

From a Single Cell to a Whole Human Liver: Disease Modeling and Transplantation

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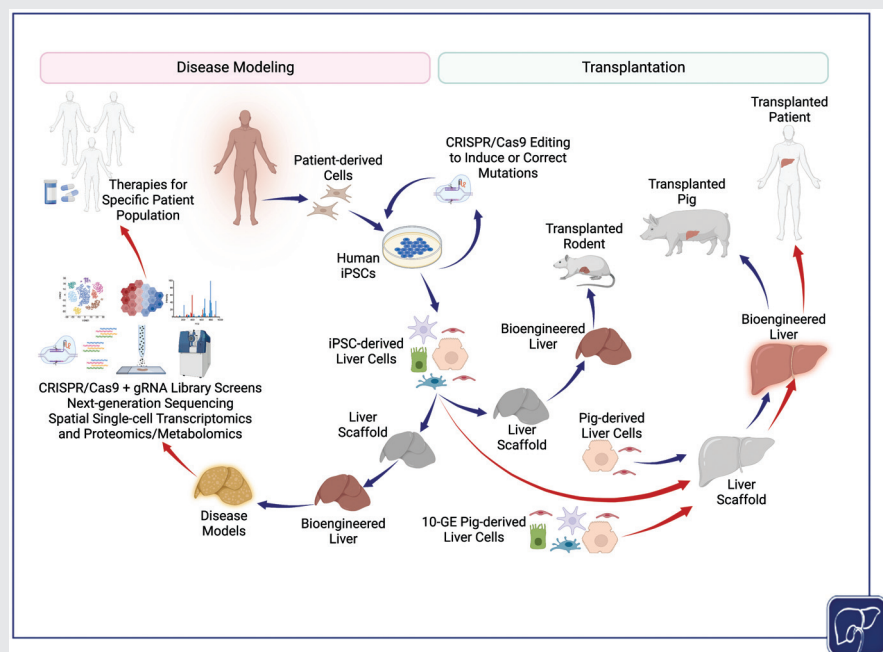
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Graphical Abstract



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Abstract

Although the underlying cause may vary across countries and demographic groups, liver disease is a major cause of morbidity and mortality globally. Orthotopic liver transplantation is the only definitive treatment for liver failure but is limited by the lack of donor livers. The development of drugs that prevent the progression of liver disease and the generation of alternative liver constructs for transplantation could help alleviate the burden of liver disease. Bioengineered livers containing human induced pluripotent stem cell (iPSC)-derived liver cells are being utilized to study liver disease and to identify and test potential therapeutics. Moreover, bioengineered livers containing pig hepatocytes and endothelial cells have been shown to function and survive after transplantation into pig models of liver failure, providing preclinical evidence toward future clinical applications. Finally, bioengineered livers containing human iPSC-derived liver cells have been shown to function and survive after transplantation in rodents but require considerable optimization and testing prior to clinical use. In conclusion, bioengineered livers have emerged as a suitable tool for modeling liver diseases and as a promising alternative graft for clinical transplantation. The integration of novel technologies and techniques for the assembly and analysis of bioengineered livers will undoubtedly expand future applications in basic research and clinical transplantation.

Keywords

- ▶ bioengineered liver
- ▶ disease modeling
- ▶ transplantation
- ▶ liver disease
- ▶ induced pluripotent stem cells

Liver disease affects more than four million people¹ and contributes to tens of thousands of deaths each year² in the United States. The term encompasses a number of diseases and disorders with various etiologies and include alcohol-induced liver disease, fatty liver disease, genetic liver disease, autoimmune liver disease, drug-related liver disease, virus-induced liver disease, and idiopathic liver disease.³ Because the liver is responsible for life-sustaining processes such as nutrient metabolism and storage, drug detoxification, and serum protein synthesis, liver diseases that progress to advanced stages are often lethal or require lifelong management.⁴

Despite significant progress in science, technology, and medicine, orthotopic liver transplantation (OLT) remains to be the only definitive treatment for many cases of end-stage liver disease.⁵ However, OLT is limited by the availability of donor livers. In the United States, more than 12,500 patients are added to the liver transplant waiting list every year but only about 9,000 of these patients will receive a transplant in the same year. In other words, approximately 3,500 of these patients either pass away or are carried over to the transplant waitlist for the next year, further extending the gap between the number of liver recipients and donors.^{6,7}

Various approaches are being utilized to help alleviate the burden of liver disease. Some are aimed at reversing or limiting the progression of liver disease before it progresses to a stage that can only be treated by OLT, while others are focused on generating alternative liver-like grafts that could be used for clinical OLT. The first approach relies on the availability of appropriate disease model systems to identify drug targets and test the effectiveness of candidate pharmacological therapies that could prevent disease progression. The second approach depends on the generation of liver constructs that are able to recapitulate the vital functions of the liver. Both approaches could benefit from

the development of bioengineered livers, artificial liver constructs that mimic the liver architecture, composition, and function, which are generated by the assembly and reorganization of various liver cell types (hepatocytes, endothelial cells, and other nonparenchymal cells) following their perfusion into a decellularized liver scaffold. The current applications, limitations, and potential improvements in the development and use of bioengineered livers for disease modeling and transplantation are discussed in this review.

Bioengineered Livers for Disease Modeling

The use of bioengineered livers for disease modeling relies on the availability of cells that are able to recapitulate pathogenesis and manifest disease phenotypes. Primary human hepatocytes are considered to be the gold standard in vitro system for studying liver disease development and for identifying and assessing the effectivity of novel pharmacological therapies.⁸ However, these cells are hard to obtain because of the scarcity of liver sources. Moreover, primary human hepatocytes suffer from limited proliferative capacity and rapid loss of liver-specific gene expression and function in conventional culture systems.^{9–11} The groundbreaking discovery that adult cells could be reprogrammed back into a stem cell state^{12,13} has led to innovative approaches to disease modeling. These cells called induced pluripotent stem cells (iPSCs) are suitable for such purposes because they can be produced from any diploid cell in the body, have tremendous proliferative capacity, and have the ability to differentiate into almost any cell type.¹⁴ The rapid development of iPSC technology and liver cell differentiation techniques within the past 15 years has led to a dramatic increase in the use of iPSC-derived hepatocyte-like cells (iHeps) for modeling a variety of liver diseases.^{15–31}

Normal iHeps

Experimental studies on the cellular mechanisms of hepatitis C virus (HCV) and hepatitis B virus (HBV) infection have previously been limited by the lack of appropriate *in vitro* models.^{32,33} Recently, however, a study reported HCV pseudoparticle entry and HCV RNA replication in iHeps indicating that the mechanisms for HCV infection and replication are present in iHeps.³⁴ Although HCV infections can now be effectively treated with direct-acting antiviral (DAA) therapy, this HCV model remains useful for studying the molecular mechanisms of HCV infection and for developing alternative treatments in case DAA-resistant viral strains emerge. A similar study demonstrated the production of HBV antigens and HBV-derived RNAs after HBV infection of iHeps indicating successful infection of iHeps by HBV.³⁵ In addition, the study showed that entecavir and Myrludex-B were able to decrease HBV viral infection in iHeps. These studies demonstrate that iHeps could be used as a replacement for primary human hepatocytes for studying the mechanism of HCV/HBV infection and for testing the effectiveness of potential drug therapies.

iHeps from Patients with Genetic Liver Disease

iPSC-based disease models have also been developed for a number of inherited metabolic liver disorders. One report published the generation of an iPSC library from patients with alpha-1-antitrypsin deficiency (AATD), familial hypercholesterolemia (FH), glycogen storage disease (GSD), and Crigler-Najjar syndrome.²³ More importantly, the study demonstrated that iHeps generated from these iPSCs exhibited the pathological characteristics of each disease such as the accumulation of misfolded alpha-1-antitrypsin polymers in AATD-derived iHeps, the impaired ability to incorporate low-density lipoprotein (LDL) in FH iHeps, and the excessive lipid accumulation and excessive production of lactic acid in GSD iHeps.²³

Subsequent studies that model genetic liver diseases using iHeps focused on cellular mechanisms that lead to disease progression. In all patients with the classical form of AATD, the PIZ mutation causes a misfolding of the mutant alpha-1-antitrypsin protein but while some patients exhibit severe liver disease necessitating OLT, others develop lung disease without manifestations of liver disease.³⁶ To address this variability in disease phenotypes, a study generated iHeps using iPSCs derived from AATD patients with severe liver disease and those without severe liver disease.²⁶ iHeps in both groups showed dilated rough endoplasmic reticulum but globular inclusions were observed only in iHeps from severe liver disease patients. In addition, iHeps from patients with severe liver disease exhibited slower rates of mutant protein degradation compared to those from patients with no liver disease. The study provides evidence that genetic modifiers have a significant contribution in determining liver disease phenotypes and suggests that putative modifiers of pathways involved in mutant protein degradation may serve as potential therapeutic targets in AATD.²⁶ In Wilson disease (WD), mutations in the copper transporter ATP7B cause the toxic buildup of copper in the liver. It has

been hypothesized that the H1069Q mutation in ATP7B inhibits the trafficking of the transporter from the ER to the lysosomes.³⁷ However, this theory has not been tested in an appropriate model system until a study analyzed iHeps derived from WD patients carrying the H1069Q mutation.²² The study found, for the first time, that loss of transporter function associated with this mutation was in fact predominantly due to ER-associated rapid degradation of the mutant protein.²² Nonalcoholic fatty liver disease (NAFLD) is a disease characterized by the accumulation of fat in hepatocytes that is not due to excessive alcohol intake. It is historically thought to be solely caused by consumption of high caloric diet and lack of exercise.³⁸ More recently, however, a study that generated and compared iHeps derived from NAFLD patients and healthy controls showed that NAFLD iHeps accumulated higher levels of lipids and upregulated genes associated with cell injury and death compared to control iHeps.³⁹ Since the study was done *in vitro* and without any impact from external factors such as diet or exercise, these findings suggest the presence of a genetic component to the development of NAFLD.³⁹ This, and many other studies, clearly establishes that NAFLD is a systemic, metabolic, and multifactorial disorder driven and influenced by the interaction of genetic and environmental components. Indeed, a recent initiative has proposed renaming NAFLD to metabolic dysfunction-associated fatty liver disease.^{40–42} These reports highlight the use of iHeps from patients with liver disease as an innovative platform for uncovering the pathogenesis of liver disease and identification of drug targets.

Genetically Edited iHeps

The field of genome editing has rapidly evolved since the development of technologies using zinc finger nucleases, transcription activator-like effector nucleases, and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9).⁴³ Using these tools, it has now been possible to target specific loci to either insert, delete, or mutate gene segments.⁴⁴ CRISPR/Cas9 editing has been shown to be exceptionally efficient at generating targeted genetic modifications and has now been widely utilized in a variety of cell types.^{45–48} The application of this system has been demonstrated in two publications reporting the genetic correction of iPSCs from patients with FH. CRISPR/Cas9 technology was used to correct the three-base pair c.654_656delTGG pathogenic deletion in one FH patient-derived iPSC line⁴⁹ and the c.901G > T mutation in another FH patient-derived iPSC line.⁵⁰ Upon differentiation, the corrected iHeps expressed mature LDLR protein after statin treatment and exhibited receptor-mediated LDL internalization.^{49,50} These studies demonstrate that CRISPR/Cas9 correction of genetic defects in iPSCs results in the restoration of hepatocyte function in differentiated iHeps.

In terms of disease modeling, CRISPR/Cas9 technology can be used to introduce known disease-causing mutations in normal iPSCs to generate iPSC disease models. This is especially useful for studying rare liver diseases where access to patient samples is challenging because of the low frequency

of disease occurrence. One such disease that has been modeled using this approach is mitochondrial DNA depletion syndrome type 3 (MTDPS3). MTDPS3 results from mutations in the deoxyguanosine kinase (DGUOK) gene that encodes for mitochondrial deoxyguanosine kinase, an enzyme that is involved in mitochondrial DNA (mtDNA) biogenesis and repair.^{51–53} The clinical presentations of MTDPS3 often vary and seem to be dependent on specific DGUOK mutations. Oftentimes, patients develop hepatic failure with more severe forms exhibiting multiple organ involvement.⁵³ There is currently no cure for the condition and current treatments involve lifelong management of symptoms or OLT. In order to model the disease and identify potential therapies for the condition, a study introduced DGUOK frameshift deletions in iPSCs using CRISPR/Cas9 and differentiated them into iHeps.⁵⁴ DGUOK iHeps exhibited many characteristics of the condition including loss of mtDNA, downregulation of mitochondrial proteins involved in the electron transport chain, decreased ATP production, increased levels of reactive oxygen species, and increased lactate production. High throughput screening of drugs that increased ATP production identified several candidate drugs including nicotinamide adenine dinucleotide (NAD), which upon further investigation was shown to restore mitochondrial activity. Treatment of MTDPS3-null rats with the NAD precursor nicotinamide riboside led to a significant increase in ATP production and electron transport chain complex activity.⁵⁴ These findings showcase the utility of CRISPR/Cas9-engineered mutant iPSCs for modeling and identifying drug treatments for rare diseases.

The use of CRISPR/Cas9 technology to introduce mutations in iPSCs could be further extended to the analysis of suspected susceptibility variants of modifier genes, genes that are not the primary cause of a disease but influence the eventual disease severity or onset. This approach was employed in a study that identified modifier genes in long QT syndrome type 2 (LQT2).⁵⁵ LQT2 is caused by mutations in potassium voltage-gated channel subfamily H member 2 that encodes for the potassium channel hERG. The disorder causes arrhythmias but while some patients exhibit severe disease, others develop mild disease.^{55,56} Analysis of induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) generated from an LQT2 family carrying the hERG R752W mutation showed that iPSC-CMs from patients with severe disease exhibited prolonged action potentials compared to iPSC-CMs from relatives with mild disease.⁵⁵ Further analysis revealed that while iPSC-CMs derived from all patients with hERG R752W mutation displayed lower I_{Kr} amplitude, only iPSC-CMs from severely affected patients exhibited greater L-type Ca^{2+} current. Whole exome sequencing identified several potential genetic modifiers of LQT2 including the GTP-binding protein REM2. CRISPR/Cas9 editing of REM2 from the severe disease-associated variant to the protected variant in iPSC-CMs led to the reversion of L-type Ca^{2+} current and action potential to wild-type levels indicating that REM2 is a genetic modifier for LQT2. The study showcases the use of iPSC technology, next-generation exome sequencing, and

CRISPR/Cas9 gene editing to identify genetic modifiers in LQT2.⁵⁵ This strategy can be applied to identify gene modifiers of monogenic liver diseases that exhibit variability in disease severity and onset, such as AATD.

In combination with large guide RNA (gRNA) libraries, CRISPR/Cas9 can also be used for genome-wide screens to identify genes whose altered expression causes alleviation or exacerbation of disease phenotypes. This approach was demonstrated in a recent report to determine senescence-promoting genes in human mesenchymal cells differentiated from human embryonic stem cells carrying the mutation for either Werner syndrome or Hutchinson-Gilford progeria syndrome.⁵⁷ After deep sequencing, the study identified genes whose deficiency led to a delay in senescence phenotype. This approach demonstrated that CRISPR/Cas9-based genetic screening is an effective method for systematically identifying genes that could be attractive therapeutic targets.⁵⁷

The abundance of studies that utilize human iHeps for disease modeling and drug testing showcases its remarkable utility and versatility in the field. However, most of these studies have been done using conventional two-dimensional (2D) cultures that fail to duplicate the liver architecture, the cellular heterogeneity, and the cell–cell and cell–extracellular matrix (ECM) interactions that occur *in vivo*.^{9–11} These limitations make 2D culture systems unsuitable for studying complex and multifactorial diseases such as NAFLD, alcoholic liver disease, and primary biliary cholangitis. To circumvent this problem, a few studies made use of three-dimensional (3D) *in vitro* systems for modeling NAFLD. In one study, hepatic organoids were differentiated from normal human embryonic stem cells and human iPSCs and were subsequently incubated in free fatty acid-enriched medium.⁵⁸ Organoids cultured in this way showed an accumulation of lipid droplets and increased overall triglyceride levels. Further analysis of the organoids showed the loss of the bile canaliculi network and the development of ductal reaction, structural hallmarks consistent with progression of fibrosis in NAFLD. Finally, gene expression analysis revealed a transcriptome signature similar to those from liver tissues of patients with nonalcoholic steatohepatitis (NASH), a severe form of NAFLD.⁵⁸ In another study, genetically engineered human iPSCs with controllable sirtuin 1 expression were differentiated into iHeps.⁵⁹ The iHeps, together with vascular endothelial cells, mesenchymal cells, fibroblasts, and macrophages were then seeded into decellularized rat liver scaffolds via portal vein perfusion recirculation and cultured in organ bioreactors under constant flow. Upon downregulation of sirtuin 1 expression, the human iPSC-derived bioengineered liver exhibited macrovesicular steatosis, acquired a proinflammatory phenotype, and developed a lipid and metabolic profile that is similar to that from human livers with NASH and terminal liver failure.⁵⁹ Although the former study induced NASH using environmental factors while the latter study induced NASH using genetic manipulation, both studies demonstrate the ability of 3D culture systems containing various liver cell types to model complex liver diseases.

Bioengineered Livers for Clinical Transplantation

Various liver assist devices have been developed in order to provide hepatic support for patients with failing livers, including an ex vivo perfusion system and a hybrid artificial liver support system containing spheroids of primary porcine hepatocytes, primary human hepatocytes or even human hepatoma cell lines.^{60,61} While many of these have been evaluated in clinical trials and have been shown to be effective for bridging patients awaiting OLT, these systems are not designed to provide long-term support and, in their current form, are not realistic alternatives for OLT.

Many studies have considered bioengineered livers as alternative grafts for clinical OLT.^{62–64} The system is tractable and allows for the introduction of multiple liver cell types that are essential for the maintenance of hepatic function.^{65–67} In addition, the use of decellularized liver scaffolds provides a liver-specific ECM microenvironment that is ideal for the survival, engraftment, and expansion of perfused liver cells.^{68,69} Finally, the vascular structures within the decellularized liver scaffolds are preserved facilitating surgical anastomosis with recipient blood vessels and ensuring adequate blood flow to liver cells.⁶⁷

Human-Scaled Bioengineered Livers

Numerous proof-of-concept studies have reported the transplantation of bioengineered livers into small animals. However, the grafts failed to survive and function for more than 3 days, most of the grafts contained only hepatocytes, and the transplants were not performed in models of hepatic dysfunction. Two groups that aimed to address some of these issues in order to advance the clinical application of bioengineered livers discuss their experience in their recent publications.^{65,66}

In the first study, decellularized pig liver scaffolds were first perfused with human umbilical vein endothelial cells (HUVECs) through the inferior vena cava followed by the portal vein.⁶⁵ After the HUVECs were allowed to engraft and proliferate, primary pig hepatocytes were perfused through the bile duct. Analysis of the bioengineered liver confirmed the engraftment of endothelial cells in the periphery of large vessels as well as parenchymal capillaries and hepatocytes within the liver parenchyma similar to cellular localization observed in native liver. The bioengineered liver was able to perform ammonia clearance and urea production and was able to maintain blood flow after short-term continuous perfusion at physiologic pressures providing evidence of hepatic function and vascular patency. Transplantation of the bioengineered liver into an acute ischemia pig model of hepatic failure resulted in the maintenance of cell viability and functional marker expression. Although the transplanted pigs were all sacrificed within 2 days post-transplant, the animals exhibited improved ammonia detoxification compared to controls that only received a portocaval shunt. The study presents a protocol for generating a preclinical bioengineered liver containing pig hepatocytes and endothelial cells that survives and functions

for up to 2 days after transplantation in a pig liver failure model.⁶⁵

In the second study, decellularized pig liver scaffolds were perfused with pig primary hepatocytes and pig aorta-derived endothelial cells through the portal vein or inferior vena cava following a stepwise protocol.⁶⁶ Analysis of samples from the bioengineered liver showed engraftment of hepatocytes within the parenchymal compartment and endothelial cells in the vascular network. The bioengineered liver secreted increasing levels of albumin, urea, and coagulation factors V, VII, and IX over 4 days indicating maintenance of hepatic function. Most importantly, transplantation of the bioengineered liver into a 60% hepatectomy pig model of liver failure resulted in graft survival for up to 28 days. Analysis of blood samples taken throughout the course of the transplant experiment also revealed lower levels of total bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and ammonia in the transplant group compared to controls in majority of the timepoints, indicating that the graft was able to compensate for the deficiency in hepatic function. All in all, the study established a protocol for generating a preclinical bioengineered liver containing hepatocytes and endothelial cells that exhibits hepatic function and survives for up to 28 days after transplantation in a pig liver failure model.⁶⁶

By demonstrating vascular patency, survival, and function of transplanted human-scaled bioengineered livers for up to a month in a large animal model of liver failure, the studies above present significant breakthroughs in the field of liver bioengineering. Nevertheless, further studies have to be conducted to reconstruct the biliary system in the grafts, extend the survival of transplanted grafts, and generate grafts with reduced immunogenicity. Recent reports that kidney and heart from transgenic pigs survived, functioned, and did not induce hyperacute immune rejection after xenotransplantation in human recipients have generated considerable excitement in the scientific and medical community.^{70,71} The 10-Gene-Edited pigs (10-GE pigs) used in these studies carried ten genetic modifications including the knockout of three pig carbohydrate genes involved in recipient immune rejection (GGTA1, β 4GalNT2, CMAH) and the pig growth hormone receptor gene, as well as the targeted insertion of two human complement inhibitor genes (hDAF, hCD46), two human anticoagulant genes (hTBM, hEPCR), and two immunomodulatory genes (hCD47, hHO1). Because of decreased immunogenicity, it is reasonable to imagine that future liver bioengineering studies would utilize liver cells obtained from 10-GE pigs as it would facilitate future clinical applications.

Bioengineered Livers with Human iHeps

Relative to other organs, the liver is considered immune-privileged because the incidence of antibody-mediated rejection following whole organ transplantation is estimated to be lower than the heart or the kidney.^{72–75} Nevertheless, acute cellular rejection following liver transplantation remains a major concern that alters long-term survival, the incidence of which can be as high as 10%.^{76,77} To circumvent

potential issues related to immunogenicity, autologous iPSC-derived grafts present an attractive solution. iPSCs can be created from somatic cells of a patient and can be differentiated into hepatocytes, cholangiocytes, vascular endothelial cells and other liver cells that can then be used for the assembly of a bioengineered liver. Hypoimmunogenic iPSC lines generated by CRISPR/Cas9-mediated inactivation of major histocompatibility complex class I and class II genes and lentiviral-mediated expression of immunomodulatory factors CD47 and/or PD-L1 have also been recently developed that can provide an “off-the-shelf” source of liver cells that should not induce recipient adaptive immune response after transplantation.^{78–80} Three reports describe the generation and analysis of bioengineered livers containing human iPSC-derived liver cells.^{27,81,82}

One study generated a hybrid bioengineered liver by perfusing a rat liver scaffold with human iHeps through the biliary duct.⁸¹ After 2 days of continuous perfusion, the iHeps were able to relocate to the parenchymal space of the scaffold. Analysis of samples from the recellularized liver revealed protein expression of albumin, AFP, HNF4a, and CYP3A4 as well as secretion of albumin indicating intact hepatic function.⁸¹ A different approach was followed in a more recent study where human iPSCs were first introduced into a rat liver scaffold prior to the initiation of hepatic differentiation.⁸² Analysis of the bioengineered liver showed increased expression of mature hepatocyte markers albumin, CYP1A2, CYP2E1, CYP2D6, and CYP2C9 as well as decreased expression of fetal markers AFP and CYP3A7 compared to controls. More importantly, the recellularized liver secreted albumin and urea indicating hepatic functionality. The results suggest that the native 3D liver microenvironment plays an essential role during hepatogenesis and that this concept could be utilized to drive iHeps towards a more mature phenotype.⁸² Lastly, a study that aims to generate a transplantable and functional human bioengineered liver recellularized the hepatic parenchyma, vascular system, and bile duct network of a rat liver scaffold with iPSC-derived cells.²⁷ This was accomplished by sequential reperfusion of a rat liver scaffold with human iHeps, iPSC-derived endothelial cells, and iPSC-derived cholangiocytes as well as human primary liver-derived fibroblast and mesenchymal stem cells. The human iPSC-derived bioengineered livers resembled the structure of human liver and upon auxiliary transplantation in immunodeficient rats, survived for up to 4 days. Hepatocyte-associated gene expression and function was enhanced in bioengineered livers with all the liver cell types compared to those that contained only iHeps indicating that the integration and interaction of iHeps with other hepatic cell types and the ECM led to improved maturation and function. This study emphasizes the importance of anatomical structure, vascular flow, and cell–cell/cell–ECM interactions for liver maturation and function.²⁷

While the above-mentioned studies provide encouraging results that support the potential use of iPSC-based bioengineered livers for transplantation, key issues regarding tumorigenicity, functional maturity, scalability, vascular patency, and assessment of long-term graft survival and func-

tion have to be addressed before clinical application. The effectivity of cell surface marker selection for excluding potentially tumorigenic undifferentiated or incompletely differentiated cells has to be evaluated.^{83–86} In addition, hepatocyte differentiation protocols need to be further optimized to generate cells that exhibit hepatocyte function that is comparable to that seen in primary human hepatocytes. Differentiation protocols must also be scaled up in order to generate the number of liver cells required to produce a bioengineered liver that could support the metabolic needs of a patient. Limited cell survival after implantation of iPSC-based bioengineered livers²⁷ indicates that, in addition to seeding with human endothelial cells, vascular growth factors must also be supplemented to induce the formation of durable vascular networks.⁸⁷ Most importantly, long-term survival and function of iPSC-derived bioengineered livers have to be assessed in an appropriate large-animal model of liver failure in order to establish their ability to rescue hepatic function.

The optimal cell source for the generation of clinically transplantable bioengineered livers is currently debatable. Each of the discussed cell sources have their advantages and limitations. 10-GE pig liver cells have mature function, no tumorigenic potential, and are easier to scale up, but their long-term immunogenic potential post-transplantation has to be carefully assessed and the reconstruction of a functional biliary network has to be demonstrated. On the other hand, autologous and hypoimmunogenic iPSC-derived liver cells have decreased immunogenicity, but more studies have to be done to eliminate potentially tumorigenic cells, induce mature hepatocyte function, and optimize scalability of differentiation. We believe that studies using either cell sources should be continued in parallel in order to address their respective deficiencies. The issue of graft function is more challenging to resolve compared to the issue of graft rejection, which can be managed through immunosuppressive treatment. Thus, we anticipate that bioengineered livers containing 10-GE pig liver cells will reach clinical use sooner than those with iPSC-derived liver cells. We also expect that initial clinical trials will utilize bioengineered livers as a bridge until a suitable donor liver becomes available for transplantation.

Blastocyst complementation, which employs the principles of organogenesis, is an alternative approach that can be used to generate transplantable livers. The technology can be used to produce chimeric animals in which cells, tissues, or even organs are derived solely from donor pluripotent stem cells.⁸⁸ In this technique, the gene/s required for the early development of a specific organ is/are knocked out in an animal blastocyst leading to an inability to grow the said organ. The blastocyst is then microinjected with donor pluripotent stem cells that have the capacity to generate the organ such that the donor cells are able to complement the missing organ as the embryo develops. This eventually leads to a chimeric offspring that carries an entire organ originating from the microinjected donor cells. Blastocyst complementation has already been applied for the generation of various tissues and organs such as blood vessels,

blood, pancreas, kidney, brain, lung, and liver in rodents, pigs, and even nonhuman primates.^{89–95} Using this technique, it is possible to generate a liver that is derived entirely from donor patient-specific iPSCs including the hepatic parenchyma, vasculature, biliary network, and gallbladder, if a blastocyst that lacks multiple developmental pathways can be created. This technology should theoretically generate organs that are nonimmunogenic after transplantation. However, it is possible that chimeric organs may contain animal cells that could potentially induce immune responses and thus, further studies are necessary to determine the immunogenicity of such chimeric organs after transplantation. A recent study demonstrating that synthetic embryos can be assembled and grown to an advanced stage of organogenesis in an ex utero system⁹⁶ opens exciting possibilities for this approach of generating transplantable livers.

Conclusion

The rapid development of tools for bioengineering, human iPSC research, hepatic differentiation, and gene editing has facilitated the generation of bioengineered livers that could be used for disease modeling, drug target discovery, and drug testing for various monogenic and complex liver diseases. With currently available technologies, it is now possible to model complex liver disease to analyze not only the changes within cells but also the alterations in cell–cell and cell–ECM interactions. Future studies would likely integrate the latest technologies such as spatial single cell transcriptomics⁹⁷ and spatial single cell proteomics/metabolomics^{98,99} to the analysis of bioengineered liver models in order to identify and validate potential therapeutic targets for complex liver diseases.

Although promising, the use of bioengineered livers containing iPSC-derived cells for clinical transplantation is still a long way from becoming a reality. The generation of iPSCs and the differentiation towards liver cells need to be optimized in order to generate the number of cells required to fully recellularize larger liver scaffolds appropriate for clinical applications. In addition, the ability to provide adequate liver function for an extended period of time and the immunogenicity of such bioengineered livers need to be carefully evaluated. It is highly likely that bioengineered livers repopulated with liver cells isolated from 10-GE transgenic pigs^{70,71} would reach clinical evaluation sooner than bioengineered livers containing iPSC-derived cells with initial studies testing bioengineered livers as a bridge to transplantation, and later on, as an alternative permanent liver graft.

Lay Summary

A lot of people around the world suffer and die from liver disease. Liver transplantation is only effective treatment for severe liver disease but there is a shortage of donor livers. The development of drugs that prevent the progression of liver disease and the production of artificial livers for transplantation could help decrease the burden of liver disease.

Artificial livers containing human stem cell-derived liver cells are being used for studying liver disease to identify and test potentially curative drugs. Artificial livers containing pig liver cells or human stem cell-derived liver cells can survive and function after transplantation but need further improvement and testing before clinical use. Thus, artificial livers are an important tool for studying liver diseases and a promising alternative graft for transplantation. The use of newly developed technologies and techniques for producing and studying artificial livers will surely improve their use in research and transplantation.

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Conflict of Interest

A.S.-G. is an inventor on a patent application that describes the use of transcription factors to treat chronic liver failure (US20140249209). E.N.T. and A.S.-G. are inventors on a provisional patent application related to methods to enhance hepatic functions in human failing livers (PCT/US2020/055500). A.S.-G. is a co-founder and has a financial interest in Von Baer Wolff, Inc. a company focused on biofabrication of autologous human hepatocytes from stem cell technology. A.S.-G., and A.O. are co-founders and have a financial interest in Pittsburgh ReLiver Inc. a company focused on programming liver failure. All interests are managed by the Conflict-of-Interest Office at the University of Pittsburgh in accordance with their policies.

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References

- Villarreal MA, Blackwell DL, Jen A. Tables of Summary Health Statistics for U.S. Adults: 2018 National Health Interview Survey. National Center for Health Statistics; 2019. Accessed on Sept 28, 2022, at: <http://www.cdc.gov/nchs/nhis/SHS/tables.htm>
- Centers for Disease Control and Prevention, National Center for Health Statistics. National Vital Statistics System, Mortality 1999–2020 on CDC WONDER Online Database, released in 2021. Data are from the Multiple Cause of Death Files, 1999–2020, as compiled from data provided by the 57 vital statistics jurisdictions through the Vital Statistics Cooperative Program. Accessed on Sept 28, 2022, at: <http://wonder.cdc.gov/ucd-icd10.html>
- Sharma A, Nagalli S. Chronic Liver Disease. Treasure Island (FL): StatPearls; 2020
- Malarkey DE, Johnson K, Ryan L, Boorman G, Maronpot RR. New insights into functional aspects of liver morphology. *Toxicol Pathol* 2005;33(01):27–34

- 5 Farkas S, Hackl C, Schlitt HJ. Overview of the indications and contraindications for liver transplantation. *Cold Spring Harb Perspect Med* 2014;4(05):4
- 6 Kwong A, Kim WR, Lake JR, et al. OPTN/SRTR 2018 Annual Data Report: Liver. *Am J Transplant* 2020;20(Suppl 1):193–299
- 7 Kwong AJ, Kim WR, Lake JR, et al. OPTN/SRTR 2019 Annual Data Report: Liver. *Am J Transplant* 2021;21(Suppl 2):208–315
- 8 Guo L, Dial S, Shi L, et al. Similarities and differences in the expression of drug-metabolizing enzymes between human hepatic cell lines and primary human hepatocytes. *Drug Metab Dispos* 2011;39(03):528–538
- 9 Heslop JA, Rowe C, Walsh J, et al. Mechanistic evaluation of primary human hepatocyte culture using global proteomic analysis reveals a selective dedifferentiation profile. *Arch Toxicol* 2017;91(01):439–452
- 10 LeCluyse EL, Witek RP, Andersen ME, Powers MJ. Organotypic liver culture models: meeting current challenges in toxicity testing. *Crit Rev Toxicol* 2012;42(06):501–548
- 11 Richert L, Liguori MJ, Abadie C, et al. Gene expression in human hepatocytes in suspension after isolation is similar to the liver of origin, is not affected by hepatocyte cold storage and cryopreservation, but is strongly changed after hepatocyte plating. *Drug Metab Dispos* 2006;34(05):870–879
- 12 Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007;131(05):861–872
- 13 Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 2007;318(5858):1917–1920
- 14 Tapia N, Schöler HR. Molecular obstacles to clinical translation of iPSCs. *Cell Stem Cell* 2016;19(03):298–309
- 15 Cayo MA, Cai J, DeLaForest A, et al. JD induced pluripotent stem cell-derived hepatocytes faithfully recapitulate the pathophysiology of familial hypercholesterolemia. *Hepatology* 2012;56(06):2163–2171
- 16 Cayo MA, Mallanna SK, Di Furio F, et al. A drug screen using human iPSC-derived hepatocyte-like cells reveals cardiac glycosides as a potential treatment for hypercholesterolemia. *Cell Stem Cell* 2017;20(04):478–489.e5
- 17 Chen YF, Tseng CY, Wang HW, Kuo HC, Yang VW, Lee OK. Rapid generation of mature hepatocyte-like cells from human induced pluripotent stem cells by an efficient three-step protocol. *Hepatology* 2012;55(04):1193–1203
- 18 Choi SM, Kim Y, Shim JS, et al. Efficient drug screening and gene correction for treating liver disease using patient-specific stem cells. *Hepatology* 2013;57(06):2458–2468
- 19 Fattahi F, Asgari S, Pournasr B, et al. Disease-corrected hepatocyte-like cells from familial hypercholesterolemia-induced pluripotent stem cells. *Mol Biotechnol* 2013;54(03):863–873
- 20 Gough A, Soto-Gutierrez A, Vernetti L, Ebrahimkhani MR, Stern AM, Taylor DL. Human biomimetic liver microphysiology systems in drug development and precision medicine. *Nat Rev Gastroenterol Hepatol* 2021;18(04):252–268
- 21 Overeem AW, Klappe K, Parisi S, et al. Pluripotent stem cell-derived bile canaliculi-forming hepatocytes to study genetic liver diseases involving hepatocyte polarity. *J Hepatol* 2019;71(02):344–356
- 22 Parisi S, Polishchuk EV, Allocca S, et al. Characterization of the most frequent ATP7B mutation causing Wilson disease in hepatocytes from patient induced pluripotent stem cells. *Sci Rep* 2018;8(01):6247
- 23 Rashid ST, Corbiveau S, Hannan N, et al. Modeling inherited metabolic disorders of the liver using human induced pluripotent stem cells. *J Clin Invest* 2010;120(09):3127–3136
- 24 Si-Tayeb K, Noto FK, Nagaoka M, et al. Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. *Hepatology* 2010;51(01):297–305
- 25 Song Z, Cai J, Liu Y, et al. Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells. *Cell Res* 2009;19(11):1233–1242
- 26 Tafaleng EN, Chakraborty S, Han B, et al. Induced pluripotent stem cells model personalized variations in liver disease resulting from α 1-antitrypsin deficiency. *Hepatology* 2015;62(01):147–157
- 27 Takeishi K, Collin de l'Hortet A, Wang Y, et al. Assembly and function of a bioengineered human liver for transplantation generated solely from induced pluripotent stem cells. *Cell Rep* 2020;31(09):107711
- 28 Vosough M, Omidinia E, Kadivar M, et al. Generation of functional hepatocyte-like cells from human pluripotent stem cells in a scalable suspension culture. *Stem Cells Dev* 2013;22(20):2693–2705
- 29 Wilson AA, Ying L, Liesa M, et al. Emergence of a stage-dependent human liver disease signature with directed differentiation of alpha-1 antitrypsin-deficient iPSCs. *Stem Cell Reports* 2015;4(05):873–885
- 30 Yusa K, Rashid ST, Strick-Marchand H, et al. Targeted gene correction of α 1-antitrypsin deficiency in induced pluripotent stem cells. *Nature* 2011;478(7369):391–394
- 31 Zhang S, Chen S, Li W, et al. Rescue of ATP7B function in hepatocyte-like cells from Wilson's disease induced pluripotent stem cells using gene therapy or the chaperone drug curcumin. *Hum Mol Genet* 2011;20(16):3176–3187
- 32 Fukuhara T, Matsuura Y. Role of miR-122 and lipid metabolism in HCV infection. *J Gastroenterol* 2013;48(02):169–176
- 33 Okuyama-Dobashi K, Kasai H, Tanaka T, et al. Hepatitis B virus efficiently infects non-adherent hepatoma cells via human sodium taurocholate cotransporting polypeptide. *Sci Rep* 2015;5:17047
- 34 Yoshida T, Takayama K, Kondoh M, et al. Use of human hepatocyte-like cells derived from induced pluripotent stem cells as a model for hepatocytes in hepatitis C virus infection. *Biochem Biophys Res Commun* 2011;416(1-2):119–124
- 35 Sakurai F, Mitani S, Yamamoto T, et al. Human induced-pluripotent stem cell-derived hepatocyte-like cells as an in vitro model of human hepatitis B virus infection. *Sci Rep* 2017;7:45698
- 36 Nelson DR, Teckman J, Di Bisceglie AM, Brenner DA. Diagnosis and management of patients with α 1-antitrypsin (A1AT) deficiency. *Clin Gastroenterol Hepatol* 2012;10(06):575–580
- 37 D'Agostino M, Lemma V, Chesi G, et al. The cytosolic chaperone α -crystallin B rescues folding and compartmentalization of misfolded multispan transmembrane proteins. *J Cell Sci* 2013;126(Pt 18):4160–4172
- 38 Ayonrinde OT. Historical narrative from fatty liver in the nineteenth century to contemporary NAFLD - reconciling the present with the past. *JHEP Rep* 2021;3(03):100261
- 39 Duwaerts CC, Le Guillou D, Her CL, et al. Induced pluripotent stem cell-derived hepatocytes from patients with nonalcoholic fatty liver disease display a disease-specific gene expression profile. *Gastroenterology* 2021;160(07):2591–2594.e6
- 40 Eslam M, Sanyal AJ, George J. International Consensus Panel. MAFLD: a consensus-driven proposed nomenclature for metabolic associated fatty liver disease. *Gastroenterology* 2020;158(07):1999–2014.e1
- 41 Méndez-Sánchez N, Díaz-Orozco LE. Editorial: International Consensus Recommendations to Replace the Terminology of Non-Alcoholic Fatty Liver Disease (NAFLD) with Metabolic-Associated Fatty Liver Disease (MAFLD). *Med Sci Monit* 2021;27:e933860
- 42 Mendez-Sanchez N, Arrese M, Gadano A, et al. The Latin American Association for the Study of the Liver (ALEH) position statement on the redefinition of fatty liver disease. *Lancet Gastroenterol Hepatol* 2021;6(01):65–72
- 43 Zhang HX, Zhang Y, Yin H. Genome editing with mRNA encoding ZFN, TALEN, and Cas9. *Mol Ther* 2019;27(04):735–746
- 44 Gupta RM, Musunuru K. Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9. *J Clin Invest* 2014;124(10):4154–4161
- 45 Cong L, Ran FA, Cox D, et al. Multiplex genome engineering using CRISPR/Cas systems. *Science* 2013;339(6121):819–823

- 46 Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 2012;337(6096):816–821
- 47 Mali P, Yang L, Esvelt KM, et al. RNA-guided human genome engineering via Cas9. *Science* 2013;339(6121):823–826
- 48 Jinek M, East A, Cheng A, Lin S, Ma E, Doudna J. RNA-programmed genome editing in human cells. *eLife* 2013;2:e00471
- 49 Omer L, Hudson EA, Zheng S, Hoying JB, Shan Y, Boyd NL. CRISPR correction of a homozygous low-density lipoprotein receptor mutation in familial hypercholesterolemia induced pluripotent stem cells. *Hepatology* 2017;1(09):886–898
- 50 Okada H, Nakanishi C, Yoshida S, et al. Function and immunogenicity of gene-corrected iPSC-derived hepatocyte-like cells in restoring low density lipoprotein uptake in homozygous familial hypercholesterolemia. *Sci Rep* 2019;9(01):4695
- 51 Brahimi N, Jambou M, Sarzi E, et al. The first founder DGUOK mutation associated with hepatocerebral mitochondrial DNA depletion syndrome. *Mol Genet Metab* 2009;97(03):221–226
- 52 Johansson K, Ramaswamy S, Ljungcrantz C, et al. Structural basis for substrate specificities of cellular deoxyribonucleoside kinases. *Nat Struct Biol* 2001;8(07):616–620
- 53 Dimmock DP, Zhang Q, Dionisi-Vici C, et al. Clinical and molecular features of mitochondrial DNA depletion due to mutations in deoxyguanosine kinase. *Hum Mutat* 2008;29(02):330–331
- 54 Jing R, Corbett JL, Cai J, et al. A screen using iPSC-derived hepatocytes reveals NAD⁺ as a potential treatment for mtDNA depletion syndrome. *Cell Rep* 2018;25(06):1469–1484.e5
- 55 Chai S, Wan X, Ramirez-Navarro A, et al. Physiological genomics identifies genetic modifiers of long QT syndrome type 2 severity. *J Clin Invest* 2018;128(03):1043–1056
- 56 Bianchi L, Priori SG, Napolitano C, et al. Mechanisms of I(Ks) suppression in LQT1 mutants. *Am J Physiol Heart Circ Physiol* 2000;279(06):H3003–H3011
- 57 Wang W, Zheng Y, Sun S, et al. A genome-wide CRISPR-based screen identifies KAT7 as a driver of cellular senescence. *Sci Transl Med* 2021;13(575):13
- 58 Ramli MNB, Lim YS, Koe CT, et al. Human pluripotent stem cell-derived organoids as models of liver disease. *Gastroenterology* 2020;159(04):1471–1486.e12
- 59 Collin de l'Hortet A, Takeishi K, Guzman-Lepe J, et al. Generation of human fatty livers using custom-engineered induced pluripotent stem cells with modifiable SIRT1 metabolism. *Cell Metab* 2019;30(02):385–401.e9
- 60 Nakazawa K, Ijima H, Fukuda J, et al. Development of a hybrid artificial liver using polyurethane foam/hepatocyte spheroid culture in a preclinical pig experiment. *Int J Artif Organs* 2002;25(01):51–60
- 61 Riordan SM, Williams R. Extracorporeal support and hepatocyte transplantation in acute liver failure and cirrhosis. *J Gastroenterol Hepatol* 1999;14(08):757–770
- 62 Vishwakarma SK, Lakkireddy C, Bardia A, et al. Bioengineered functional humanized livers: an emerging supportive modality to bridge the gap of organ transplantation for management of end-stage liver diseases. *World J Hepatol* 2018;10(11):822–836
- 63 Pullen LC. Bioengineered organs: not a matter of “if”. *Am J Transplant* 2022;22(01):1–2
- 64 Dai Q, Jiang W, Huang F, Song F, Zhang J, Zhao H. Recent advances in liver engineering with decellularized scaffold. *Front Bioeng Biotechnol* 2022;10:831477
- 65 Anderson BD, Nelson ED, Joo D, et al. Functional characterization of a bioengineered liver after heterotopic implantation in pigs. *Commun Biol* 2021;4(01):1157
- 66 Higashi H, Yagi H, Kuroda K, et al. Transplantation of bioengineered liver capable of extended function in a preclinical liver failure model. *Am J Transplant* 2022;22(03):731–744
- 67 Shaheen MF, Joo DJ, Ross JJ, et al. Sustained perfusion of revascularized bioengineered livers heterotopically transplanted into immunosuppressed pigs. *Nat Biomed Eng* 2020;4(04):437–445
- 68 Watt FM, Huck WT. Role of the extracellular matrix in regulating stem cell fate. *Nat Rev Mol Cell Biol* 2013;14(08):467–473
- 69 Yagi H, Fukumitsu K, Fukuda K, et al. Human-scale whole-organ bioengineering for liver transplantation: a regenerative medicine approach. *Cell Transplant* 2013;22(02):231–242
- 70 Porrett PM, Orandi BJ, Kumar V, et al. First clinical-grade porcine kidney xenotransplant using a human decedent model. *Am J Transplant* 2022;22(04):1037–1053
- 71 Wang W, He W, Ruan Y, Geng Q. First pig-to-human heart transplantation. *Innovation (Camb)* 2022;3(02):100223
- 72 Chih S, Chruscinski A, Ross HJ, Tinkam K, Butany J, Rao V. Antibody-mediated rejection: an evolving entity in heart transplantation. *J Transplant* 2012;2012:210210
- 73 Demetris AJ, Bellamy CO, Gandhi CR, Prost S, Nakanuma Y, Stolz DB. Functional immune anatomy of the liver-as an allograft. *Am J Transplant* 2016;16(06):1653–1680
- 74 Gloor J, Stegall MD. Sensitized renal transplant recipients: current protocols and future directions. *Nat Rev Nephrol* 2010;6(05):297–306
- 75 Lee BT, Fiel MI, Schiano TD. Antibody-mediated rejection of the liver allograft: An update and a clinico-pathological perspective. *J Hepatol* 2021;75(05):1203–1216
- 76 Chen Y, Zhang W, Bao H, He W, Chen L. High mobility group box 1 contributes to the acute rejection of liver allografts by activating dendritic cells. *Front Immunol* 2021;12:679398
- 77 Mao JX, Guo WY, Guo M, Liu C, Teng F, Ding GS. Acute rejection after liver transplantation is less common, but predicts better prognosis in HBV-related hepatocellular carcinoma patients. *Hepatology* 2020;74(03):347–361
- 78 Deuse T, Hu X, Gravina A, et al. Hypoimmunogenic derivatives of induced pluripotent stem cells evade immune rejection in fully immunocompetent allogeneic recipients. *Nat Biotechnol* 2019;37(03):252–258
- 79 Han X, Wang M, Duan S, et al. Generation of hypoimmunogenic human pluripotent stem cells. *Proc Natl Acad Sci U S A* 2019;116(21):10441–10446
- 80 Xu H, Wang B, Ono M, et al. Targeted disruption of HLA genes via CRISPR-Cas9 generates iPSCs with enhanced immune compatibility. *Cell Stem Cell* 2019;24(04):566–578.e7
- 81 Minami T, Ishii T, Yasuchika K, et al. Novel hybrid three-dimensional artificial liver using human induced pluripotent stem cells and a rat decellularized liver scaffold. *Regen Ther* 2019;10:127–133
- 82 Acun A, Oganessian R, Jaramillo M, Yarmush ML, Uygun BE. Human-origin iPSC-based recellularization of decellularized whole rat livers. *Bioengineering (Basel)* 2022;9(05):9
- 83 Kuroda T, Yasuda S, Sato Y. In vitro detection of residual undifferentiated cells in retinal pigment epithelial cells derived from human induced pluripotent stem cells. *Methods Mol Biol* 2014;1210:183–192
- 84 Tano K, Yasuda S, Kuroda T, Saito H, Umezawa A, Sato Y. A novel in vitro method for detecting undifferentiated human pluripotent stem cells as impurities in cell therapy products using a highly efficient culture system. *PLoS One* 2014;9(10):e110496
- 85 Tateno H, Onuma Y, Ito Y, et al. Elimination of tumorigenic human pluripotent stem cells by a recombinant lectin-toxin fusion protein. *Stem Cell Reports* 2015;4(05):811–820
- 86 Sekine K, Tsuzuki S, Yasui R, et al. Robust detection of undifferentiated iPSC among differentiated cells. *Sci Rep* 2020;10(01):10293
- 87 Jang S, Collin de l'Hortet A, Soto-Gutierrez A. Induced pluripotent stem cell-derived endothelial cells: overview, current advances, applications, and future directions. *Am J Pathol* 2019;189(03):502–512
- 88 Kobayashi T, Yamaguchi T, Hamanaka S, et al. Generation of rat pancreas in mouse by interspecific blastocyst injection of pluripotent stem cells. *Cell* 2010;142(05):787–799
- 89 Matsunari H, Nagashima H, Watanabe M, et al. Blastocyst complementation generates exogenous pancreas in vivo in pancreatic cloned pigs. *Proc Natl Acad Sci U S A* 2013;110(12):4557–4562

- 90 Goto T, Hara H, Sanbo M, et al. Generation of pluripotent stem cell-derived mouse kidneys in Sall1-targeted anephric rats. *Nat Commun* 2019;10(01):451
- 91 Hamanaka S, Umino A, Sato H, et al. Generation of vascular endothelial cells and hematopoietic cells by blastocyst complementation. *Stem Cell Reports* 2018;11(04):988–997
- 92 Mori M, Furuhashi K, Danielsson JA, et al. Generation of functional lungs via conditional blastocyst complementation using pluripotent stem cells. *Nat Med* 2019;25(11):1691–1698
- 93 Fu R, Yu D, Ren J, et al. Domesticated cynomolgus monkey embryonic stem cells allow the generation of neonatal interspecies chimeric pigs. *Protein Cell* 2020;11(02):97–107
- 94 Das S, Koyano-Nakagawa N, Gafni O, et al. Generation of human endothelium in pig embryos deficient in ETV2. *Nat Biotechnol* 2020;38(03):297–302
- 95 Ruiz-Estevez M, Crane AT, Rodriguez-Villamil P, et al. Liver development is restored by blastocyst complementation of HHEX knockout in mice and pigs. *Stem Cell Res Ther* 2021;12(01):292
- 96 Tarazi S, Aguilera-Castrejon A, Joubran C, et al. Post-gastrulation synthetic embryos generated ex utero from mouse naive ESCs. *Cell* 2022;185(18):3290–3306.e25. doi: 10.1016/j.cell.2022.07.028
- 97 Longo SK, Guo MG, Ji AL, Khavari PA. Integrating single-cell and spatial transcriptomics to elucidate intercellular tissue dynamics. *Nat Rev Genet* 2021;22(10):627–644
- 98 Taylor MJ, Liyu A, Vertes A, Anderton CR. Ambient single-cell analysis and native tissue imaging using laser-ablation electrospray ionization mass spectrometry with increased spatial resolution. *J Am Soc Mass Spectrom* 2021;32(09):2490–2494
- 99 Taylor MJ, Lukowski JK, Anderton CR. Spatially resolved mass spectrometry at the single cell: recent innovations in proteomics and metabolomics. *J Am Soc Mass Spectrom* 2021;32(04):872–894