

Induction of Hepatocyte Differentiation in Human Pluripotent Stem Cells

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ABSTRACT

Culturing of hepatocytes is used in both experimental and clinical procedures. Human induced pluripotent stem (hiPS) cells can be established from the somatic cells of individual patients. The *In vitro* production of hepatocytes from these hiPS cells would

be advantageous in that it could resolve the ethical problems associated with stem cell research and could eliminate the need for administration of immunosuppressants in hepatocyte transplantation. To establish a method for the production of hepatocytes from hiPS cells, it is necessary to understand both the mechanism of hepatocyte differentiation as well as the current techniques for culturing of primary hepatocytes. Meanwhile, hepatocytes can also be differentiated from endodermal cells upon stimulation with growth factors that induce expression of specific transcriptional regulators. Current protocols for the generation of hepatocytes from hiPS cells include the sequential application of growth factors to mimic the environment of fetal liver. Initially, cells are treated with activin and bone morphogenetic proteins, followed by the application of hepatocyte growth factor and, at the final differentiation stage, Oncostatin M. Expression of transcription factors is necessary for hepatocyte differentiation. However, because the efficiency at which vectors expressing these factors can be transfected into hiPS cells is disappointingly low, adenoviral vectors have been used, which have a transduction efficiency of 100%. This method is used to sequentially introduce sex-determining region Y (Sry) HMG box (Sox) 17, hematopoietically expressed homeobox, and hepatocyte nuclear factor-4 α into hiPS cells. These factors, in concert with the stimulation with growth factors, promote differentiation of these cells to hepatocytes. Despite the development of these procedures, however, the resulting cells remain immature and only perform certain hepatocyte functions. In this review, we describe each of the above points, and then discuss novel approaches and future directions for the *In vitro* production of hepatocytes.

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Key words: Transcription factor; Growth factor; Drug metabolism; Transplantation

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INTRODUCTION

The liver is a single large organ, and hepatocytes account for approximately 70-80% of its cells. These liver-specific cells play a major role in protein synthesis, glucose metabolism, and detoxification. Notably, even when a portion of the hepatocytes are lost, the remaining cells maintain the ability to proliferate^[1]. Indeed, one of the ideal methods for the treatment of loss of liver tissue is transplantation of hepatocytes^[2].

Human induced pluripotent stem (hiPS) cells are generated with cellular reprogramming factors^[3], and have the potential to differentiate into a variety of cell types. Because hiPS cells can be generated from each patient individually, the use of these cells for treating liver ailments could avoid potential ethical complications as well as the development of graft-versus-host disease. Therefore, hiPS cells may comprise an ideal cell source for patients needing transplantation of liver tissues. As a result, the development of methods for producing hepatocytes from hiPS cells is currently underway.

In this review, we begin by discussing current methods for the culturing of primary hepatocytes. This information is also applicable to the maintenance of hepatocytes differentiated from hiPS cells. Subsequently, we discuss the applications for cultured hepatocytes, as these applications could provide potential uses for the hiPS cells. The mechanism governing hepatocyte differentiation has been applied to the *In vitro* production of hepatocytes from hiPS cells. This cellular mechanism as well as the current protocols for *In vitro* differentiation of hiPS cells into hepatocytes will therefore be summarized. Despite the development of these protocols, however, the production of functioning hepatocytes from hiPS cells remains technically challenging. As such, the limitations of these differentiation methods will be discussed. Lastly, we present novel approaches for *In vitro* hepatocyte production.

CULTURING OF PRIMARY HEPATOCYTES

The culturing of hepatocytes can be applied to drug development, cell-mediated therapies, and *In vitro* disease models. Indeed, primary hepatocyte culturing is an ideal *In vitro* model for investigating drug metabolism and toxicology, and primary hepatocytes can be transplanted into patients experiencing liver failure^[4]; however, the culturing of these cells remains technically difficult. Hepatocytes are typically isolated from a fragment of resected donor liver using a 2-step collagenase perfusion technique^[4]. Fetal hepatocytes (107 cells) have been transplanted into patients with hepatic encephalopathy^[2]. While there was improvement in liver function there was no observed increase in patient survival times. While the reason for this was unclear, it was speculated that the number of cells transplanted was insufficient to enhance patient survival^[5]. There are several disadvantages to utilizing primary hepatocytes, however. Once isolated, these cells are prone to apoptosis and damage^[6], and exhibit low levels of proliferation in culture^[7]. The use of hiPS cells, however, could overcome several of the difficulties associated with the *In vitro* culturing of primary hepatocyte.

OTHER SOURCES OF HEPATOCYTES

There are three cell types that can be utilized to generate transplantable hepatocytes: bone marrow stem cells, mesenchymal stem cells, and adipose stem cells. Bone marrow stem cells have the capacity for self-renewal, differentiation, and homing. Indeed, bone marrow stem cells harvested from independent donors have been shown to home to recipient bone marrow tissues^[8]. Surprisingly, a previous study demonstrated that mouse bone marrow stem cells, which express CD34 and SCA-1, successfully differentiated into liver epithelial cells *In vitro*^[9]. Likewise, autopsy analyses demonstrated that transplanted bone marrow cells differentiated into hepatocytes in humans^[10]. Notably, these transplanted bone marrow cells reduced liver fibrosis in mice^[11]. Hepatocyte-like cells have also been produced from human umbilical mesenchymal stem cells through the introduction of hepatocyte nuclear factor-4 α ^[12,13]. Meanwhile, adipose stem cells can be purified, cultured, and subjected to sequential stimulation with growth factors, thereby mimicking the *in vivo* hepatocyte differentiation process^[14]. However, none of these three cell sources is considered feasible for clinical use. This is largely due to the fact that so called hepatocyte from these stem cells are far from the functional hepatocytes because these stem cell populations do not exhibit sufficient pluripotency to differentiate into hepatocytes. As such, hiPS cells are considered an ideal source for the *In vitro* production of hepatocytes as they are pluripotent.

PUTATIVE APPLICATIONS OF HEPATOCYTES DIFFERENTIATED FROM HIPS CELLS

Successful differentiation of hepatocytes from hiPS cells would be beneficial for both medical practice and biological analyses. Potential applications would be as follows: (1) Transplantation into patients with hepatic insufficiency; (2) A method for providing support to patients with hepatic insufficiency; for use in bioartificial liver devices; (3). Toxicology analyses of drugs; (4) *In vitro* models of liver disease; (5) *In vitro* model of hepatocyte infection with hepatitis viruses; (6) *In vitro* model of hepatocyte differentiation. Each of these applications will be presented in detail.

1. Transplantation into patients with hepatic insufficiency

One of the most important applications of hepatocytes generated from hiPS cells would be transplantation into patients with hepatic insufficiency caused by fulminant hepatitis. Because this disease is the result of a significant loss of functional hepatocytes, transplantation of healthy cells could be an ideal therapeutic option. Hepatic progenitor cells have the potential to differentiate into both mature hepatocytes and bile duct epithelial cells. Furthermore, hepatic progenitor cells could be expected to construct normal liver structures, including hepatic lobules and bile ducts. Hepatic progenitor cells derived from mouse embryonic stem (ES) cells were previously shown to engraft in host liver tissues and to differentiate into hepatocytes when transplanted into partially hepatectomized mice^[15]. Likewise, hepatocytes engrafted in mice suffering from acute liver failure caused by carbon tetrachloride intoxication^[16]. This promising finding suggests that hepatocytes from pluripotent cells are transplantable. Indeed, hepatocytes have been differentiated from human ES cells and then successfully transplanted^[17]. While a major disadvantage associated with the use of human ES cells is the possibility of inducing graft-versus-host disease, the ability to treat patients with hepatocytes derived from their own tissues would

eradicate this limitation. Therefore, this approach would allow for successful treatment of patients suffering from acute liver failure. Hepatocyte-like cells derived from mouse iPS cells have been shown to improve acute liver failure caused by carbon tetrachloride^[18]. These cells were transplanted through peritoneal injection, which led to significant reduction in the extent of liver necrosis. The authors concluded that the observed hepatoprotective effects were due to antioxidant activities of the transplanted cells. Meanwhile, transplanted hepatocytes generated from human iPS cells maintain their proliferative capacity, as evidenced by immunostaining with Ki67-specific antibodies^[19]. This report is promising in that it indicates that transplanted cells can proliferate and compensate for lost liver tissue in patients experiencing acute liver failure.

2. A method for providing support to patients with hepatic insufficiency

Hepatocytes are utilized in bioartificial liver systems of patients suffering from liver failure. While these systems currently employ either primary hepatocytes or hepatocellular carcinoma cells^[20], which can be rejected by the host, Iwamuro *et al* recently tested the use of hepatocytes derived from mouse iPS cells in bioartificial liver systems^[21]. In these experiments, the mouse cells produced albumin and expressed enzymes involved in the urea cycle, consistent with hepatocyte-like cells. It is expected that similar results could be obtained using hiPS cells.

3. Drug screening for toxicology

The liver is the primary organ affected by drug toxicity^[22]. Primary hepatocytes and hepatocellular cancer cells have been used to assess drug-induced liver injury^[23-25]. Again, the major limitations of using primary hepatocytes are the technical difficulties associated with obtaining and maintaining these cells. In addition, the innate limitation of hepatocellular carcinoma cells is that they are distinct from hepatocytes. Hepatocytes derived from hiPS cells are expected to overcome these limitations. Indeed, hepatocyte-like cells were recently used for analyzing the toxicity of particular compounds to the liver^[26]. In this study, the authors used cell viability, nuclear shape and other markers to assess hepatotoxicity, and succeeded in efficiently screening a panel of 240 compounds to identify those associated with high and low toxicity.

4. *In vitro* models of liver disease

Similar to studies performed in mice, hepatocytes could be produced from patients with metabolic diseases and utilized to characterize these disorders^[27]. While it is not possible to harvest mature hepatocytes from human patients, hiPS cell populations could be established. Once established from a patient(s) with a given metabolic liver disease, these hiPS cells could then be differentiated into hepatocytes^[28], which would provide an ideal model system for studying the disorder. Furthermore, the disease of interest could potentially be cured by transplanting functioning hepatocytes into the patient, as these cells play pivotal roles in metabolism.

High plasma levels of low-density lipoprotein cholesterol (LDL-Chol) are known to cause cardiovascular disease. As such, successful reduction of LDL-Chol levels may prevent these diseases. Furthermore, hiPS cells derived from patients with familial hypercholesterolemia (FH), which is caused by mutations in the gene encoding the LDL receptor, could provide an effective model for analyzing the mechanism of this condition^[29]. Wilson's disease is a congenital metabolic disorder that results in excess copper accumulation in hepatocytes due to a mutation in the ATP7B gene

^[30]. Hepatocytes were recently established from hiPS cells originated from a patient with Wilson's disease^[31]. The hepatocytes from hiPS cells consistently express the mutant ATP7B gene. These findings therefore demonstrate that *In vitro* differentiation of hepatocytes can be utilized to characterize liver diseases and may be useful for developing novel therapies to treat these conditions.

5. *In vitro* model of hepatocyte infection with hepatitis viruses

Hepatitis C virus (HCV) causes liver cirrhosis and hepatocellular carcinoma (HCC). Meanwhile, unlike hiPS cells, which are not permissive to HCV infection, hepatocyte-like cells derived from hiPS cells can be infected by HCV^[32,33]. Hepatocyte-like cells produced from iPS cells were transplanted into mice and were viable for greater than three months^[34]. Furthermore, these hepatocyte-like cells were infected with the HCV virus, indicating that this mouse model is useful for investigating HCV infections. Likewise, hepatocyte-like cells derived from hiPS cells exert an inflammatory response to infection^[35], and may therefore provide a suitable *In vitro* model for studying the mechanism of human HCV infection. Such a model could lead to innovative strategies for inhibiting HCV replication or survival, which would help to prevent both HCV-mediated liver cirrhosis and HCC.

Hepatitis B virus (HBV) is another significant causative agent of viral hepatitis, liver cirrhosis, and HCC. Hepatocytes differentiated from hiPS cells are also permissive to HBV infection and are therefore a useful model for studying human HBV-mediated illnesses^[36].

6. *In vitro* model of hepatocyte differentiation

It is very hard to obtain primary hepatocytes from liver tissues because the culturing of these cells is associated with technical challenges. Knowledge of hepatocyte differentiation, however, would improve the methods for generating hepatocytes from hiPS cells *In vitro*. Because it is not possible to investigate human hepatocyte differentiation using an *in vivo* model, our knowledge of this process could be significantly enhanced by an *In vitro* model, such as the differentiation of hepatocytes from hiPS cells^[27]. As discussed above, hepatocytes differentiated from hiPS cells are a promising and useful tool for basic research and clinical applications; however, differentiation protocols have yet to be established.

HEPATOCTYE DIFFERENTIATION

Before moving on to describing current methods for the generation of hepatocytes from human and mouse iPS cells, we will discuss the cellular mechanism that governs hepatocyte differentiation^[37].

1. Growth factors and hepatocyte differentiation

Activin A, a member of the transforming growth factor- β superfamily, mimics Nodal, binds Activin receptors, and activates Smad2 by phosphorylation^[38]. Furthermore, activin A enhances differentiation of human ES cells to endodermal cells^[39]. The differentiation of iPS cells to endodermal cells requires the Wnt/ β -catenin signaling pathway^[40]. Indeed, Wnt/ β -catenin modulates the patterning and organogenesis of the endoderm^[41]. Wnt/ β -catenin signaling also directs liver specification of cells in the foregut during liver development^[42], and is essential for proliferation of hepatoblasts and differentiation of hepatoblasts to both bile duct epithelial cells and mature hepatocytes in mice^[43]. Lastly, activin A and Wnt3A function synergistically to promote efficient differentiation of hiPS cells into endodermal cells *In vitro*^[44].

Basic fibroblast growth factor (bFGF) sustains the self-renewal characteristics of human ES cells^[45]. bFGF is also secreted from the developing heart during liver development^[46]. bFGF causes the foregut endoderm to develop into the liver. Meanwhile, bone morphogenetic proteins (BMPs) are members of the TGF- β superfamily and are involved in spinal development^[47]. BMP4 is secreted from the septum transversum during embryonic development, and it induces the expression of liver-specific genes in endodermal cells^[48]. In addition, BMP4 stimulates the differentiation of hepatic stem cells to mature hepatocytes^[49].

Hepatocyte growth factor (HGF) is secreted from sinusoidal endothelial cells in the liver^[50]. HGF is a strong mitogen of hepatocytes and plays an important role in liver regeneration^[51,52]. Indeed, HGF was found to promote differentiation of murine ES cells to hepatocytes^[53]. Meanwhile, Oncostatin M (OncoM), a member of the interleukin-6 cytokine family that is required for differentiation of hepatoblasts to mature hepatocytes, is secreted from hematopoietic stem cells in fetal liver^[54,55].

Dexamethasone and insulin-transferrin-selenium (ITS) are used to maintain the *In vitro* function of hepatocytes. These compounds promote differentiation of hepatoblasts^[56], and are used to promote differentiation of hiPS cells to the hepatic lineage^[57].

2. Transcription factors and hepatocyte differentiation

Transcription factors play essential roles during hepatocyte differentiation by controlling the expression levels of liver-specific genes^[58]. The forkhead box A family of transcription factors consists of three members: FOXA1, FOXA2, and FOXA3^[59]. These proteins, which are also known as hepatic nuclear factor 3 (HNF3) alpha (A1), beta (A2) and gamma (A3), are involved in both liver development and metabolism. Liver development is initiated with the formation of a liver bud, which is mediated by the FoxA1 and GATA4 transcriptional regulators^[58]. A previous report demonstrated that FoxA1 is up-regulated in response to retinoic acid, and down-regulates Nanog^[60]. This report also suggests that FoxA1 suppresses a gene strongly involved in maintenance of pluripotency while at the same time promoting differentiation toward hepatocytes. It has been predicted that induction of the expression of this transcription factor promotes differentiation of hiPS to hepatocytes. Meanwhile, a FoxA2 deficiency was lethal in mice due to severe defects in liver and pancreatic development^[61]. FoxA2 and GATA4 are the first proteins that were demonstrated to bind to the albumin gene enhancer sequence in embryonic liver precursor cells^[62]. These proteins relax compacted chromatin structures, and initiate hepatocyte differentiation.

FoxA3 contributes to hepatocyte differentiation and regulates the expression of liver-specific genes^[63]. Together, GATA4, FoxA1, and FoxA3 induce transdifferentiation of mouse fibroblasts to functional hepatocyte-like cells^[64]. In addition to the FoxA (HNF3) proteins, HNF4 α is required for proper liver development^[65]. Indeed, mice that are deficient in HNF4 α are not viable due to the lack of normal gastrulation^[66]. Furthermore, introduction of *Hnf4 α* into knockout mice with HNF4 α revealed that this compound is essential for differentiation of hepatocytes to cells with liver-specific metabolic functions. Furthermore, HNF4 α regulates genes involved in drug metabolism such as P450 and uridine 5'-diphospho (UDP)-glucuronosyltransferases^[65]. Interestingly, the human hepatocytes are rich with binding sites for both HNF1 α and HNF4 α ^[67]. These results support the conclusion that HNF1 α and HNF4 α are essential for hepatocyte differentiation.

The disruption of hematopoietically expressed homeobox (Hex)

expression resulted in embryonic lethality that was attributable to insubstantial liver formation; thus, Hex is essential for liver organogenesis^[68]. Moreover, Hex is essential for hepatoblast differentiation and bile duct formation^[69].

The expression of sex-determining region Y (Sry) HMG box (Sox) 17 is restricted to the nascent primitive endodermal epithelium, and the absence of Sox17 leads to premature delamination and migration of the parietal endoderm^[70].

CCAAT/enhancer binding protein α (C/EBP α) is a basic leucine zipper transcription factor. C/EBP α is enriched in liver cells where it governs the expression of liver specific genes, but is down-regulated in patients after partial hepatectomy^[71,72]. Hepatocytes of knockout mice with C/EBP α are positive for A6 and albumin^[73]. A6 is an antigen of biliary epithelial cells^[74]. Albumin is a marker of hepatocytes. These results suggest that C/EBP α (-/-) hepatocytes exhibit characteristics of both hepatocytes and bile duct epithelial cells. Interestingly, suppression of C/EBP α expression in hepatoblasts promotes differentiation to bile duct epithelial cells^[75]. These results suggest that C/EBP α promotes the differentiation of hepatoblasts to mature hepatocytes.

PROTOCOLS FOR DIFFERENTIATION OF HIPS CELLS INTO HEPATOCYTES

Growth factors and transcription factors play essential roles in hepatocyte differentiation. As such, the application of growth factors is critical for *In vitro* production of hepatocytes from hiPS cells and is therefore included in all current protocols. We will first describe the *In vitro* methods developed for the generation of hepatocytes using growth factors. Notably, the differentiation of hiPS cells to hepatocytes or cholangiocytes requires the application of distinct growth factors. A previous study demonstrated that cholangiocyte differentiation requires growth hormone, epidermal growth factor, interleukin-6, and sodium taurocholate^[76]. Additional report suggests that the mechanism of hepatocyte differentiation from hepatoblasts is distinct from that of cholangiocytes^[73,77]. Transcription factors are also essential for hepatocyte differentiation, and transfection reagents or electroporation are conventionally used to introduce genes of interest into iPS cells^[78,79]; however, it remains difficult to effectively introduce transcription factors into hiPS cells using these methods^[80]. Therefore, methods for hepatocyte differentiation using adenoviral vector-mediated expression of transcription factors will be discussed.

1. Generation of hepatocytes from iPS cells using growth factors

Most protocols have adopted the method of stepwise addition of growth factors to resemble the *in vivo* hepatocyte differentiation process during liver development^[81-84] (Table 1). This process involves the progression of endodermal cells to immature hepatocytes (often referred to as hepatoblasts), and finally to mature hepatocytes. All current differentiation protocols include the use of activin A, which was shown to maintain the pluripotency of the iPS cells by inducing the expression of Nanog^[85,86]; however, these reports are controversial. In these protocols, a high concentration of activin A (100 ng/mL) is applied to hiPS cells to induce hepatocyte differentiation, while 10 ng/mL is used to maintain pluripotency. Notably, the morphology of hiPS cells exposed to 10 ng/mL activin A is different from that of cells treated with 100 ng/mL^[87]. It is speculated that activin A exerts different effects on iPS cells at higher and lower concentrations; however, the mechanism of this activity is unknown. Subsequently, LY294002 (a specific inhibitor of phosphatidylinositol 3 phosphatase), B27 supplement, or bFGF are added, depending on

Table 1 Protocols for the differentiation of human induced pluripotent stem (hiPS) cells into hepatocytes using growth factors.

Author	Day 0-5	Day 5-10	Day 10-15	Day 15-20
DeLaForest <i>et al.</i> ^[80]	Activin A, LY294002	BMP4, FGF2	HGF	OncoM
Si-Tayeb <i>et al.</i> ^[81]	Oxygen: 20%	Oxygen: 4%	Oxygen: 4%	Oxygen: 20%
	Activin A	BMP4, FGF2	HGF	OncoM
Song <i>et al.</i> ^[82]	Day 0-3	Day 4-7	Day 8-13	Day 14-18
	Activin A	FGF4, BMP3	HGF, KGF	OncoM
Chen <i>et al.</i> ^[87]	Day 1-3	Day 4-7	Day 8-12	Day 19-21
	RPMI1640/B27	KO/DMEM	IMDM	OncoM, Dex
	Activin A, Wnt3a, HGF	Glutamine, NEAA, 2-mercaptoethanol, DMSO	OncoM, Dex, ITS	

BMP: bone morphogenetic protein; FGF: fibroblast growth factor; HGF: hepatocyte growth factor; OncoM: oncostatin M; KGF: keratinocyte growth factor; dex: dexamethasone; RPMI1640: Roswell Park Memorial Institute 1640; B27: B27 supplement; KO/DMEM: knockout Dulbecco's Modified Eagle's medium; IMDM: Iscove's Modified Dulbecco's medium; NEAA: non-essential amino acids; ITS: insulin-transferrin-selenium. Brackets indicate reference numbers.

the purpose of the research. After 3-5 days of culturing, the iPS cells differentiate into endodermal cells. From days 5-10, a combination of BMP2 or 4 and either FGF2 or 4 is applied. FGF2 was shown to sustain the self-renewal characteristics of human ES cells^[45]. Contrary to this phenomenon, however, FGF2 is added to hiPS to mediate differentiation to hepatocytes.

Chen *et al.*^[88] proposed another multistep protocol in which growth factors, but not transcription factors, are utilized. Using this method, they succeeded in differentiating hiPS cells into mature hepatocytes within only 12 days, which is significantly faster than the time required to generate hepatocytes in other studies. In their protocol, activin A (100 ng/mL) and HGF (10 ng/mL) are added from on days 1 to 3. Furthermore, hepatocyte-like cells were successfully derived when HGF was added at the first step of differentiation^[88]. Sox17 and FoxA2 expression, which are induced by activin A, are important markers of endodermal differentiation. HGF and activin A may produce synergistic effects on the differentiating cells.

Similar to Chen *et al.*, Kondo and co-workers generated hepatocyte-like cells by sequential stimulation with growth factors^[89]. However, while Chen and co-workers utilized IMDM medium from day 8 to 12, the Kondo protocol requires the use of Modified LaFord medium from day 12 to 21. Using their protocol, Kondo *et al.* were successful in generating hepatocyte-like cells from two different hiPS cell lines. Furthermore, these hepatocyte-like cells are useful for drug screening as they express CYP3A4. This report was also informative in that the results indicate that the culture medium used might affect the differentiation process.

Yanagida and co-workers successfully generated hepatic progenitor-like cells upon sequential stimulation of hiPS cells with cytokines^[90]. The stimulated cells were subjected to separation by flow cytometry using CD13- and CD133-specific antibodies, and the resulting cell population were found to express both hepatocyte and cholangiocyte markers. This approach is useful in that hepatic progenitor-like cells are thought to produce hepatocytes and cholangiocytes for development of the liver architecture.

Javed *et al.* established a method by which hiPS cells harvested from patients can be utilized to generate hepatocytes^[91]. In this method, Javed and co-workers sequentially stimulated the hiPS with growth factors, including activin A and bFGF. In contrast to the other protocols, however, they co-cultured the hiPS cells with non-parenchymal stellate cells of liver in embryoid bodies. Embryoid bodies have also been used for the differentiation of hepatocytes from mouse ES cells^[55], and stellate cells are the source of growth factors required for liver regeneration^[92,93]. This approach is reasonable in that the hiPS cells mature toward hepatocytes under more physiologically-relevant conditions.

In a separate study, Nakamura *et al.*^[94] derived mature hepatocytes from human ES and hiPS cells under feeder- and serum-free

conditions. They also succeeded in producing cholangiocytes and proliferating progenitor cells. While indocyanine green was taken up by 30% of the resulting hepatocytes, 80% of the cells exhibited glycogen storage. The cells also exhibited CYP3A4 metabolic activity. These results indicate that the group successfully generated active hepatocytes.

Human serum contains multiple growth factors. Notably, in a previous study, sera from hepatectomised patients promoted hepatocyte differentiation from iPS cells^[95]. As a result, this approach would be a promising method for *In vitro* culturing of hepatocytes. In addition, the application of proteomic approaches would elucidate the mechanism of hepatocyte differentiation upon exposure to these sera^[96].

2. Generation of hepatocytes from iPS cells using transcription factors

Transcription factors play important roles in liver development and hepatocyte differentiation^[97]. However, only a few protocols using transcription factors have been developed. The primary reason for this is that the introduction of genes into iPS cells is technically difficult. While transfection of human or mouse iPS cells with plasmids is generally ineffective, adenoviral vectors provide highly efficient transduction of hiPS cells^[98]. Inamura *et al.*^[99] transduced hES and hiPS cells with a Hex expression vector resulting in efficient production of hepatoblasts (Table 2). Previously, this group demonstrated that transduction of hepatoblasts with an HNF4 α expression vector yielded differentiation into mature hepatocytes *In vitro*^[84]. In this protocol however, hepatoblasts were first incubated with activin A. Sox17 was then introduced to promote differentiation, followed by introduction of HNF4 to induce terminal differentiation of the hepatoblasts into hepatocytes. Mature hepatocytes appeared at approximately 20 days after the initiation of the differentiation process. These reports are interesting in that they suggest that the *In vitro* differentiation process of hiPS cells into hepatocytes resembles that which occurs *in vivo*.

Table 2 Protocol for the differentiation of human induced pluripotent stem (hiPS) cells into hepatocytes using transcription factors.

Author	Day 0-3	Day 3-6	Day 7-9	Day 10-20
Takayama <i>et al.</i> ^[83]	Activin A	Activin A	BMP4, FGF4	HGF, OncoM, Dex
	Day3: Ad-Sox17	Day 5: passage Day6: Ad-Hex	Day 9: Ad-HNF4 α	

BMP4: bone morphogenetic protein 4; FGF4: fibroblast growth factor 4; HGF: hepatocyte growth factor; OncoM: oncostatin M; Dex: dexamethasone; Ad-Hex, Sox17, and HNF4A: adenovirus vector transducing Hex, Sox17, and HNF4A, respectively; Hex: hematopoietically expressed homeobox; Sox17: sex-determining region Y (Sry) box 17; HNF4 α : hepatocyte nuclear factor 4 α . Brackets indicate reference numbers.

In a separate study, Sekine *et al.*^[100] treated cells with the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 as well as 100 ng/mL activin A. Either HGF or keratinocyte growth factor (KGF) was then applied from days 10-14. In this study, while FoxA2 and Sox17 expression were observed, the expression of alpha-fetoprotein (AFP) and albumin were not analyzed. These results indicate that PI3Ks may control the differentiation of iPS cells into endodermal cells; however, other factors would still be required to mediate this process *In vitro*.

LIMITATIONS ASSOCIATED WITH HEPATOCYTES DERIVED FROM HIPS CELLS

Cells cultured using the protocols mentioned above are referred to as hepatocyte-like cells. This is due to the fact that these cells exhibit only certain similarities to primary hepatocytes. First, hepatocyte-like cells have been shown to remain in an immature state^[101]. Furthermore, the detoxification activity of these cells is lower than that of primary hepatocytes in culture^[82,84]. Hepatocytes derived from hiPS cells also exhibit lower levels of FoxA1, FoxA2, FoxA3, and HNF1 α expression, and Takayama *et al.*^[84] therefore speculated that *In vitro* growth of hepatocytes requires additional factors that have yet to be identified. Meanwhile, hepatocytes differentiated from hiPS cells express certain mRNA molecules that are normally not detected in fetal or adult liver tissues^[81]. Notably, hepatocytes are differentiated more efficiently from hepatoblast-derived hiPS cells than from adult hepatocytes, suggesting that the efficiency of differentiation may depend on the origin of the iPS cells. Indeed, differentiation potential depends on the hiPS cells used^[102], and there are differences between hiPS cell populations in the efficiency of differentiation to hepatocytes, as evidenced by differences in albumin expression levels^[103]. The reprogramming factors themselves affect the differentiation of iPS cells to specific cell fates^[104]. Although the results originate from mouse iPS cells, there are differences in the methylation sites between iPS cells reprogrammed with different factors. As a result, given that the previously mentioned liver-specific genes are important for clinical and pharmacological applications, these protocols need to be further developed before the resulting cell populations can be used in a medical setting.

NOVEL APPROACHES FOR THE GENERATION OF HEPATOCYTES FROM IPS CELLS

Despite the protocols described above, it is still challenging to obtain mature hepatocytes *In vitro*. One of the reasons for these difficulties is that the iPS cells utilized in these studies have been cultured two-dimensionally on plates or dishes^[105]. To overcome this limitation, novel approaches are under investigation that can be divided into three categories: the extracellular matrix, 3D culture, and cell sheet approaches.

An extracellular matrix (ECM) provides conditions suitable for cultured cells to differentiate into hepatocytes. In one study, coculturing of mouse ES cells with M15 cells, a mesonephric cell line, induced differentiation of the ES cells into the hepatocyte lineage^[106]. Eighty percent of the mouse ES cells cultured with M15 expressed AFP, and 9% expressed albumin. Interestingly, even fixed M15 cells promoted mouse ES cell differentiation. Meanwhile, Shiraki *et al.* reported that a synthesized basement membrane composed of human recombinant laminin 511^[107] induced differentiation of mouse ES cells into hepatocyte lineages.

A 3D culture system composed of gelatin and an ECM derived from Swiss 3T3 cells^[108] preserved the functions of hepatocyte-like cells differentiated from hiPS cells. The most important ECM component was determined to be type 1 collagen. In a separate study, 3D culturing promoted the maturation of hepatocytes derived from hiPS cells^[109].

For this culturing technique, cells are grown in hollow fibers similar to embryoid bodies. Hollow fibers are useful because the efficiency of embryoid body formation is low compared to that of mouse ES cells, which also differentiate into hepatocytes within hollow fibers^[110]. This organoid culture system allows efficient formation of mouse ES cell aggregates in their lumen, and the liver-specific functions of the mouse ES cells are comparable with those of primary hepatocytes.

Takebe *et al.* cultured hiPS cells with human mesenchymal stem cells and human umbilical vascular endothelial cells^[111]. The mixed cell populations formed three-dimensional structures that were transplantable to mice. The transplanted complexes were vascularized and resembled an organ-like structure similar to the liver. This method is promising in that the liver-like structure could be utilized for future therapeutic strategies.

Du *et al.* assembled hepatocytes and endothelial cells in hydrogel fibers to form three-dimensional cultures^[112]. Notably, the hepatocytes and endothelial cells were both differentiated from iPS cells. Their report therefore suggests that three-dimensional structures could be generated from hiPS cells harvested from patients, which could then be transplantable.

Primary rat hepatocytes have been successfully cultured for 200 days on temperature-responsive sheets^[113]. Furthermore, primary rat hepatocytes maintained liver-specific functions for up to 28 days on the temperature-responsive sheets when cultured with endothelial cells^[114]. These sheets attach to the bottoms of culture dishes at 37°C and detach at 25°C, and provide a system for easy culturing and handling of cells. Likewise, this system enables easy manipulation of hiPS cells and may be used to promote differentiation of these cells into hepatocytes.

Valproic acid (VPA) is a histone deacetylase inhibitor^[115], which enables efficient generation of iPS cells^[116]. Indeed, Kondo *et al.* utilized this compound to promote differentiation of hepatocytes from iPS cells^[117]. Their protocol essentially involves multistep stimulation with growth factors such as activin A, HGF, OncoM, and dexamethasone. Importantly, the expression levels of liver-specific genes and of albumin were higher in the cell populations treated with VPA than in the untreated populations. Likewise, genes involved in drug-metabolism were highly expressed in the VPA-treated cells. While the mechanism is unclear, the stage at which VPA is administered and the duration of VPA treatment affected the efficiency of hepatocyte differentiation.

For practical application of hepatocytes derived from iPS cells, it is necessary to produce these cells on a large scale. Stirred-suspension bioreactors are useful for scalable *In vitro* generation of hepatocytes^[118]. Vosough *et al.* initiated hepatocyte differentiation by treating a stirred-suspension culture with rapamycin and activin A. The resulting cells exhibited hepatocyte characteristics such as albumin secretion and urea production. Although these cells required further maturation, this strategy provides a method with the potential to generate sizable quantities of hepatocytes from iPS cells *In vitro*.

The application of iPS cell-derived hepatocytes to the screening of drugs for liver toxicity requires enhanced expression of CYP3A and other genes that are important for detoxification. HNF6 up-regulates CYP3A4 in iPS cells^[119], suggesting that *In vitro* generation

of hepatocyte-like cells that perform specific functions similarly to mature hepatocytes is an option for overcoming the current limitations associated with hepatocyte-like cells that are described above. In addition, it has been suggested that transcription factors are key players in the generation of mature hepatocytes from iPS cells. Indeed, FoxA1 and HNF1 α promote differentiation of hiPS cells to hepatocytes that exhibit mature functions^[120].

DIRECT PRODUCTION OF HEPATOCYTES FROM FIBROBLASTS

In addition to the production of hepatocytes derived from iPS cells, the generation of hepatocytes directly from fibroblasts has been reported. In a previous publication, ectopic expression of HNF1 α , HNF4 α , and FoxA3 in human fibroblasts using a lentiviral expression system resulted in the differentiation to hepatocytes that, upon transplantation, enhanced the survival of mice with liver damage^[121]. In a separate report, HNF1A, HNF4A, HNF6, ATF5, PORX1, and C/EBP α were expressed in human fibroblasts, and the resulting hepatocytes exhibited expression of genes involved in drug metabolism, such as CYP3A4, at levels comparable to those observed in freshly-isolated human primary hepatocytes^[122]. Surprisingly, hepatocytes generated from human fibroblasts were capable of repopulating mouse livers^[123]. With further refinement, this approach could circumvent the need for the differentiation of hiPS cells to hepatocytes. Meanwhile, the limitation of this approach is that, because they are primary cells, the ability to passage and propagate fibroblasts is limited.

THE TUMORIGENICITY OF IPS CELLS

A significant concern associated with the transplantation of iPS cell-derived hepatocytes is the risk of tumorigenicity^[124]. This tumorigenicity was initially attributed to genomic integration of viral vectors^[125]. Therefore, to reduce this risk, plasmid vectors, Sendai virus vectors, and RNA synthesized from the cDNA of the four reprogramming transcription factors have been used to induce hepatocyte differentiation^[126-128]. Additionally, combinations of reprogramming factors have been investigated^[129]. Despite these efforts, however, the risk of tumorigenicity has yet to be eliminated. The link between pluripotency and tumorigenicity was first reported in 1960 in a study of teratocarcinoma^[130]. Both pluripotency and tumorigenicity involve mechanisms of self-renewal, proliferation, and active telomerases^[131]. As a result, it is difficult to eliminate the risk of tumorigenicity due to residual hiPS cells that persist in the transplanted material. It is therefore necessary to develop methods for eradicating iPS cells within differentiated somatic cell populations.

PURIFICATION OF HEPATOCYTES FROM IPS CELLS

Flow cytometry, heat shock, suicidal gene therapy, and toxic materials have been utilized for purification of hepatocytes. Yamamoto *et al* introduced green fluorescent protein (GFP), driven by an albumin promoter/enhancer, into mouse ES cells^[16]. Albumin is also expressed in endodermal cells^[132]. Meanwhile, the genetic knock-in of a marker gene at the albumin locus is expected to enable purification of hepatocytes differentiated from hiPS cells^[133]. The flow cytometry with albumin promoter/enhancer may isolate the other cells than hepatocytes, such as endodermal cells.

Immunolabeling of delta-like 1 homolog (DLK1) has been

utilized for the isolation of hepatoblasts by flow cytometry [134]. The drawback of this approach, however, is that DLK1 is not expressed in the adult human liver^[135]. Meanwhile, sublethal heat shock treatment induced apoptosis in human ES cells^[136]; however, this approach might also damage the hepatocytes. In addition, the thymidine kinase gene, driven by the Nanog promoter, was introduced into hiPS cells^[137]. The cells were subsequently ablated with ganciclovir treatment. This method is ideal for selecting differentiated hepatocytes because they do not express Nanog. One potential limitation of this approach, however, is that ganciclovir might be toxic to hepatocytes. Likewise, while, benzethonium chloride, methylbenzethonium, and N-oleoyl serinol induce apoptosis in human iPS and mouse ES cells^[138,139], these compounds may also damage hepatocytes. Thus, efficient purification methods that do not result in damage to hepatocytes have yet to be developed.

HEPATOCYTE SELECTION MEDIUM

Glucose is an important energy source for cell growth and survival. Glucose deprivation aids in the purification of hepatocytes because, unlike their precursor cell types, they are capable of producing this monosaccharide^[140]. Pyruvate, which is the end product of glycolysis, is incorporated into the citric acid cycle, and removal of pyruvate and glucose from *In vitro* growth media resulted in neuronal cell death^[141]. Meanwhile, galactose enters glycolysis as a substrate for galactokinase, which is expressed in the liver and kidney (Figure 1)^[142,143]. Therefore, it is predicted that hepatocytes can survive in a medium containing galactose but lacking glucose and pyruvate^[144,145].

Of the amino acids, arginine deprivation is least tolerated by cells cultured *In vitro*^[146]. Arginine is produced through the urea cycle, which is exclusive to hepatocytes (Figure 2). Indeed, the removal

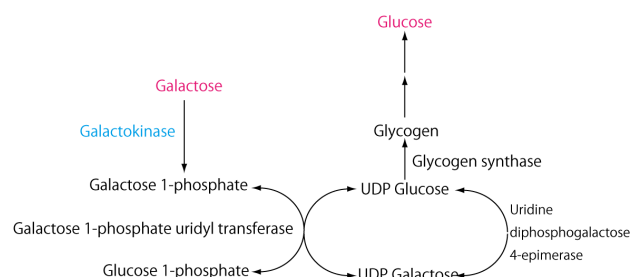


Figure 1 Graphic depiction of the production of glucose from galactose. Galactose is metabolized to galactose-1-phosphate by galactokinase, which is expressed in hepatocytes, ultimately resulting in the production of glucose.

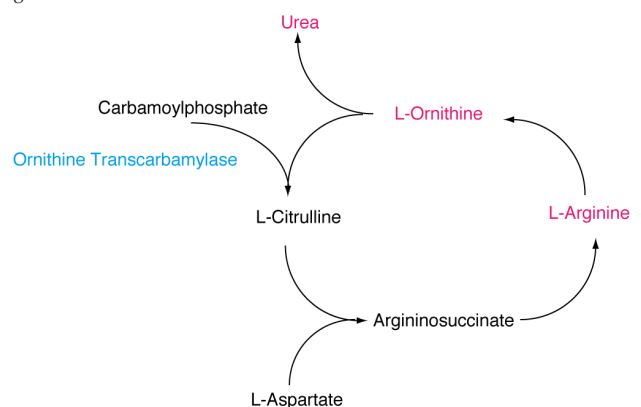


Figure 2 Graphic depiction of the urea cycle. In the urea cycle, ornithine is generated directly from arginine and then metabolized to produce urea. Alternatively, ornithine transcarbamylase, which is expressed in the liver, can metabolize ornithine to form citrulline, thereby feeding back into the cycle.

of arginine led to the development of the first medium for purifying hepatocytes^[140]. Subsequently, hepatocytes were purified from hiPS cells by culturing in a medium lacking both arginine and tyrosine, referred to as hepatocyte selection medium (HSM)^[147]. HSM is advantageous in that it does not contain toxic reagents and consists only of common media components. As a result, this medium is not harmful to hepatocytes.

CONCLUSIONS

Human iPS cells are a promising source of hepatocytes, which could be utilized for drug screening, for cell transplantation, and as a model for studying human diseases. While several protocols for the differentiation of hiPS cells into hepatocytes have been published, the cell populations derived using these methods exhibit limited hepatocyte characteristics. In the future, as more sophisticated methods are developed, novel applications of these cells will be realized.

CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

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