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Review

Application of Induced Pluripotent Stem Cells in Liver Diseases

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Tens of millions of patients are affected by liver disease worldwide. Many of these patients can benefit from therapy involving hepatocyte transplantation. Liver transplantation is presently the only proven treatment for many medically refractory liver diseases including end-stage liver failure and inherited metabolic liver disease. However, the shortage in transplantable livers prevents over 40% of listed patients per year from receiving a liver transplant; many of these patients die before receiving an organ offer or become too sick to transplant. Therefore, new therapies are needed to supplement whole-organ liver transplantation and reduce mortality on waiting lists worldwide. Furthermore, the remarkable regenerative capacity of hepatocytes in vivo is exemplified by the increasing number of innovative cell-based therapies and animal models of human liver disorders. Induced pluripotent stem cells (iPSCs) have similar properties to those of embryonic stem cells (ESCs) but bypass the ethical concerns of embryo destruction. Therefore, generation of hepatocyte-like cells (HLCs) using iPSC technology may be beneficial for the treatment of severe liver diseases, screening of drug toxicities, basic research of several hepatocytic disorders, and liver transplantation. Here we briefly summarize the growing number of potential applications of iPSCs for treatment of liver disease.

Key words: Induced pluripotent stem cells (iPSCs); Liver diseases; Cell therapy; Hepatocyte transplantation

INTRODUCTION

Tens of millions of patients are affected by liver disease worldwide. Many of these patients can benefit from therapy involving biologically active living cells, either by treatment of their liver disease or by prevention of their disease phenotype. Liver transplantation is presently the only proven treatment for many medically refractory liver diseases including end-stage liver failure and inherited metabolic liver disease. However, the shortage in transplantable livers prevents over 40% of listed patients per year from receiving a liver transplant; many of these patients die before receiving an organ offer or become too sick to transplant (www.unos.org). Therefore, new therapies are needed to supplement whole-organ liver transplantation and reduce mortality on waiting lists worldwide. Furthermore, the remarkable regenerative capacity of hepatocytes in vivo is exemplified by the increasing number of innovative cell-based therapies and animal models of human liver disorders.This review article highlights the growing number of potential applications of induced pluripotent stem cells (iPSCs) for treatment of liver disease. We must stress that they are potential applications since full differentiation of iPSCs into mature hepatocytes has yet to be reported.

Before describing iPSCs, we will identify the cell type that they were modeled after, embryonic stem cells (ESCs). James Thomson's group at University of Wisconsin first reported the enormous potential of human ESCs to differentiate into all three germ layers and multiple tissues and cells types (69). This observation raised the hopes of curing many previously untreatable diseases and rectifying the shortage of transplantable tissues. However, after a decade of research, progress with ESC development was limited by issues related to ethical concerns of embryo disruption and immune incompatibility with donor–recipient combinations of ESC transplants.

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These concerns were addressed by iPSC technology, which brings together the potential benefits of ESCs (i.e., self-renewal, pluripotency) and addresses the concerns of embryo disruption and immune compatibility inherent with ESCs: iPSCs bypass the ethical concerns of embryo destruction since they are produced from somatic cells in vitro without embryonic tissues or oocytes, and immune-compatibility concerns, since they have the potential for generating patient-specific cell types for cell replacement therapy.

Differentiated cell types produced from a patient's iPSCs have many potential therapeutic applications, including their use in tissue replacement and gene therapy. Our previous study (82) and others' (61) reporting the potential benefits of hepatocyte-like cells (HLCs) generated from iPSCs have described their secretion of human albumin, synthesis of urea, and expression of cytochrome P450 (CYP) enzymes in vitro. More recently, investigators have reported that HLCs differentiated from human iPSCs (hiPSCs) of patients with the inherited metabolic conditions may be used to model inherited liver diseases (56). Transplantation of HLCs derived from hiPSCs could represent an alternative to liver transplantation for the treatment of acute liver failure (ALF), liver cirrhosis, viral hepatitis, and the correction of inherited metabolic liver disorders resulting from genetically deficient states. These studies indicate the many potential applications of iPSCs in liver diseases (18,31).

DEVELOPMENT IN METHODS OF REPROGRAMMING IPSCs

Significant efforts have been made to understand the reprogramming process involved with the generation of iPSCs. iPSCs currently have been obtained from multiple species such as mouse (66), human (93), rat (34), pig (14), monkey (39), and marmoset (70). To date, several strategies have been identified for making iPSCs, and the advantages and limitations of each strategy are outlined in Table 1.

Initial reports of iPSC generation used γ retroviruses to express the four defined factors {octamer-binding transcription factor 4 [*OCT4* (O)], sex-determining region Y box 2 [*SOX2* (S)], Kruppel-like factor 4 [*KLF4* (K)], and *c-MYC* (M), so-called OSKM cocktail} to induce pluripotency in somatic cells (66). It took two decades to make human ESCs after the discovery of mouse ESCs, while in 2007, only 1 year after mouse iPSCs were made, the first hiPSCs were generated using a lentivirus with O, S, *NANOG* (N), and *LIN28* (L) (so-called OSNL) (81) or OSKM (65). A major limitation of the use of retroviral, including lentiviral, strategy is associated with spontaneous reactivation of the viral transgenes, and the virus genes integrate into the genome, which is a risk of tumor formation (19). The search for ways to induce pluripotency suitable for clinical application and without incurring genetic change has thus become the focus of intense research effort. Toward this end, iPSCs have been derived via adenoviral vectors (63) and Sendai vectors (SeV) (16), which express exogenous genes transiently and cannot integrate into the host genome (63). In 2008, production of virus-free iPSCs by simple plasmid transfection technique addressed a critical safety concern of iPSC use in clinical application (80). iPSCs have also been derived from serial protein transduction using recombinant proteins to eliminate genome integration by DNA method (90). Another strategy to avoid insertional mutagenesis by viral vectors is the application of small molecules to replace reprogramming transgenes (20). In 2008, Woltjen et al. (75) reported a new approach using piggyBac (PB) transposition to generate iPSCs without vector integration. In 2010, Yakubovet al. (76) and Warren et al. (74) described a simple, nonintegrating strategy for reprogramming cell fate based on administration of synthetic messenger RNA (mRNA) modified to overcome innate antiviral responses. In 2008, Lin et al. (36) used microRNA (miR-302) to generate hiPSCs from a human skin cancer cell line. This technology was a significant advance in safety and effectiveness in reprogramming clinically useful iPSCs. Recently, Obokata et al. (51) reported a surprisingly simple, faster, and more efficient method called stimulus-triggered acquisition of pluripotency (STAP), which requires neither nuclear transfer nor the introduction of transcription factors. Exposure to stress, including a transient low pH, was sufficient to make iPSCs. These iPSCs are even more malleable than those made by traditional methods mentioned above. Their findings indicate that epigenetic fate determination of mammalian cells can be markedly converted in a context-dependent manner by strong environmental cues. However, this technique proved to be too good to be true as the paper has been retracted due to an inability to replicate—among other problems.

The typical factors used in reprogramming cocktails are OSKM or OSNL. To produce safer patient-specific iPSCs for clinical application, one or more reprogramming factors are also designed to be removed from the cocktail because of their tumorigenicity; c-MYC and KLF4 are most frequently removed. Nakagawa et al. used only three factors (OSK) to generate mouse and human iPSCs, thus eliminating the oncogene risk of c-MYC (47). Huangfu et al. (20) and Meng et al. (45) also generated iPSCs in which the c-MYC and KLF4 factors were removed. In 2009, Kim et al. reprogrammed mouse neural stem cells to iPSCs after introducing OCT4 and KLF4 (27), or just OCT4 (26). Liu et al. (41) also reported reprogramming with a single factor, OCT4, yielding iPSC production comparable to a four-virus transduction protocol. Attempts to minimize genome integration were also made by combining the DNA of all four factors into

Table 1. Multiple Reprogramming Methods to Make iPSCs

Strategy Species	Genes	Species	Cell Type	Efficiency	Years	Reference	Description
Retrovirus	OSKM	Mouse	Embryonic fibroblasts	0.1%	2006	(66)	The first report of iPSCs.
	OSK	Mouse	Fibroblasts	0.01%	2008	(47)	Retroviruses integrate into the genome and are associated with spontaneous reactivation of the viral transgenes and a risk of tumor formation.
		Human	Adult dermal fibroblasts	0.001%	2008		
	OSK+ valproic acid (VPA)	Human	Fibroblasts	1%	2008	(20)	
	OS + VPA	Human	Fibroblasts	0.001%	2008		Small molecule compounds have been shown to dramatically promote the efficiency of reprogramming in both human and mouse cells, whether induced by two, three, or four factors.
	OS	Mouse	Neural stem cells	0.14%	2008	(28)	
	OK	Mouse	Neural stem cells	0.11%	2009	(27)	
	O	Mouse	Neural stem cells	0.1%	2009	(26)	
		Human	Amniotic fluid cells ($CD34^+$ huAFCs)	NA	2012	(41)	
	microRNA (miRNA-302)	Human	Skin cancer cell line	NA	2008	(36)	miRNA strategies offer a very simple, efficient, and safe method to produce iPSCs.
Lentivirus	OSNL	Human	Embryonic fibroblasts IMR 90	0.01%	2007	(81)	Lentivirus transduction does not shut down following transformation into iPSCs. Therefore, iPSCs generated from lentiviruses cannot be used safely in clinical application.
	OSKMNL	Human	Newborn foreskin fibroblasts	0.1%	2008	(35)	
	OSKM	Human	Fibroblasts	0.01%	2007	(65)	
	OS	Human	Cord blood $CD34^+$ cells	0.5–2%	2012	(45)	
	GFP	Human	Fibroblasts	NA	2010	(23)	
Adenovirus	OSKM	Mouse	Hepatocyte	0.0006%	2008	(63)	The first instance of iPSC generation without virus integration. It was difficult to maintain sufficiently high levels of the reprogramming factors with adenoviral method, so the reprogramming efficient is very low.
		Human	Embryonic fibroblasts IMR 90	0.0002%	2009	(91)	
Sendai virus (SeV)	OSKM	Human	Fibroblast BJ Fibroblast HDF	1%	2009	(16)	The SeV vector is a single-strand RNA virus having no DNA phase and without virus integration. Use of Sendai virus requires stringent steps to purge reprogrammed cells of replicating virus, and the sensitivity of the viral RNA replicase to transgene sequence content may limit the generality of this reprogramming vehicle.
		Mouse	Embryonic fibroblasts	0.83%	2011	(48)	
		Human	Cord blood $CD34^+$ cells	0.2%	2011	(50)	
Plasmid transfection	OSKM	Mouse	Embryonic fibroblasts	0.0015%	2008	(53)	This method is very inefficient, but removed one of obstacles concerning gene integration to clinical applications of hiPSCs. More transcriptional factors could achieve a much higher efficiency level.
	OSKMNL	Human	Human foreskin fibroblasts	0.1%	2009	(80)	
PiggyBac (PB) transposition	OSKM	Mouse	Embryonic fibroblasts	NA	2009	(75)	PB transposon/transposase technology only requires the inverted terminal repeats that flank the targeted transgene and the transient expression of the transposase enzyme to catalyze insertion or excision events. It still carries the risk of trans-genes remaining in the genome

(*continued*)

Table 1. (*Continued*)

Strategy Species	Genes	Species	Cell Type	Efficiency	Years	Reference	Description
Recombinant protein	OSKM	Human	Fibroblasts	0.001%	2009	(25)	This technology eliminates genetic manipulation. However, reprogramming human cells by DNA-free recombinant proteins is currently an inefficient process, and reprogramming efficiencies decrease after removing any reprogramming factor.
	OSKM+VPA	Mouse	Embryonic fibroblasts	0.0006%	2009	(90)	
	OSK+VPA	Mouse	Embryonic fibroblasts	0.0002%			
	Retrovirus SKM+ protein O	Mouse	Embryonic fibroblasts	NA	2010	(68)	
Messenger RNA (mRNA)	OSKM+VPA	Human	Fibroblasts	0.6–1.4%	2010	(74)	mRNA reprogramming method is efficient and avoids any DNA integration events and the associated genomic damage. The main disadvantage includes the innate antiviral response activated by the introduction of large amounts of long RNAs, and mRNA is degraded quickly, and thus multiple transfections are required.
	OSKML+VPA	Human	Fibroblasts	2–4.4%	2010		
	OSNL	Human	Fibroblasts	0.05%	2010	(76)	

iPSCs, induced pluripotent stem cells; OSKMNL, octamer-binding transcription factor 4 [OCT4 (O)], sex-determining region Y box 2 [SOX2 (S)], Kruppel-like factor 4 [KLF4 (K)], c-MYC (M), NANOG (N), and LIN28 (L); GFP, green fluorescent protein.

one polycistronic vector (22). Cre-Lox recombination is known as a site-specific recombinase technology. The system consists of a single enzyme, Cre recombinase, which recombines a pair of short target sequences called the Lox sequences. Placing Lox sequences appropriately allows genes to be inserted or exchanged for other genes. More recently, several groups have reported the derivation of iPSCs by integration of the four reprogramming factors with the use of plasmids and lentiviruses followed by Cre–loxP excision of the inserted genes after switching on the reprogramming process (22,55). In fact, Kane et al. have generated iPSCs without transcription factors, by merely transducing human fibroblasts with lentivectors expressing a single green fluorescent protein (23). Based on the above research, more and safer reprogramming approaches have been developed to generate iPSCs for the clinical application.

INFLUENCE OF CELL SOURCE FOR MAKING IPSCs

Does the origin of the cell source influence the characteristics of its corresponding hiPSCs? Likely, though Liu has reported that in vivo liver regeneration can result from hiPSCs of diverse origins (37). So far, somatic fibroblasts are widely applied, and they are highly efficient, but there is an invasive procedure to get them. Blood cells are easily accessible, but have low efficiency. It has been demonstrated that iPSCs retain certain gene expressions of the parent cells, and this suggests that iPSCs of different origins may possess different capacities to differentiate (43). Prior work with iPSCs derived from fibroblasts suggests that mitochondrial numbers returned to their prereprogrammed state or lower levels after redifferentiation (2,64).

According to K. Kim and colleagues, hiPSCs derived from cord blood cells showed a hematopoietic differentiation advantage when compared with hiPSCs derived from keratinocytes (29). Therefore, the ability to reprogram human hepatocytes may be crucial for developing liver disease models with iPSCs, especially for certain liver diseases involving acquired somatic mutations that occur only in hepatocytes of patients and not in other cell types. Liu et al. reported their generation of endoderm-derived hiPSCs from primary hepatocytes (38). Interestingly, iPSCs from stomach or liver cells harbor fewer integrated proviruses than fibroblasts, a feature which might indicate that lower expression levels of reprogramming factors may be required to achieve pluripotency (1). However, there is also strong evidence that the epigenetic memory of hiPSC lines is lost over time, making it equally feasible to generate hepatocytes from hiPSCs regardless of its tissue of source (49). Further investigation is needed to verify both sides of the controversy.

LARGE EXPANSION SYSTEM OF IPSCs

One of the technical hurdles that must be overcome firstly before iPSCs can be clinically implemented is their scalable, reproducible production and their differentiated progeny. All of the iPSC lines established thus far have been generated and expanded with static tissue culture protocols, which are time consuming and suffer from

batch-to-batch variability. Additionally, monolayer cultures provide limited iPSCs only sufficient for research, so a large-scale expanding system for culturing and maintenance is required for further research as well as future clinical applications.

Shafa et al. reported on the expansion and long-term maintenance of iPSCs in a stirred suspension bioreactor (SSB) (58). Their study showed that murine iPSCs can be maintained and expanded in SSB without loss of pluripotency over a long-term period. Kehoe et al. also reported scalable SSB culture of hiPSCs (24). They demonstrated SSB cultured iPSCs as aggregates, and the iPSC aggregates retained the ability to express pluripotency markers, as well as the potential for multilineage differentiation in vitro and in vivo. Chen et al. describes microcarriers (MCs) in bioreactors, another three-dimensional (3D) suspension culture for iPSC cultivation (6). Such a culture system represents an efficient process for the large-scale expansion and maintenance of iPSCs, which is an important first step in their clinical application.

HEPATIC DIFFERENTIATION OF IPSCs IN VITRO

It was shown that hepatic cells could be generated from iPSCs. So far, neither ESCs nor iPSCs can differentiate to fully mature hepatocytes in vitro. Researchers have then termed such populations of cells derived from iPSCs or ESCs as HLCs; HLCs indicate some of the properties of mature hepatocytes. For the first time, Song et al. (62) demonstrated that hiPSCs can be directly induced to HLCs by the administration of various growth factors in a time-dependent manner. Subsequent reports optimized this method with minor modifications, which usually includes Stage 1 - endoderm induction [activin A, wingless-type mouse mammary tumor virus (MMTV) integration site family, member 3A, (Wnt3a)], Stage 2 - hepatic specification [fibroblast growth factor 2 (FGF2), FGF4, bone morphogenetic protein 4 (BMP4)], Stage 3 - hepatoblast expansion [hepatocyte growth factor (HGF)], and Stage 4 - hepatic maturation (oncostatin M). In addition to growth factors, a variety of protocols have been used for the differentiation of hiPSCs toward the hepatic lineage. The application of small molecules [dimethyl sulfoxide (DMSO), dexamethasone] has been used for the hepatic differentiation from iPSCs. It has been demonstrated that these molecules can regulate specific target(s) in signaling and epigenetic mechanisms and can manipulate cell fate without genetic alterations. The characteristics of stem cell-derived hepatocytes produced from various differentiation protocols have been critically reviewed (4). For example, Zhang et al. induced functional hepatocytes from mouse iPSCs by a combination of cytokines and sodium butyrate (85). These protocols are time consuming, usually requiring more than 20 days. In contrast, Chen et al. (7) reported rapid generation of HLCs from hiPSCs by an efficient three-step protocol. For the clinical application, the differentiated cells should be assessed by comparing them with primary liver-derived cells for morphology and the expression of a set of proteins such as α-fetoprotein (AFP) and albumin. Further investigation revealed that iPSC-derived hepatic progenitor-like cells maintain potential of bipotent differentiation toward hepatocytic cells and cholangiocytic cells (77), and HLCs from iPSCs have the functional and proliferative capabilities needed for liver regeneration in mice (13). In Si-Tayeb's studies (61), HLCs differentiated from hiPSCs were injected directly into the hepatic parenchyma of newborn mice and exhibited human albumin expression 7 days later. However, ultimately, in vitro proof that mature HLCs have been produced need demonstration of functional hepatocyte properties such as nutrient processing, detoxification, plasma protein synthesis, and engraftment after transplantation into a suitable animal model (4). Our previous study (82) shows that, although HLCs generated from hiPSCs have the properties of primary hepatocytes, they were not as mature as primary hepatocytes because they presented lower albumin, incomplete urea cycle activity, lower CYP activity, immature mitochondria, and lower oxygen consumption than primary hepatocytes. HLCs also show persistent expression and high levels of AFP production (82), suggesting that HLCs exhibit an inability to turn off early stage gene(s) as the mechanism of persistent immature phenotype (78). Since the undifferentiated iPSCs have the potential to form teratomas, it is important to optimize the differentiation protocol, increase the differentiation efficiency, and remove the undifferentiated iPSCs before they are turned to clinical use. More importantly, iPSC–HLCs have demonstrated a capacity to pass stringent in vivo test as engrafting and maturing in animal models, as an ideal proof for their identity and functionality (78).

Furthermore, it is also required to produce HLCs in a large scale for clinical application. As mentioned above, generation of HLCs from iPSCs is a time-consuming procedure, and the monolayer culture of iPSCs and then HLCs cannot meet the need of future clinical application. Vosough et al. (72) reported their generation of functional HLCs from hiPSCs in a scalable suspension culture with rapamycin for "priming" phase and activin A for induction. After they were transplanted into the spleens of mice with acute liver injury, these HLCs could increase the survival rate, engrafted successfully into the liver, and continued to present hepatic function (i.e., albumin secretion after implantation). This amenable scaling up and outlined enrichment strategy provides a new platform for generating HLCs. This integrated approach may open new windows in clinical application or pharmaceutical industries.

To address the issue of maturation of HLCs from iPSCs, Ogawa et al. (52) showed that the combination of 3D cell aggregation and cAMP signaling enhanced the maturation of hiPSC-derived hepatoblasts to a hepatocyte-like population that displays expression profiles and metabolic enzyme levels comparable to those of primary human hepatocytes. Importantly, they also demonstrated that generation of the hepatoblast population capable of responding to cAMP is dependent on appropriate activin/nodal signaling in the definitive endoderm at early stages of differentiation. Together, these findings provide new insights into the pathways that regulate maturation of iPSC-derived HLCs and in doing so provide a simple and reproducible approach for generating metabolically functional hepatocytes.

Shan et al. (59) used a screening approach involving two different classes of small molecules to identify factors that induce the proliferation of mature primary human hepatocytes or induce the maturation of HLCs from hiPSCs. The first class induced functional proliferation of primary human hepatocytes in vitro. The second class enhanced hepatocyte functions and promoted the differentiation of iPSC-derived hepatocytes toward a more mature phenotype than what was previously obtainable. Gene expression profiles showed that small molecule-treated HLCs more closely resembled mature hepatocytes and marked increases in the amount of albumin and CYP, family 3, subfamily A (CYP3A) than untreated cells. Of particular interest was that AFP was largely absent. The identification of these small molecules can help address a major challenge affecting many facets of liver research and may lead to the development of new therapeutics for liver diseases.

ENGINEERING LIVER

Because even a small number of undifferentiated cells can result in teratoma formation, a key goal is inducing iPSC differentiation into target cell types without producing large numbers of undifferentiated cells. To date, no iPSC-derived differentiation protocol has succeeded in yielding full function transplantable and high-purity hepatocytes that fulfill both criteria: functional engraftment and response to proliferative stimuli in the diseased liver. Zhao et al. produced mice using iPSCs and tetraploid complementation (88), which can provide the liver organ for engineering liver, but tumor formation potential has not been completely eliminated.

Takebe and his team grew the liver organ using iPSCs, created by reprogramming human skin cells (67). The researchers first placed the iPSCs on growth plates in a specially designed medium; after 9 days, analysis showed that they contained a biochemical marker of hepatocytes. At that key point, Takebe added two more types of cell known to help to recreate organ-like function in animals: endothelial cells, which line blood vessels, taken from an umbilical cord; and mesenchymal cells. Two days later, the cells assembled into a 5-mm-long, 3D tissue that the researchers labeled a liver bud—an early stage of liver development (67).The liver bud transplants were examined histologically at multiple time points, and 32.9% of cells from the hiPSC-derived liver buds were albumin positive. These buds quickly hooked up with nearby blood vessels and continued to grow vigorously after transplantation. The vascular networks of liver buds were similar in density and morphology to those of adult livers and performed liver functions. The team says that the tissue's 3D will give it advantages over simple cell replacement therapies. It could be used for long-term replacement or short-term graft while the recipient waits for a suitable liver donor or in cases in which doctors anticipate that the native liver will eventually regain its function. Engineering liver technology takes iPSCs from research to the clinic.

THERAPY POTENTIALITY OF IPSCs IN LIVER DISEASES

In Hereditary Liver Disease

The liver is a vital organ that represents a promising target for cell therapy because of its ability to functionally integrate transplanted hepatocytes. A major indication for hepatocyte transplantation is inherited metabolic liver diseases in children. Hepatocyte transplantation has been performed as a treatment for inherited liver diseases, either for bridging to whole organ transplantation or for long-term correction of the underlying metabolic deficiency (15). Development of iPSCs from adult somatic tissues may provide a unique approach to create patient and disease-specific treatment for inherited liver diseases.

In the case of monogenic diseases, in which all the cells from the body initially carry the disease-causing mutation in their genomic DNA, a gene correction approach should be considered to generate disease-free autologous cells. This ex vivo gene correction step can be before or after (somatic cell) reprogramming. Thus, a combination of cell and gene therapy is used. Various differentiation methods for gene correction have been critically reviewed (17,63). iPSC-based cell therapies have been applied in several animal models of liver-based metabolic disorders, with encouraging results (11). Yusa et al. performed targeted gene correction of α1-antitrypsin deficiency (A1ATD) in iPSCs (84). They showed that a combination of zinc finger nucleases (ZFNs) and PB technology in hiPSCs can achieve biallelic correction of a point mutation (Glu342Lys) in the *A1AT* gene that is responsible for A1ATD. Genetic correction of hiPSCs restored the structure and function of A1AT in subsequently derived liver cells in vitro and in vivo. This approach is significantly

more efficient than any other gene-targeting technology that is currently available and crucially prevents contamination of the host genome with residual nonhuman sequences. Their results provide the first proof of principle for the potential of combining hiPSCs with genetic correction to generate clinically relevant cells for autologous cell-based therapies. Zhang et al. (86) describe the generation of iPSCs from a Chinese patient with Wilson's disease (WD) that bears the R778L Chinese hotspot mutation in the ATPase, Cu^{++} transporting, β polypeptide (ATP7B) gene. These iPSCs were pluripotent and could be readily differentiated into HLCs that displayed abnormal cytoplasmic localization of mutated ATP7B and defective copper transport. They performed gene correction using a self-inactivating lentiviral vector that expresses codon-optimized ATP7B, or treatment with the chaperone drug curcumin could reverse the functional defect in vitro. Hence, their work describes an attractive model for studying the pathogenesis of WD that is valuable for screening compounds or gene therapy approaches aimed to correct the abnormality. In principle, transplantation with genetically corrected iPSC-derived differentiated cells could be applied to any disease in humans for which the underlying mutation is known and that can be treated by cell transplantation (11).

In Liver Failure

The potential cases for cell-based therapies are ALF, acute on chronic liver failure, and acute decompensation after liver resection. Acute-on-chronic liver failure is defined as an acute deterioration of a chronic liver disease. HLCs derived from patient-specific iPSCs provide the unlimited source of hepatocytes to treat liver failure. Isobe et al. reported that in vivo transplantation of HLCs derived from hiPSCs can rescue rodents from lethal drug-induced ALF (21), and iPSC-derived HLCs have both the functional and proliferative potential for liver regeneration after transplantation in an ALF model (13).

Hepatocyte transplantation has become a tangible alternative for the treatment of end-stage liver disease. A major obstacle has always been the poor engraftment of the transplanted cells. Liu et al. (40) hypothesized that cotransplantation of iPSC-derived HLCs and mesenchymal stem cells (MSCs) could be an ideal choice for the treatment of end-stage liver disease, based on the facts of homogeneous differentiation of HLCs from iPSCs and paracrine effects by MSCs. The paracrine effects by MSCs have been proven to play an important role in liver regeneration and repair, and the effects can be used as an enhancer for HLC engraftment. Therefore, MSCs can be the best choice for a partner with iPSC-derived HLCs for cotransplantation. To some extent, iPSC-derived HLCs also induce MSCs into hepatocytes, and they may support each other. This hypothesis requires additional supporting data to be verified.

However, time to make, mature, and expand a patient's cells into iPSCs and then hepatocytes may be prohibitive for ALF treatment. Allogeneic source of hepatocytes is more practical, especially for use in ALF treatment.

In Bioartificial Liver (BAL)

The BAL system removes toxins by filtration or adsorption (artificial liver) while performing biotransformation and synthetic functions of biochemically active hepatocytes. BAL is an extracorporeal supportive therapy developed to bridge patients with liver failure to liver transplantation or to recovery of the native liver. To date, the different cell types that have been used in various BALs include primary porcine or human hepatocytes, cell lines, fetal liver cells, and stem cell-derived cells. Primary human hepatocytes are not available in sufficient amounts needed for clinical BAL usage and often retain their differentiated functions for a short duration in vitro. In addition, immunogenic reactions resulting from the xenogenecity of porcine hepatocyte products and the possibility of xenozoonotic retroviral infection of patients with porcine endogenous retrovirus (PERV) are concerns (but risk has never been quantified since no cases exist) using these cells. Continuous cell lines, as the other cell source, often lose functions that cells possess in vivo. Human ESCs and iPSCs, however, show great promise as cell sources for BAL devices. Optimization of the current hepatic differentiation protocols can allow the iPSCs to substitute for presently used cell sources. However, since a successful BAL depends on full maturity and functionality of hepatocytes, no reports of clinical BAL therapy from iPSC-derived HLCs yet exist.

In Liver Cirrhosis

Since chronic liver failure is caused by both hepatocellular damage or loss and fibrosis-induced cirrhosis, the therapeutic strategy should be based on replacing the damaged hepatocytes or hepatocyte regeneration and the correction of imbalanced fibrogenesis. Reports suggest that iPSC-differentiated HLCs enhanced liver regeneration in mice (13), reduced liver fibrosis in a mouse model (3), and stabilized chronic liver disease (9). Liver cirrhosis is also due to the disruption of tissue architecture and progressive accumulation of inflammatory cells and fibrosis. While "new" HLCs derived from iPSCs may temporarily support the impaired liver function, they would hardly be able to restore the original liver structure and eliminate collagen deposition. Hopefully, further precisely designed studies could bring about a practicable approach by which fibrogenic cells can be reprogrammed into hepatic parenchymal cells in the cirrhotic liver (8). A success of cell-based therapy in cirrhosis also includes

collagen deposition and readsorption, and the development of new antifibrotic agents, combined with effective antiviral agents for patients with viral hepatitis.

In Liver Disease Model

Liver tissue from patients is difficult to obtain and only reveals the disease aftermath, so several genetic disorders have been modeled in rodents and large animals (4). Although these models of human congenital and acquired diseases are invaluable, they provide a limited representation of human pathophysiology (54), and most animal models of human diseases are imperfect (4). Using diseased human primary hepatocyte cultures may overcome these problems in some sense and represent a platform. However, since under monolayer culture conditions primary hepatocytes lose their liver-specific functions within a few days, disease-specific iPSCs generated from patients who have inherited liver diseases and HLCs derived from such iPSCs represent a more rigorous platform and can be used as instruments to study the pathogenesis, disease mechanism(s), and possible cures for inherited liver disorders.

Although current animal models, including the toxic milk mouse, ATP7B2/2 mouse, and Long–Evans Cinnamon rat, have provided very useful representation concerning the pathogenesis of WD, physiological differences between species limit the conclusions. Zhang et al. reported establishment of an in vitro disease model using iPSCs from WD patients (86).

Monogenic metabolic disorders of the liver are an ideal platform to explore the complex gene–environment interactions and the role of genetic variation in the onset and progression of liver disease, and hiPSC technology provides an unprecedented opportunity to generate live cellular models of disease for therapeutic candidate discovery and cell replacement therapy. Rashid et al. derived iPSCs from skin fibroblasts of patients with A1ATD, glycogen storage disease type 1a, familial hypercholesterolemia, Crigler–Najjar syndrome type 1, and hereditary tyrosinemia. The iPSCs were then differentiated into a hepatic phenotype and were characterized with special attention to the phenotypic properties specific to the corresponding diseases (56). Ordonez and Goldstein used hiPSCs to model monogenic metabolic disorders of the liver (54). Despite some limitations, the large array of iPSC lines created in Ordonez's study should permit more in-depth studies of disease phenotypes, and the patient-derived HLCs from iPSCs can be used as suitable specific models to study the pathogenesis, mechanism(s), and possible treatment for inherited liver disorders.

To exclude compensation by hepatocytes not derived from iPSCs, Espejel et al. transferred wild-type mouse iPSCs into the embryos of fumarylacetoacetate hydrolase (FAH)-deficient mice to generate chimeric mice and demonstrated the ability of iPSCs to develop into HLCs in vivo and to protect the FAH-deficient mice from developing hepatic failure (13). In doing so, the authors were able to avoid the current limitations of in vitro hepatocyte differentiation protocols by allowing the iPSCs to undergo normal ontogenic development into mature hepatocytes in vivo. Although the correction of a metabolic disease by blastocyst injection of iPSCs cannot be translated to the treatment of human metabolic diseases, this study clearly demonstrates that iPSCs can give rise to fully functional somatic cells in vivo.

Research on hepatitis B virus (HBV) or hepatitis C virus (HCV) has been hampered by the difficulty in culturing human primary hepatocytes, which tend to differentiate and lose hepatic function after a limited period of in vitro culture. Thus, alternative models have been used. In vitro models using animal hepatocytes, human hepatocellular carcinoma (HCC) cell lines, or in vivo transgenic mouse models have contributed invaluably to understanding the pathogenesis of HBV and HCV (92). However, host tropism of HBV or HCV is limited to humans and chimpanzees. HBV and HCV infection have never been fully understood because there are few conventional models for hepatotropic virus infection. Yoshida's group (79) investigated the entry and genomic replication of HCV in iPSC-derived HLCs by using HCV pseudotype virus (HCVpv) and HCV subgenomic replicons, respectively. They showed that iPSC-derived HLCs, but not iPSCs, were susceptible to infection with HCVpv. The iPSC-derived HLCs expressed HCV receptors, and HCV RNA genome replication occurred in the iPSC-derived HLCs. Anti-cluster of differentiation 81 (CD81) antibody, an inhibitor of HCV entry, and interferon, an inhibitor of HCV genomic replication, dose-dependently attenuated HCVpv entry and HCV subgenomic replication in iPSC-derived HLCs, respectively. These findings suggest that iPSC-derived HLCs are an appropriate model for HCV infection.

Schwartz et al. reported that hiPSC-derived HLCs support the entire life cycle of HCV (57). iPSC-derived HLCs are also a promising system for drug discovery for HCV infection. The iPSC-derived HLCs can be used with popular models of HCV infection: HCV subgenomic replicons and HCVpv. Their findings will contribute to our understanding of the mechanisms of HCV infection and to the identification of novel targets for HCV therapy by means of iPSC technology.

Taken together, the generation of disease-specific iPSC lines potentially enhances the research and development of strategies for liver diseases.

In Drug Discovery and Hepatotoxicity Screening

An added benefit of iPSCs is that they can be used for drug-screening research. A multitude of potential new

drugs need to be efficiently screened for hepatotoxicity every year, while a major challenge for drug discovery is to develop appropriate preclinical models. Human primary hepatocytes have become a major liver model for hepatotoxicity tests. Unfortunately, as mentioned above, there is also a shortage of primary hepatocytes, and it is difficult to culture the hepatocytes in vivo without losing their special function. Today approximately 70% of the top 20 pharmaceutical companies utilize stem cells in their research, and among these, 64% use human ESCs or their derivatives. Human ESCs and their derivatives do not encompass all the variances within a population or all ethnicities. Alternatively, such cells for drug screening could be obtained from iPSC-derived HLCs. Since cells from many patients with different metabolism phenotypes can be tested, hiPSC-derived HLCs have been expected to be used for individual new drug screening and optimization of patient-effective therapy and side effects of compounds during the drug discovery process (44,56).

Moreover, the potential to make genetically corrected hiPSCs from diverse genetic subtypes from any number of diseases also allows for the development of reliable models for studying the development and progression of genetic diseases in vitro (10,93). For example, disease-causing gene correction in hiPSCs would make a "perfect control" for pharmaceutical in vitro comparative studies.

In Liver Cancer

Moriguchi et al. (46) first generated the patient-specific HLCs differentiated from hiPSCs of the patients with HCC and found the hiPSCs could transform into human hepatoma-like cells again during the differentiation induction process to normal HLCs through the knockdown of cyclin-dependent kinase inhibitor 1 (p21). Then, based on their hypothesis that an aldo-ketoreductase family 1, member B10 (AKR1B10) inhibitor could be used to enhance the differentiation effects of retinoic acid, they investigated the efficacies of a cyclic retinoid plus tolestat as an AKR1B10 inhibitor therapy for the human hepatoma-like cells. Their results demonstrated a cyclic retinoid (10 μM) plus tolestat (10 μM) would be appropriate regimens for human hepatoma-like cells. However, by using the patient-specific HLCs differentiated from hiPSCs of the patients with HCC, the efficacies and toxicities of the above-mentioned combination therapy for the individual patients with HCC will be evaluated more specifically in the near future.

Lei et al. (32) showed highly reactive antigen-specific cytotoxic T lymphocytes (CTLs) can be generated from iPSCs to provide an unlimited source of functional CTLs for adoptive immunotherapy in breast cancer. In principle, this approach could be applied to liver cancer, and their study provides a valuable advance for studying and treating other cancers.

In Liver Transplantation

Liver transplantation is the only proven treatment for most end-stage inherited metabolic liver disorders and liver failure including ALF, acute-on-chronic failure of a cirrhotic liver, and acute decompensation after liver resection. Unfortunately, there is a serious shortage of transplantable donor organs so many patients die before transplantation or become too sick to transplant. Therefore, it is hoped that HLCs derived from patient-specific iPSCs will provide an autologous source of hepatocytes to treat metabolic liver disorders or liver failure, either prior to liver transplantation as an extracorporeal supportive therapy to bridge patients with liver failure to liver transplantation or to recovery of the native liver.

Alternatively, living donor liver transplantation (LDLT), utilizing a right or left lobe from a living donor, has been developed to increase the number of donor livers. Size mismatch of the graft to recipient remains a major risk factor contributing to postoperative graft dysfunction (5,17,30). The degree of graft damage is inversely related to graft size, an association recognized as small-for-size syndrome (SFSS) (12). Small-for-size liver grafts are more susceptible to reperfusion injury, including microcirculatory damage (42), severe inflammatory responses (33), and accelerated acute rejection (60). As a result, the incidence of coagulopathy, ascites, prolonged cholestasis, and encephalopathy are higher after use of small-for-size grafts. Moreover, small-for-size liver grafts show delayed and impaired regeneration required for the recovery of hepatic mass after LDLT (73,89), therefore, supporting liver function in the early posttransplant period when liver function decompensation will increase the survival (83). HLCs from patient-specific iPSCs provide the ideal cell source for liver function supporting. Our team is performing the project to treat small-for-size liver transplants with iPSC-derived HLCs with a rat model and have obtained some interesting data. If the support potential of iPSCs is verified in this model, it will be a promising alternative to treat SFSS and helpful to improve survival in transplantation settings.

Moreover, engineered donor grafts derived from iPSCs, including recellularized biomatrix (71) and liver buds produced from iPSCs (67), may someday provide organs for liver transplantation.

CONCLUSIONS AND PERSPECTIVES

iPSCs present possibilities for in vitro modeling, improving research on diseases, drug development, tissue engineering, the development of BAL, and a foundation for producing autologous cell therapies that would avoid immune rejection and enable correction of gene defects prior to cell transplantation. Therefore, iPSCs would open up new opportunities for medical advances. However, there are still several major obstacles that need to be overcome before iPSCs will reach the bedside. These

include (i) evaluation of different types of original cells and improved efficiency of iPSCs generation without viral integration, (ii) avoiding the use of animal feeders to culture hiPSCs, (iii) developing a differentiation protocol that can produce adult cell types whose maturity and functionality are comparable to their in vivo counterparts, (iv) removing undifferentiated iPSCs and other cell types that have the potential to form tumors in vivo, and (v) a quick generation and differentiation protocol for emergent use. Additionally, it has been reported recently that transplantation of undifferentiated iPSCs demonstrated a T-cell-dependent immune response in recipient syngeneic mice due to the abnormal expression of antigens following genetic manipulation (87). Therefore, critical aspects need to be further addressed, including the long-term safety, tolerability, and efficacy of the iPSC-based treatments, as well as their carcinogenic potential. It is paramount to conduct larger and well-designed clinical trials to fully establish the safety profile of such therapies and to define the target patient groups with efficacy assessed by standardized protocols. Taken together, the human iPSC–hepatocytes represent a promising tool in many aspects of research in liver disease and regenerative medicine.

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