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PLURIPOTENT STEM CELLS (J ROY-CHOWDHURY, SECTION EDITOR)

Induced Pluripotent Stem Cells as a Source of Hepatocytes

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Abstract During the past decade, a series of discoveries has established the potential of the so-called terminally differentiated cells to transition to more primitive progenitor cells. The dramatic demonstration of the ability to reprogram differentiated somatic cells to induced pluripotent stem cells (iPSC) that can then give rise to cells of all three germ layers has opened the possibility of generating virtually any cell type in culture, from any given individual. Taking advantage of these concepts, researchers have generated iPSC by reprogramming a wide variety of somatic cells. In addition to their practical implications, these studies have provided crucial insights into the mechanism of cell plasticity that underlies the transition from one cell type to another. Using concepts derived from research on embryological development, investigators have differentiated iPSC to cells resembling hepatocytes in many ways. Such hepatocyte-like cells could be of enormous value in disease modeling, drug discovery and regenerative medicine. However, the currently available methods do not yield cells that fully reproduce the characteristics of adult primary hepatocytes. Thus, generating hepatocytes from iPSC is very much a work in progress. In addition to chronicling these exciting developments, this review will discuss the emergent new approaches to generating iPSC, improving their differentiation to hepatocyte-like cells, and maintaining the hepatocyte-like cells in culture for longer survival and better function.

Keywords iPS · Reprogramming · Hepatocyte-like cells · Directed differentiation

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Introduction

In mammals, the liver plays a pivotal role for diverse functions, including protein synthesis, metabolism, detoxification and excretion. Reproducing all or most of these functions in isolated liver cells is a major challenge. Availability of viable, functional hepatocytes would have been highly beneficial for pharmacological evaluation, creating cellular models for pathophysiological analysis of diseases, generating bioartificial liver support and regenerative therapy of the liver. Orthotopic liver transplantation can replace virtually all liver functions and rescue patients with acute and chronic liver failure, as well as monogenic liver diseases, such as Crigler–Najjar Syndrome type 1, alpha-1 antitrypsin deficiency, primary hyperoxaluria, etc. Because liver transplantation is a formidable and expensive procedure, and is dependent on the immediate availability of livers, hepatocyte transplantation is being explored as a minimally invasive alternative to organ transplantation for many of these disorders. However, the severe shortage of donor livers, which are normally prioritized for organ transplantation, drastically limits the availability of usable

livers for isolating primary hepatocytes. The problem is compounded by the fact that primary hepatocytes rapidly deteriorate in function in culture and their viability after cryopreservation is extremely variable. Therefore, there is a great need for alternative renewable sources of human hepatocytes. Tissue stem cells, such as mesenchymal and hematopoietic stem cells, liver progenitor cells and pluripotent stem cells (PSC), are being evaluated as sources of human hepatocytes. This review will focus on the different approaches for generating induced pluripotent stem cells (iPSC) from normal or patient-specific somatic cells and their differentiation into hepatocyte-like cells.

Pluripotent Stem Cells for Disease Modeling and Regenerative Medicine

PSC can give rise to all cell types of the body, and therefore offer great promise in disease modeling, drug development and regenerative medicine (Fig. 1). Much progress has been made taking advantage of the unlimited proliferation capacity, plasticity and pluripotency of human embryonic stem cells (hESC) [1•]. The landmark work of Takahashi and Yamanaka [1•, 2•] that led to the reprogramming of somatic cells to iPSC has provided several potential advantages over using ESC. First, ethical concerns are mitigated, as no embryo needs to be destroyed for generation of these cells. Second, iPSC can be generated from individual patients, permitting the development of 'personalized' cellular models of genetic diseases. Finally, autologous cells differentiated from iPSC from patients with monogenic disorders could be used for regenerating organs after correction of the genetic defect, thereby circumventing the need for immune suppression.

Reprogramming Somatic Cells to iPSC

Since Yamanaka and associates generated the first iPSC from mouse fibroblasts in 2006, various additional methods have been reported for reprogramming somatic cells to iPSC. Typically, these methods include expression of the four so-called Yamanaka pluripotency factors, Oct3/4, Sox2, Klf4 and c-Myc (OSKM). As described later in this section, recently an alternative approach has been described based on expression of specific microRNAs that may affect the transcription of multiple genes simultaneously.

Somatic Cells for Reprogramming

Investigators have used many different somatic cell sources for reprogramming into iPSC (Fig. 2). Skin fibroblasts have been used most commonly [2•], but other cells such as peripheral blood cells [3, 4], chord blood endothelia [5], adipose-derived stem cells [6], neural stem cells [7], hepatocytes [8], keratinocytes [9], pancreatic β-cells [10•], amniotic cells [11] or renal tubular epithelial cells shed in the urine [12] can also be used for reprogramming into iPSC. Issues to be considered in selecting the somatic cell source include the ease of collecting the cells, epigenetic memory that may lead to retention of some characteristics of the somatic cells in the derived iPSC, and the possibility of acquired genetic mutations or rearrangement in the somatic

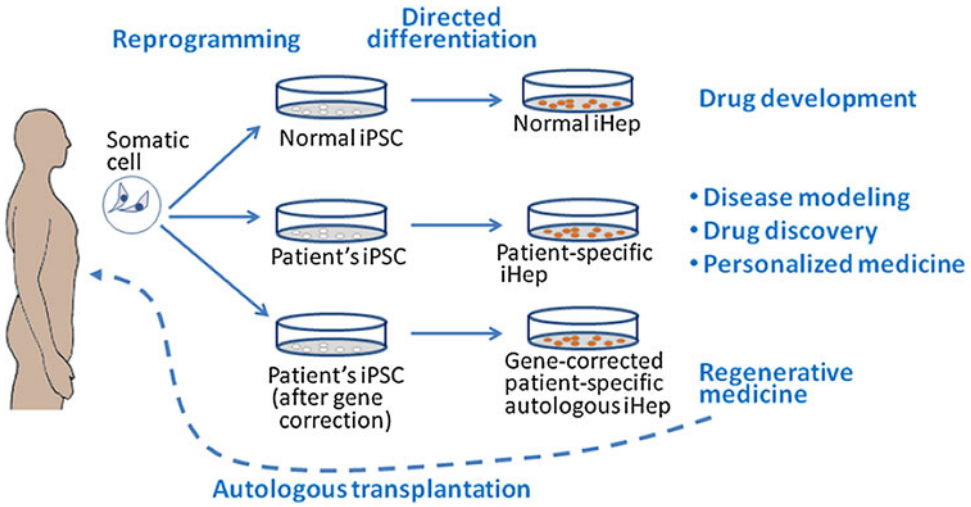

Fig. 1 Generation of iPSC and differentiation to hepatocyte-like cells (iHeps). Somatic cells are collected from normal subjects or patients with liver diseases with known or unknown genetic basis by biopsy, blood drawing, hair plucking or urine collection. The somatic cells are reprogrammed to iPSC by approaches summarized in Fig. 2. The iPSC are differentiated to iHeps with or without gene correction, as indicated, by methods summarized in Fig. 3. The procedures and putative uses are shown in *blue font* (Color figure online)

cells. For example, umbilical chord blood epithelial cells would be a highly desirable cell type for reprogramming; however, such cells are usually not available in many patients by the time the diagnosis of an inherited disease is made. Tissue stem cells derived from adipose tissue and bone marrow are also excellent cells to reprogram, but invasive procedures are required to obtain them. A less invasive source is venous blood. However, somatic genetic recombination, such as the random combinations of the variable, diverse, and joining gene segments [V(D)J recombination] in T and B lymphocytes remains a concern. Therefore, investigators are exploring the use of peripheral blood hematopoietic stem cells, which are normally present in very small numbers. Skin fibroblasts are the most common cells used for iPSC generation. These cells are obtained by skin biopsy, which is a micro invasive procedure. An additional concern relates to the life-long exposure of the skin cells to environmental pollutants, chemicals and UV rays that may potentially cause somatic cell mutations. The incidence of this has not been determined systematically. Recently, renal tubular epithelial cells shed in the urine have been utilized for iPSC generation. Collection of these cells is completely non-invasive. It should be noted that all somatic cells can acquire somatic mutations, which can persist in the derived iPSC, even after removing the epigenetic marks. To what extent this may affect the characteristics of the iPSC, in terms of their ability to be differentiated to desired target cells, remains to be determined.

Approaches to Reprogramming

Two basically different approaches have been used by a number of investigators for reprogramming somatic cells to iPSC (Fig. 2). The original approach is based on the landmark work by Takahashi and Yamanaka [2•], which showed that expression of only four transcription factors, OCT3/4, KLF4, SOX2 and MYC, which are now known as Yamanaka factors (Y4), can reprogram somatic cells to iPSC. A number of strategies have been used to achieve the expression of these factors. A completely different approach is based on expression of specific microRNAs, which affect the expression of multiple genes simultaneously. These approaches are discussed in brief below.

Reprogramming Based on Expression of Yamanaka Factors

In their original study, the Yamanaka laboratory used Moloney's murine leukemia retroviral vectors, which integrate into the cellular genome. Gamma-retroviruses, including the Moloney Leukemia virus, are replication-defective because of the deletion of genes encoding proteins for virus replication and packaging. These vectors infect only dividing cells [13], which reduces the efficiency of reprogramming. Subsequently, expression of the exogenous transgenes is silenced, but in the meantime the reprogrammed cells continue to express endogenous pluripotency factors. However,

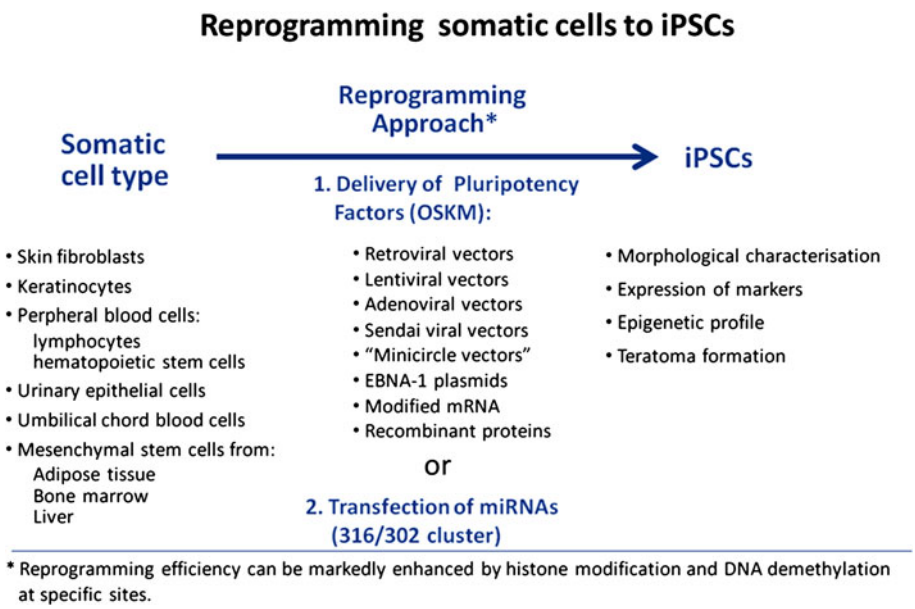

Fig. 2 The different types of somatic cells reprogrammed to iPSC by various investigators are listed. It appears that any dividing cell has the potential to be reprogrammed. The original reprogramming method is based on delivery of the four Yamanaka pluripotency factors, OCT3/4, SOX2, KLF4 and MYC. The means to achieve this has been listed. An alternative approach is based on the expression of microRNA 316/302 cluster (see text). Once colonies with morphological characteristics and cell surface phenotype of pluripotent cells are obtained, these are passaged multiple rounds and tested for expression of marker genes and teratoma formation

the transgenes remain integrated into the host cellular genome at random sites. In contrast, vectors based on a different class of retroviruses, termed lentiviruses, can infect non-dividing cells and have been shown to successfully generate iPSC [14]. To reduce the possibility of activation of oncogenes by the randomly integrated proviral genome, vectors have been designed to permit the removal of the exogenous genes using cre recombinase [15]. However, this system may result in incomplete recombination, owing to insufficient expression of Cre recombinase. Another highly efficient integration-based method utilizes the piggyback (PB) transposons. The advantage of this system is that it requires only an active transposase and 13 bp inverted terminal repeats (ITR) for insertion and excision of the reprogramming cassette. The transgenes can be eliminated seamlessly after reprogramming, without leaving any residual nucleic acid sequences in the genome [16].

Despite these design improvements, random integration of proviruses is considered to increase the risk of tumor development in future clinical applications [17]. Therefore, to improve the safety, other methods have been designed that should provide transgene-free iPSC. These methods include transfection of modified mRNAs, delivery of recombinant transcription factor proteins, infection with recombinant episomal viruses (e.g. adenoviruses and Sendaï viruses), and transfection of "minicircles" from which the bacterial component of plasmids is removed [18] or episomal plasmids containing Epstein–Barr viral sequences.

Plews et al. [19] first achieved reprogramming by transfecting in vitro transcribed modified mRNA encoding five pluripotency factors. To increase the half-life of the transfected mRNA by avoiding the cellular interferon response that normally results after RNA transfection, cytidine and uridine residues are replaced with 5-methylcytidine and pseudouridine, respectively. In addition, an interferon receptor mimetic, B18R/B19R, is expressed to reduce interferon binding to its receptor. To further improve the efficiency of this system, other groups have added IRES sequences and strong translational initiation signals in the 5′UTR, and a polyA signal at the 3′UTR [20]. However, despite the mRNA modification to inhibit RNA degradation, this method requires repeated transfection, which may be harmful for more sensitive primary cells.

Reprogramming has also been achieved by direct delivery of reprogramming proteins (OSKM). The major hurdle in this strategy is delivering the proteins across the cell membrane. Peptides rich in arginine or lysine, termed cell penetrating peptides (CPP) [21, 22], such as a peptide fragment of the human immunodeficiency virus transactivator of transcription (HIV-TAT), have been tagged on to the transcription factors to achieve transmembrane delivery. The reprogramming efficiency of this method was low [7], probably because of the need to transfer large amounts of the recombinant transcription factors and a relatively short dwell time of the proteins in the dividing cells.

Recombinant adenoviruses can transduce a large variety of cells from various species and can be generated at high transduction efficiency [23]. Adenoviral vectors are episomal, and integration of the transgene into the host genome is extremely infrequent, but not inexistent [24]. Being episomal, adenoviral vectors are rapidly lost in dividing cells, and repeated infection is needed. Unfortunately, the efficiency for generating iPS cells from primary human cells is much lower compared to mouse fibroblasts [25], which may be related in part to the species difference in the cell surface expression of the adenoviral receptor (Coxsackie adenovirus receptor) [26].

Another non-integrative strategy using recombinant Sendaï virus (aka Hemagglutinating Virus of Japan, HVJ) was first reported by Li et al. [27]. The Sendaï virus is a single-stranded RNA virus of the paramyxovirus family, which differs from other viral vectors in that its entire replication cycle occurs within the cytosol, virtually eliminating the possibility of integration into the genome. A single infection with recombinant Sendaï viruses expressing the pluripotency factors results in a high frequency of reprogramming of human primary somatic cells [28, 29, 30, 31•].

Episomal plasmid vectors offer an efficient integration-free method of somatic cell reprogramming. Conventional plasmids are diluted and lost from dividing cells after transfection, requiring repeated transfection and resulting in a low efficiency of reprogramming [32, 33•, 34]. To overcome this shortcoming, episomal vectors containing oriP/EBNA1 (Epstein–Barr nuclear-antigen 1) have been developed that can replicate during cell division for about six cycles [35]. The Yamanaka laboratory has refined the episomal vector system by developing a set of three plasmids that express OCT3/4, SOX2, KLF4, L-Myc and Lin28, in addition to a shRNA that suppresses p53 expression [33•].

Reprogramming Methods Based on Overexpression of microRNAs

MicroRNAs (miRNAs) can affect the expression of multiple genes in a coordinated manner, and are emerging as important regulators of cell function. Somatic cell reprogramming with miRNAs represent the first alternative to overexpression of transcription factors for generating iPSC. miRNAs that are expressed preferentially in ESC are thought to help maintain the pluripotent cell phenotype [36•, 37•]. Several microRNAs (miRNAs), including the *miR302/367* cluster, have been reported to enhance the efficiency of transcription factor-based reprogramming of somatic cells [38•]. The *miR302/367* cluster, which is

highly expressed in pluripotent cells, consists of five miRNAs located in intron 8 of the Larp 7 gene on chromosome 3 that are transcribed as a single polycistronic transcript [39•]. Four of these microRNAs (*miR301a, b, c and d*) have identical seed sequences. The *miR302/367* cluster is highly conserved across species, and its expression is induced by the pluripotency transcription factors, OCT3/4 and SOX2. In turn, expression of the *mir302/367* cluster activates endogenous OCT3/4 expression following reprogramming. Pathway analysis highlighted potential relevant effectors, including mesenchymal-to-epithelial transition, cell cycle, and epigenetic regulators. The *miR302-367* targets TGFβ receptor 2, promotes E-cadherin expression, accelerates mesenchymal-to-epithelial transition and promotes cell division [40•]. In fact, retrovirus-mediated expression of this gene cluster alone has been reported to be sufficient in reprogramming both mouse and human somatic cells [41•]. It is now well recognized that chromatin remodeling is essential in reprogramming. In this context, several laboratories have reported a potent and cooperative role of the inhibition of histone deacetylase 2 by valproic acid in *miR302/367*-mediated reprogramming [38•, 40•, 41•].

Differentiation of iPS into Hepatocytes

Generation of iPS-derived hepatocytes not only serves as a source for potential therapeutic application in human liver diseases, but also enables the understanding of inherited liver diseases by providing cell-based pathophysiological models in vitro. iPSC generated from individual patients with several monogenic liver diseases have been shown to reflect several aspects of the pathologic phenotype of patients, and can potentially provide deeper insights of disease processes and expose new therapeutic targets [42].

Comprehension of the molecular correlates of liver development during embryogenesis has contributed tremendously to the understanding of the differentiation processes [43, 44]. In mammalian embryos, the ventral foregut endoderm is the tissue from which the liver originates. Thus, stepwise induction of definitive endoderm, followed by hepatic progenitors, and finally mature hepatocyte-like cells are the three essential consecutive processes through which (PSC) must pass in order to attain hepatocyte-like phenotypes (Fig. 3). A number of protocols have been established to produce hepatocyte-like cells from both human ESC and iPSC [45•]. The initial step of generating with the phenotype of definitive endoderm has turned out to be crucial in hepatocyte differentiation. Critical requirement for this step is exposure to the transforming growth factor β (TGF-β) superfamily members activin A and bone morphogenic protein 4 (BMP4) [46–49]. A short exposure to Wnt3a, which is expressed at critical stages of human liver development and specifically interacts with activin A [50], enhances the production of definitive and hepatic endoderm. Fibroblast growth factors (FGF), in combination with BMP4, contribute to the definitive endoderm cell commitment at a later stage of embryonic development [51, 52]. An additional important discovery was that the effect of all factors that stimulate early mammalian development is inhibited in the presence of fetal bovine serum in culture medium [53]. It is now clear that the interplay of activin A, Wnt3a, FGF2 and BMP4 plays a major role in determining the early cell fate of pluripotent stem cells toward definitive endoderm. Specific marker genes that are expressed during embryonic development of definitive endoderm include SRY (sex determining region Y)-box 17 (Sox17) and forkhead box A2 (Foxa2). Expression of these genes specifies foregut endoderm, which subsequently gives rise to pancreatic and hepatic cells [54].

The next step toward hepatocyte generation is the developmental induction of hepatic progenitor cells or hepatoblasts from the definitive endoderm. In cell culture, this is accomplished by adding specific growth factors, of which hepatocyte growth factor (HGF) appears to be the most important [55, 56]. Hepatocyte nuclear factor 4 alpha (HNF4α) is a transcription factor that is expressed initially in the developing hepatic diverticulum. HNF4α expression increases during liver development, and marks the differentiation toward hepatocyte lineage during in vitro differentiation [57]. Another important marker of hepatic

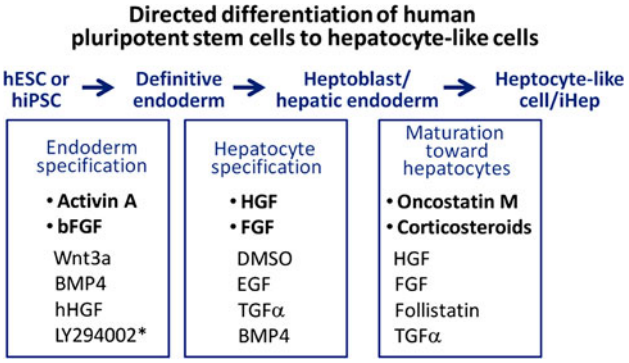

Fig. 3 The general strategy for directed differentiation of iPSC to hepatocyte-like cells is to emulate the stages of ontogenic development of the liver. Pluripotent cells (hESC and hiPSC) may be differentiated in monolayer cultures or after conversion to embryoid bodies. The first critical step is differentiation to definitive endoderm-like cells. The next step is to achieve hepatocyte specification, which gives rise to hepatoblast-like cells. Finally, attempt is made to induce maturation to cells as close to primary hepatocytes as possible. Factors that are considered to be critical by multiple investigators are *bulleted* and *bold faced*. Other factors that have been reported to be helpful are also listed. LY294002 is an inhibitor of phosphatidylinositide 3-kinases (PI3-kinase)

progenitor cells is the serum glycoprotein alpha-fetoprotein (AFP), which is expressed in primitive hepatocytes [58].

The final step of the differentiation process is the induction of hepatocyte maturation. In culture this has been achieved by adding oncostatin M (OSM), an interleukin-6 family cytokine, in combination with glucocorticoids [59] to the cell culture medium. Differentiation of hepatocyte-like cells is associated with assumption of hepatocyte-like morphology and intracellular glycogen accumulation. The strategy for changing the liver lineage cells from a hepatoblast/fetal hepatocyte phenotype to cells with adult primary hepatocyte-like characteristics, involves mimicking the molecular/physiological changes that occur during perinatal and neonatal life. Some hepatocellular functions, such as albumin and apolipoprotein synthesis, and urea production, are already near the adult level during late fetal life. Other functions, such as bilirubin glucuronidation and alpha-1 antitrypsin synthesis, are at a very low level at birth, and are stimulated after birth by various factors, including perinatal hormonal changes, increase of portal blood flow and blood oxygen tension after the closure of the ductus venosus, elevation of plasma glucagon and catecholamine levels, and possibly many other changes. Epigenetic modification of DNA during perinatal life plays an important role in the expression of adult hepatocyte-specific genes [60]. Some forms of UDP-glucuronosyltransferase and sulfotransferases reach adult levels only at adolescence, coincident with the surge of sex hormones. As the underlying mechanisms of hepatocyte maturation are not fully known, simulating the perinatal changes in vitro is challenging. On the other hand, the empirically gained knowledge of differentiating cells from the hepatoblast stage to the final hepatocyte-like cells offers a model for progression of hepatocyte maturation.

Expression of marker genes that are generally evaluated at this stage includes albumin, CK18, cytochrome p450 enzymes (CyP), α1-antitrypsin (ATT), asialoglycoprotein receptor 1 (ASGPR), C/EBPα, UGT1A1 and Prox1. In addition to the marker gene expression profile, functional in vitro assays are carried out to further evaluate the features of hepatocyte-like cells; for example, urea production, the uptake of indocyanin green and low-density lipoprotein (LDL), inducible cytochrome P450 activity, and secretion of albumin and alpha-1 antitrypsin into the medium. Primary hepatocytes are usually used for comparison in these in vitro assays. However, a high quality of primary human hepatocytes needs to be assured for this purpose [61].

For a comprehensive list of cell culture components used by various investigators for the stepwise directed differentiation of iPSC to hepatocyte-like cells, see the review by Han et al. [45•].

Improving Differentiation of iPSC-Derived Hepatocyte-Like Cells

Despite the effort of many investigators, differentiation of human iPSC to cells equivalent to primary hepatocytes have not been achieved yet. It is generally stated in literature that the iPSC-derived hepatocytes have fetal hepatocyte-like characteristics. However, although these cells have some characteristics of fetal hepatocytes, they also express some genes, such as uridinediphosphoglucuronate glucuronosyltransferase-1 (*UGT1A1*) and alpha 1-antitrypsin (*SERPINA1*). Most hepatocyte-preferred genes are expressed at much lower levels than in primary hepatocytes. Upon transplantation into the liver of immunodeficient or immunosuppressed hosts, these cells engraft with much lower efficiency than the primary hepatocytes. Moreover, as in cultured primary hepatocytes, expression of liver-specific genes in iPSC-derived hepatocyte-like cells declines rapidly. Therefore, much effort is underway to improve the differentiation and maintenance of iPSC-derived hepatocyte-like cells, some of which are discussed below.

Forced Expression of Transcription Factors

In addition to exposure to cytokines and other chemicals, developmental stage-specific transcription factors have been expressed in the cells at various steps of differentiation. Thus, adenovector-mediated expression of SOX17 [62], HEX [63] and HNF4 [64] has been used to improve the differentiation to definitive endoderm, hepatoblasts and hepatocyte-like cells, respectively. Therefore, it may be possible to use sequential transient expression of transcription factors, or perhaps microRNAs, to achieve a more desirable phenotype of hepatocyte-like cells. Until iPSC-derived hepatocytes with morphology and gene expression patterns very similar to adult primary hepatocytes are achieved, it may be necessary to pay special attention to characteristics specifically required for a given application. For example, the nuclear receptor, constitutive androstane receptor (CAR), regulates the expression of multiple gene products involved in the detoxification of endogenous metabolites, drugs and other xenobiotics. It has been reported that permanent transduction of the iPSC-derived cells at a late stage of differentiation with CAR yielded hepatocyte-like cells that exhibited high levels of xenobiotic detoxification functions [65].

Maintenance of the iPSC-Derived Hepatocyte-Like Cells in Culture

Within the liver, hepatocytes form chords with other hepatocytes and exist in the close vicinity of non-parenchymal

cells, such as hepatic sinusoidal endothelial cells, stellate cells and Kupffer cells. Clearly, the native liver matrix, the three-dimensional structure of the liver and cross-talk with non-parenchymal cells all play important roles in maintaining the viability and gene expression characteristics of hepatocytes in situ. Therefore, it is unrealistic to expect either primary hepatocytes or iPSC-derived hepatocyte-like cells to retain their function in "minimalistic" monolayer cultures. Based on this consideration, investigators are attempting to partially recreate the spatial organization of the liver to support the function and viability of iPSC-derived hepatocytes [66]. Primary hepatocytes and hepatic sinusoidal epithelial cells appear to support each other in three-dimensional co-culture [67]. These principles have been applied to iPSC-derived hepatocyte-like cells, showing that co-culturing with endothelial and stromal cells favor the maturation of hepatocyte-like cells by cell–cell contact or via paracrine factors [68]. Recently, hepatocyte-specific definitive endoderm was cultured with human umbilical vein endothelial cells and mesenchymal stem cells (MSC), resulting in three-dimensional cellular clusters in which the iPSC-derived cells expressed alpha-fetoprotein, albumin and other hepatocyte-preferred genes, indicating that cluster formation promoted maturation toward a hepatocyte phenotype. When implanted intracranially, in the small bowel mesentery, or under the renal capsule of immune-deficient mice, the clusters became vascularized and proliferated for 2 months. The engrafted cell clusters secreted human albumin and alpha-1-antitrypsin into the host plasma. Furthermore, the cells exhibited cytochrome P450 activity, and improved the survival of mice subjected to toxic hepatic injury. This model partially recreates the liver environment, but does not provide a drainage system, namely bile ducts. Other investigators have attempted to provide a more complete liver-like scaffold to isolated hepatocytes by decellularizing whole livers by detergent perfusion, and then populating the scaffold with primary human hepatocytes and endothelial cells [69, 70]. Although these scaffolds were generated with the purpose of transplanting primary hepatocytes, a similar environment could potentially enhance the differentiation and survival of iPSC-derived hepatocyte-like cells.

In summary, the advent of reprogramming somatic cells to iPSC and directed differentiation of these cells to hepatocyte-like cells offers great promise for applications in pathophysiological studies, pharmacological testing and regenerative medicine. Differentiation of the iPSC toward hepatocyte phenotype can be considered a "work in progress" at this time, but research by many groups worldwide is producing creative and original solutions for overcoming a myriad of existing hurdles, and to eventually translate these exciting developments for pharmacological, pathophysiological and therapeutic applications.

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Compliance with Ethics Guidelines

Conflict of Interest Vanessa Sauer, Namita Roy-Chowdhury, Chandan Guha and Jayanta Roy-Chowdhury declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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