased.	35)
	2013	Human iPS cell-derived HLCs	Liver fibrosis was induced by injections of 1.0 mL/kg of CCl ₄ twice weekly for 4 weeks	Intravenous injection	Survival rate increased, serum total LDH and bilirubin levels decreased.	33)
	2016	Human iPS cell-derived HLCs (cell sheet)	Acute liver injury was induced by inoculation of 3 mL/kg CCl ₄ 1 d before transplantation	Cell sheet transplantation	Survival rate increased, serum AST and ALT levels decreased.	36)
	2017	Human iPS cell-derived HLCs	Acute liver injury was induced by inoculation of 3 mL/kg CCl ₄ 1 d before transplantation	Intrasplenic transplantation	Survival rate increased.	34)
	2017	Human iPS cell-derived HLCs	Chronic liver injury was induced by inoculation of 0.6 mL/kg CCl ₄ twice weekly for 8 weeks	Intrasplenic transplantation	Gene expression levels of liver fibrosis markers decreased.	34)
	2018	Mouse iPS cell-derived HLCs	Arginase-1-deficient mice	Intrasplenic transplantation	Survival time extended.	37)
Heterotopic transplantation	2013	Human iPS cell-derived liver organoids	Ganciclovir (50 mg/kg) treated TK-NOG mice	Transplantation in mesentery	Survival rate increased.	13)
	2018	Human iPS cell-derived liver organoids	Acute liver injury was induced by administration of 1.5 μ g/kg diphtheria toxin	Subrenal capsule space transplantation	Survival rate increased, serum AST and ALT levels decreased.	38)
	2018	Human 3D-cultured iPS cell-derived HLCs	50% partial hepatectomized mice	Intraperitoneal transplantation	Body weight recovered to initial level.	39)
	2018	Human ES cell-derived HLC fibre	Fumarylacetate hydrolase (Fah $^{-/-}$) mice	Subcutaneous transplantation	Body weight loss ceased, expression levels of liver damage markers decreased.	39)
	2018	Human iPS cell-derived HLCs	Hemophilia B model mice	Subrenal capsule space transplantation	FIX activity levels improved, bleeding times decreased.	40)

that the survival rate increased from 60% to 100% and total serum LDH and bilirubin levels decreased in chronic liver injury mice after transplantation of human iPS cell-derived HLCs *via* tail vein injection.³³⁾ Takayama *et al.* showed that the survival rates of acute liver injury mice were increased from 0 to 41.6% by intrasplenic transplantation of human iPS cell-derived HLCs.³⁴⁾ In addition, Liu *et al.* performed transplantation of human iPS cell-derivatives *via* intravenous injection, and reported that not only hepatocyte transplantation but also transplantation of progenitor cells such as definitive endoderm cells and hepatoblast-like cells improved the survival rate of chronic liver injury mice.³⁵⁾ Nagamoto *et al.* performed sheet transplantation of human iPS cell-derived HLCs into acute liver injury mice.³⁶⁾ Two-layers of human iPS cell-derived HLC sheets were directly attached on the surface of mouse livers. They found that the survival rate of the sheet transplanted mice (approximately 60%) was higher than that of the intrasplenically transplanted mice (approximately 20%). Orthotopic hepatocyte transplantation has also been shown to rescue mice from metabolic liver injury. *Arginase-1*-deficient mice lacking exons 7 and 8 of this gene exhibit a lethal phenotype approximately 2 weeks after birth.³⁷⁾ The genetic error of iPS cells derived from *Arginase-1*-deficient mice was corrected using genome editing technology. The genetically edited cells were differentiated into HLCs *in vitro* and were subsequently used for transplantation into the *Arginase-1*-deficient mouse model. Survival time was prolonged after this transplantation. Therefore, orthotopic transplantation of human iPS cell-derived HLCs would be therapeutically effective for various types of liver injuries, such as acute, chronic and metabolic liver injury.

Despite the above-described attempts at orthotopic transplantation of hepatocytes, the engraftment efficiency is still not sufficiently high. Therefore, heterotopic transplantation of hepatocytes has also been performed with the aim of improving engraftment efficiency. As heterotopic transplantations, hepatocyte transplantation into cranial windows, mesenteric site, and kidney capsules have been conducted.¹⁴⁾ Transplantation of human iPS cell-derived liver organoids improved the survival rate of transgenic NOG mice expressing *herpes simplex virus type 1 thymidine kinase (HSVtk)* transgene in the liver (TK-NOG mice) compared with sham-operated mice.¹³⁾ Nie *et al.* also showed that the serum alanine transaminase (ALT) and aspartate transaminase (AST) levels in acute liver injury mice decreased after the subrenal transplantation of human iPS cell-derived liver organoids.³⁸⁾ Furthermore, Rashidi *et al.* reported that subcutaneous transplantation of human ES cell-derived HLCs can support failing liver function *in vivo*.³⁹⁾ In their experiments, human ES cell-derived HLCs were seeded on a polycaprolactone (PCL) scaffold and attached to the fibers. The large PCL fibers were subcutaneously transplanted into *fumarylacetoacetate hydrolase*-deficient (*Fah*^{-/-}) mice. After transplantation, weight loss and abnormal accumulation of lipid droplets ceased. It was also reported that heterotopic transplantation of human iPS cell-derived HLCs can rescue mice from genetic liver injury such as hemophilia B, also known as coagulation factor IX deficiency.⁴⁰⁾ After the transplantation of human iPS cell-derived HLCs under the subrenal capsule space, the gene expression level, plasma concentration, and activity of coagulation factor IX increased and the bleeding time of tail-clipped mice significantly decreased.

Therefore, not only orthotopic transplantation but also heterotopic transplantation are therapeutically effective for various liver injuries. In cases in which orthotopic liver transplantation cannot be performed, heterotopic liver transplantation may be an effective treatment.

As described above, transplantation of human iPS cell-derived HLCs was shown to exert a therapeutic effect in liver injury model mice. However, this therapeutic effect remains somewhat limited. This might be due to the low engraftment efficiency of hepatocytes or immaturity of human iPS cell-derived HLCs. Therefore, in order to treat liver diseases using human iPS cell-derived HLCs in the clinical setting, it is essential to improve their transplantation method and hepatic functions.

5. TISSUE ENGINEERING USING HUMAN iPS CELL-DERIVED HEPATOCYTE-LIKE CELLS

The previous section introduced several studies in which liver injuries were treated by the transplantation of human iPS cell-derived HLCs. Transplantation is an effective treatment for liver injury, but it is considered that human iPS cell-derived HLCs can also be used *ex vivo*. A device that can reproduce liver functions *ex vivo* has been developed. It is expected that serum proteins and other impurities such as ammonia can be detoxified using the device. This section introduces a bio-artificial liver (BAL) and a decellularized liver scaffold using human iPS cell-derived HLCs.

The BAL is a bioreactor that incorporates hepatocytes. Most of the BAL consists of hollow fibers made of semipermeable membranes.^{41,42)} After perfusing the patient plasma through the BAL, the incorporated hepatocytes remove the impurities from the plasma. It was reported that a BAL constructed of hollow fibers and porcine hepatocytes can degrade ammonia and galactose.⁴³⁾ Additionally, Mazariegos *et al.* treated patients with fulminant liver failure with a BAL constructed of hollow fibers and primary porcine hepatocytes.⁴⁴⁾ After perfusing the patient's whole blood through the BAL, approximately 80% of the ammonia and lactate were removed. However, it is difficult to maintain the therapeutic effects because the hepatocytes gradually lose their hepatic functions during long-term culture in the BAL. To maintain the hepatic functions of hepatocytes at a high level, it is necessary to develop a device that more closely reflects the *in vivo* extracellular environment (such as the extracellular matrix (ECM)).

Decellularized scaffolds have been developed as a tool that can supply the ECM necessary for maintaining the hepatic functions of hepatocytes. Decellularized scaffolds can be created by treating the liver with surfactants. Because a decellularized scaffold preserves the three-dimensional structure composed of the ECM, it is expected to be a suitable extracellular environment for long-term culture of hepatocytes. Uygun *et al.* decellularized rat liver by refluxing the sodium dodecyl sulfate (SDS) and recellularized liver scaffolds with primary rat hepatocytes.⁴⁵⁾ The decellularized liver scaffold preserved the hepatic lobular structure and vascular bed. The ALB secretion and urea synthesis capacities of the recellularized liver scaffold were similar to those of normal liver. In addition, the recellularized liver scaffold could be transplanted into rats, supporting hepatocyte survival and function with minimal

ischemic damage. Yagi *et al.* decellularized adult porcine livers to generate human-sized liver scaffolds.⁴⁶⁾ The livers were decellularized by refluxing dispase and collagenase, and then the scaffolds were recellularized with primary porcine hepatocytes. The recellularized liver scaffold showed ALB and urea synthesis capacities. Therefore, a decellularized liver scaffold might be a desirable environment for maintaining the hepatic functions of isolated hepatocytes *ex vivo*.

In order to implement these technologies in clinical practice, the scaling up of liver scaffolds and utilization of human hepatocytes are required. Therefore, human iPS cell-derived HLCs, which can be supplied on a large-scale, are expected to be an ideal cell source. Some researchers succeeded in generating a BAL consisting of decellularized liver scaffolds recellularized with human iPS cell-derived HLCs. The expression of hepatic markers such as ALB could be maintained in these devices for a long time.^{47–49)} Although the therapeutic effects of these devices have not been verified, it is expected that BALs using human iPS cell-derived HLCs will be applied clinically for the treatment of liver diseases by improving the production technology and quality control methods of BAL.

Recently, Collin de l'Hortet *et al.* have repopulated decellularized rat livers with genetically modified human iPS cell-derived HLCs and non-parenchymal cells to generate a model of human livers with non-alcoholic fatty liver disease (NAFLD).⁵⁰⁾ To reproduce the NAFLD pathology, *Sirtuin 1* (*SIRT1*) expression was knocked down in their human iPS cell-derived HLCs because metabolic derangements in hepatocytes are known to be regulated by *SIRT1*. They found that their human iPS cell-derived liver tissue developed macrosteatosis, acquired the proinflammatory phenotype, and shared a similar lipid and metabolic profile with human fatty liver. It is suggested that BAL would also be a useful tool for investigating human liver biology and disease.

6. CONCLUSION

In order to utilize human iPS cell-derived HLCs (and liver organoids) for regenerative medicine in the clinical setting, the hepatic differentiation methods, safety evaluation systems, and transplantation methods must be improved. As shown in this review, technological developments are underway by many research teams. We hope that those studies will achieve major advances, and that human iPS cell-derived HLCs (and liver organoids) will become the effective treatments for liver injuries.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

- Enosawa S, Horikawa R, Yamamoto A, Sakamoto S, Shigeta T, Nosaka S, Fujimoto J, Nakazawa A, Tanoue A, Nakamura K, Umezawa A, Matsubara Y, Matsui A, Kasahara M. Hepatocyte transplantation using a living donor reduced graft in a baby with ornithine transcarbamylase deficiency: a novel source of hepatocytes. *Liver Transpl.*, **20**, 391–393 (2014).
- Fisher RA, Bu D, Thompson M, Tisnado J, Prasad U, Sterling R, Posner M, Strom S. Defining hepatocellular chimerism in a liver failure patient bridged with hepatocyte infusion. *Transplantation*, **69**, 303–307 (2000).
- Mitry RR, Hughes RD, Aw MM, Terry C, Mieli-Vergani G, Girlanda R, Muiesan P, Rela M, Heaton ND, Dhawan A. Human hepatocyte isolation and relationship of cell viability to early graft function. *Cell Transplant.*, **12**, 69–74 (2003).
- Shiraki N, Umeda K, Sakashita N, Takeya M, Kume K, Kume S. Differentiation of mouse and human embryonic stem cells into hepatic lineages. *Genes Cells*, **13**, 731–746 (2008).
- Touboul T, Hannan NRF, Corbineau S, Martinez A, Martinet C, Branchereau S, Mainot S, Strick-Marchand H, Pedersen R, Di Santo J, Weber A, Vallier L. Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. *Hepatology*, **51**, 1754–1765 (2010).
- Toba Y, Kiso A, Nakamae S, Sakurai F, Takayama K, Mizuguchi H. FGF signal is not required for hepatoblast differentiation of human iPS cells. *Sci. Rep.*, **9**, 3713 (2019).
- Si-Tayeb K, Noto FK, Nagaoka M, Li J, Battle MA, Duris C, North PE, Dalton S, Duncan SA. Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. *Hepatology*, **51**, 297–305 (2010).
- Takayama K, Inamura M, Kawabata K, Tashiro K, Katayama K, Sakurai F, Hayakawa T, Furue MK, Mizuguchi H. Efficient and directive generation of two distinct endoderm lineages from human ESCs and iPSCs by differentiation stage-specific SOX17 transduction. *PLoS ONE*, **6**, e21780 (2011).
- Takayama K, Inamura M, Kawabata K, Katayama K, Higuchi M, Mizuguchi H. Efficient generation of functional hepatocytes from human embryonic stem cells and induced pluripotent stem cells by HNF4 α transduction. *Mol. Ther.*, **20**, 127–137 (2012).
- Takayama K, Inamura M, Kawabata K, Sugawara M, Kikuchi K, Higuchi M, Nagamoto Y, Watanabe H, Tashiro K, Sakurai F, Hayakawa T, Furue MK, Mizuguchi H. Generation of metabolically functioning hepatocytes from human pluripotent stem cells by FOXA2 and HNF1 α transduction. *J. Hepatol.*, **57**, 628–636 (2012).
- Nagamoto Y, Tashiro K, Takayama K, Ohashi K, Kawabata K, Sakurai F, Tachibana M, Hayakawa T, Furue MK, Mizuguchi H. The promotion of hepatic maturation of human pluripotent stem cells in 3D co-culture using type I collagen and Swiss 3T3 cell sheets. *Biomaterials*, **33**, 4526–4534 (2012).
- Takayama K, Kawabata K, Nagamoto Y, Kishimoto K, Tashiro K, Sakurai F, Tachibana M, Kanda K, Hayakawa T, Furue MK, Mizuguchi H. 3D spheroid culture of hESC/hiPSC-derived hepatocyte-like cells for drug toxicity testing. *Biomaterials*, **34**, 1781–1789 (2013).
- Takebe T, Sekine K, Enomura M, Koike H, Kimura M, Ogaeri T, Zhang R-R, Ueno Y, Zheng Y-W, Koike N, Aoyama S, Adachi Y, Taniguchi H. Vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature*, **499**, 481–484 (2013).
- Takebe T, Zhang R-R, Koike H, Kimura M, Yoshizawa E, Enomura M, Koike N, Sekine K, Taniguchi H. Generation of a vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nat. Protoc.*, **9**, 396–409 (2014).
- Takebe T, Enomura M, Yoshizawa E, Kimura M, Koike H, Ueno Y, Matsuzaki T, Yamazaki T, Toyohara T, Osafune K, Nakauchi H, Yoshikawa HY, Taniguchi H. Vascularized and complex organ buds from diverse tissues via mesenchymal cell-driven condensation. *Cell Stem Cell*, **16**, 556–565 (2015).
- Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*, **131**, 861–872 (2007).
- Okita K, Matsumura Y, Sato Y, Okada A, Morizane A, Okamoto S, Hong H, Nakagawa M, Tanabe K, Tezuka K, Shibata T, Kunisada T, Takahashi M, Takahashi J, Saji H, Yamanaka S. A more efficient method to generate integration-free human iPS cells. *Nat. Methods*, **8**, 409–412 (2011).

- 18) Okita K, Yamakawa T, Matsumura Y, Sato Y, Amano N, Watanabe A, Goshima N, Yamanaka S. An efficient nonviral method to generate integration-free human-induced pluripotent stem cells from cord blood and peripheral blood cells. *Stem Cells*, **31**, 458–466 (2013).
- 19) Turner M, Leslie S, Martin NG, Peschanski M, Rao M, Taylor CJ, Trounson A, Turner D, Yamanaka S, Wilmut I. Toward the development of a global induced pluripotent stem cell library. *Cell Stem Cell*, **13**, 382–384 (2013).
- 20) Morizane A, Kikuchi T, Hayashi T, Mizuma H, Takara S, Doi H, Mawatari A, Glasser MF, Shiina T, Ishigaki H, Itoh Y, Okita K, Yamasaki E, Doi D, Onoe H, Ogasawara K, Yamanaka S, Takahashi J. MHC matching improves engraftment of iPSC-derived neurons in non-human primates. *Nat. Commun.*, **8**, 385 (2017).
- 21) Takayama K, Igai K, Hagihara Y, Hashimoto R, Hanawa M, Sakuma T, Tachibana M, Sakurai F, Yamamoto T, Mizuguchi H. Highly efficient biallelic genome editing of human ES/iPS cells using a CRISPR/Cas9 or TALEN system. *Nucleic Acids Res.*, **45**, 5198–5207 (2017).
- 22) Takayama K, Hagihara Y, Toba Y, Sekiguchi K, Sakurai F, Mizuguchi H. Enrichment of high-functioning human iPS cell-derived hepatocyte-like cells for pharmaceutical research. *Biomaterials*, **161**, 24–32 (2018).
- 23) Deguchi S, Yamashita T, Igai K, Harada K, Toba Y, Hirata K, Takayama K, Mizuguchi H. Modeling of hepatic drug metabolism and responses in CYP2C19 poor metabolizer using genetically manipulated human iPS cells. *Drug Metab. Dispos.*, **47**, 632–638 (2019).
- 24) Lanza R, Russell DW, Nagy A. Engineering universal cells that evade immune detection. *Nat. Rev. Immunol.*, **19**, 723–733 (2019).
- 25) Xu H, Wang B, Ono M, Kagita A, Fujii K, Sasakawa N, Ueda T, Gee P, Nishikawa M, Nomura M, Kitaoka F, Takahashi T, Okita K, Yoshida Y, Kaneko S, Hotta A. Targeted disruption of HLA genes via CRISPR-Cas9 generates iPSCs with enhanced immune compatibility. *Cell Stem Cell*, **24**, 566–578.e7 (2019).
- 26) Kuroda T, Yasuda S, Kusakawa S, Hirata N, Kanda Y, Suzuki K, Takahashi M, Nishikawa S, Kawamata S, Sato Y. Highly sensitive *in vitro* methods for detection of residual undifferentiated cells in retinal pigment epithelial cells derived from human iPS cells. *PLOS ONE*, **7**, e37342 (2012).
- 27) Kuroda T, Yasuda S, Matsuyama S, Tano K, Kusakawa S, Sawa Y, Kawamata S, Sato Y. Highly sensitive droplet digital PCR method for detection of residual undifferentiated cells in cardiomyocytes derived from human pluripotent stem cells. *Regen. Ther.*, **2**, 17–23 (2015).
- 28) Gropp M, Shilo V, Vainer G, Gov M, Gil Y, Khaner H, Matzrafi L, Idelson M, Kopolovic J, Zak NB, Reubinoff BE. Standardization of the teratoma assay for analysis of pluripotency of human ES cells and biosafety of their differentiated progeny. *PLOS ONE*, **7**, e45532 (2012).
- 29) Kanemura H, Go MJ, Nishishita N, Sakai N, Kamao H, Sato Y, Takahashi M, Kawamata S. Pigment epithelium-derived factor secreted from retinal pigment epithelium facilitates apoptotic cell death of iPSC. *Sci. Rep.*, **3**, 2334 (2013).
- 30) Kusakawa S, Machida K, Yasuda S, Takada N, Kuroda T, Sawada R, Okura H, Tsutsumi H, Kawamata S, Sato Y. Characterization of *in vivo* tumorigenicity tests using severe immunodeficient NOD/Shi-scid IL2R^γ null mice for detection of tumorigenic cellular impurities in human cell-processed therapeutic products. *Regen. Ther.*, **1**, 30–37 (2015).
- 31) Mitsui K, Ide K, Takayama A, Wada T, Irie R, Kosai K. Conditionally replicating adenovirus prevents pluripotent stem cell-derived teratoma by specifically eliminating undifferentiated cells. *Mol. Ther. Methods Clin. Dev.*, **2**, 15026 (2015).
- 32) Kang S-J, Park Y-I, Hwang S-R, Yi H, Tham N, Ku H-O, Song J-Y, Kang H-G. Hepatic population derived from human pluripotent stem cells is effectively increased by selective removal of undifferentiated stem cells using YM155. *Stem Cell Res. Ther.*, **8**, 78 (2017).
- 33) Asgari S, Moslem M, Bagheri-Lankarani K, Pournasr B, Miryounesi M, Baharvand H. Differentiation and transplantation of human induced pluripotent stem cell-derived hepatocyte-like cells. *Stem Cell Rev. Rep.*, **9**, 493–504 (2013).
- 34) Takayama K, Akita N, Mimura N, Akahira R, Taniguchi Y, Ikeda M, Sakurai F, Ohara O, Morio T, Sekiguchi K, Mizuguchi H. Generation of safe and therapeutically effective human induced pluripotent stem cell-derived hepatocyte-like cells for regenerative medicine. *Hepatol. Commun.*, **1**, 1058–1069 (2017).
- 35) Liu H, Kim Y, Sharkis S, Marchionni L, Jang YY. *In vivo* liver regeneration potential of human induced pluripotent stem cells from diverse origins. *Sci. Transl. Med.*, **3**, 82ra39 (2011).
- 36) Nagamoto Y, Takayama K, Ohashi K, Okamoto R, Sakurai F, Tachibana M, Kawabata K, Mizuguchi H. Transplantation of a human iPS cell-derived hepatocyte sheet increases survival in mice with acute liver failure. *J. Hepatol.*, **64**, 1068–1075 (2016).
- 37) Sin YY, Ballantyne LL, Richmond CR, Funk CD. Transplantation of gene-edited hepatocyte-like cells modestly improves survival of arginase-1-deficient mice. *Mol. Ther. Nucleic Acids*, **10**, 122–130 (2018).
- 38) Nie YZ, Zheng YW, Ogawa M, Miyagi E, Taniguchi H. Human liver organoids generated with single donor-derived multiple cells rescue mice from acute liver failure. *Stem Cell Res. Ther.*, **9**, 5 (2018).
- 39) Rashidi H, Luu NT, Alwahsh SM, Ginai M, Alhaque S, Dong H, Tomaz RA, Vernay B, Vigneswara V, Hallett JM, Chandrashekrana A, Dhawan A, Vallier L, Bradley M, Callanan A, Forbes SJ, Newsome PN, Hay DC. 3D human liver tissue from pluripotent stem cells displays stable phenotype *in vitro* and supports compromised liver function *in vivo*. *Arch. Toxicol.*, **92**, 3117–3129 (2018).
- 40) Okamoto R, Takayama K, Akita N, Nagamoto Y, Hosokawa D, Iizuka S, Sakurai F, Suemizu H, Ohashi K, Mizuguchi H. Human iPS cell-based liver-like tissue engineering at extrahepatic sites in mice as a new cell therapy for hemophilia B. *Cell Transplant.*, **27**, 299–309 (2018).
- 41) Allen JW, Hassanein T, Bhatia SN. Advances in bioartificial liver devices. *Hepatology*, **34**, 447–455 (2001).
- 42) Strain AJ, Neuberger JM. A bioartificial liver—state of the art. *Science*, **295**, 1005–1009 (2002).
- 43) Sajiki T, Iwata H, Paek HJ, Tosha T, Fujita S, Ueda Y, Park YG, Zhu B, Satoh S, Ikai I, Yamaoka Y, Ikada Y. Morphologic studies of hepatocytes entrapped in hollow fibers of a bioartificial liver. *ASAIO J.*, **46**, 49–55 (2000).
- 44) Mazariegos GV, Patzer JF, Lopez RC, Giraldo M, DeVera ME, Grogan TA, Zhu Y, Fulmer ML, Amiot BP, Kramer DJ. First clinical use of a novel bioartificial liver support system (BLSS). *Am. J. Transplant.*, **2**, 260–266 (2002).
- 45) Uygun BE, Soto-Gutierrez A, Yagi H, Izamis ML, Guzzardi MA, Shulman C, Milwid J, Kobayashi N, Tilles A, Berthiaume F, Hertl M, Nahmias Y, Yarmush ML, Uygun K. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. *Nat. Med.*, **16**, 814–820 (2010).
- 46) Yagi H, Fukumitsu K, Fukuda K, Kitago M, Shinoda M, Obara H, Itano O, Kawachi S, Tanabe M, Coudriet GM, Piganelli JD, Gilbert TW, Soto-Gutierrez A, Kitagawa Y. Human-scale whole-organ bioengineering for liver transplantation: a regenerative medicine approach. *Cell Transplant.*, **22**, 231–242 (2013).
- 47) Iwamuro M, Shiraha H, Nakaji S, Furutani M, Kobayashi N, Takaki A, Yamamoto K. A preliminary study for constructing a bioartificial liver device with induced pluripotent stem cell-derived hepatocytes. *Biomed. Eng. Online*, **11**, 93 (2012).
- 48) Lorgette M, Scottoni F, Crowley C, Fiadeiro R, Maghsoudlou P, Pellegata AF, Mazzacuvu F, Gjinovci A, Lyne AM, Zulini J, Little D, Mosaku O, Kelly D, De Coppi P, Gissen P. Mouse decellularised liver scaffold improves human embryonic and induced pluripotent

- stem cells differentiation into hepatocyte-like cells. *PLOS ONE*, **12**, e0189586 (2017).
- 49) Minami T, Ishii T, Yasuchika K, Fukumitsu K, Ogiso S, Miyachi Y, Kojima H, Kawai T, Yamaoka R, Oshima Y, Kawamoto H, Kotaka M, Yasuda K, Osafune K, Uemoto S. Novel hybrid three-dimensional artificial liver using human induced pluripotent stem cells and a rat decellularized liver scaffold. *Regen. Ther.*, **10**, 127–133 (2019).
- 50) Collin de l'Hortet A, Takeishi K, Guzman-Lepe J, Morita K, Achreja A, Popovic B, Wang Y, Handa K, Mittal A, Meurs N, Zhu Z, Weinberg F, Salomon M, Fox IJ, Deng CX, Negrath D, Soto-Gutierrez A. Generation of human fatty livers using custom-engineered induced pluripotent stem cells with modifiable SIRT1 metabolism. *Cell Metab.*, **30**, 385–401.e9 (2019).