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Induced Pluripotent Stem Cells as a Source of Hepatocytes

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<http://dx.doi.org/10.5772/54552>

1. Introduction

Human induced pluripotent stem (hiPS) cells are generated with cellular reprogramming factors [1], and they have the potential to differentiate into a variety of cells. Ethical issues and graft-versus-host disease may be avoided with hiPS cells because they can be established in each patient individually. hiPS cells may therefore be an ideal cell source for patients.

The liver is a single large organ, the cells of which are 70–80% hepatocytes. These liver-specific cells play a major role in protein synthesis, glucose metabolism, and detoxification. Methods of producing hepatocytes from hiPS cells have been under development for some time. In this chapter, we will cover the following topics:

- Hepatocyte culture
- Applications of hepatocyte culture
- Production of hepatocytes from human embryonic stem (hES) cells
- Protocols for differentiation of hiPS cells into hepatocytes
- Current applications of hepatocytes differentiated from hiPS cells
- Limitations of differentiation
- Future directions

First we will discuss primary hepatocyte culture. The knowledge on primary hepatocyte culture is applicable to maintenance of hepatocytes differentiated from hiPS cells. Next, appli-

cation of hepatocyte culture will be discussed because the application would provide potential usage of hiPS cells. Then production of hepatocytes from ES cells will be presented. Methods presented in this section are prototypes of differentiation protocols of hiPS cell into hepatocytes. Sequentially, current protocols of differentiation of hiPS cells into hepatocytes will be summarized. Applications of hepatocytes from hiPS cells will be presented specific to human diseases such as hepatitis C virus. Even with the protocols above mentioned, differentiation of hiPS cells to functioning hepatocytes is difficult. Limitations of differentiation will be discussed. Finally, potential new approaches will be presented in the last section.

2. Primary hepatocyte culture

Before the era of ES cells or iPS cells, primary hepatocyte culture had been the only method to investigate differentiation and function of hepatocytes. The accumulated knowledge on hepatocytes would be applicable to maintain hepatocytes differentiated from hiPS cells. Hepatocyte culture is useful for developing drugs, cell therapies, and disease models. Primary hepatocyte culture is an ideal in vitro model of drug metabolism and toxicology, and primary hepatocytes can be transplanted into patients with liver failure [2]. Hepatocytes from patients with metabolic diseases can be used to investigate disease mechanisms. However, primary hepatocyte culture remains technically difficult. Hepatocytes are isolated from a fragment of resected donor liver with a 2-step collagenase perfusion [2]. Fetal hepatocytes (10^7 cells) have been transplanted into patients with hepatic encephalopathy [3], and while the disease improved, there was no increase in survival time. The speculated reason is that not enough cells were transplanted [4]. Isolated hepatocytes are prone to apoptosis and damage [5] and have difficulty proliferating once cultured [6]. Primary hepatocyte culture also presents ethical issues when cells are harvested from humans. Now hiPS cells could overcome problems that primary hepatocyte culture encounters.

3. Application of hepatocytes differentiated from iPS cells

If hiPS cells could differentiate into hepatocytes, they would be useful for medical practice and biological study. Potential applications would be as follows:

- Transplantation into patients with hepatic insufficiency
- A method to support patients with hepatic insufficiency such as hemodialysis
- Drug screening
- Toxicology
- In vitro model of hepatitis C virus infection
- In vitro model of hepatocyte differentiation

- In vitro model of liver diseases

One of the most important applications of hepatocytes from hiPS cells would be transplantation into patients with hepatic insufficiency caused by fulminant hepatitis. The disease could be treated perfectly with transplanted hepatocytes because it is caused by significant loss of functioning hepatocytes. Hepatic progenitor cells have potential to differentiate into mature hepatocytes and bile duct epithelial cells. Hepatic progenitor cells would be expected to construct normal liver structure such as hepatic lobule and bile ducts. Hepatic progenitor cells derived from mouse embryonic stem (ES) cells engraft in host liver tissue and differentiate into hepatocytes when transplanted into partially hepatectomized mice [7]. Hepatocytes will also engraft in mice with acute liver failure caused by carbon tetrachloride intoxication [8]. This is a promising finding that suggests that hepatocytes from pluripotent cells are transplantable. Hepatocytes have indeed been differentiated from human ES cells and transplanted [9]. One disadvantage of the use of human ES cells is that they may provoke graft-versus-host disease. This could be overcome if hepatocytes are derived from iPS cells established from the individual patient. Patients with acute liver failure could be successfully treated in this manner.

Another application of hepatocytes from hiPS cells would be metabolic diseases. The disease could be cured with transplantation of functioning hepatocytes because they play pivotal roles in metabolism. High levels of low-density lipoprotein cholesterol (LDL-Chol) in the plasma is known to cause cardiovascular disease. Successful reduction of LDL-Chol may lead to prevention of cardiovascular disease. Mutations in the LDL receptor gene result in familial hypercholesterolemia (FH); iPS cells derived from patients with FH provide a good model for analyzing the mechanism of this condition [10].

4. Differentiation of ES cells into hepatocytes

Cultured primary hepatocytes do not proliferate but disappear and lose their function quickly. Pluripotent stem cells have been focused as a cell source of hepatocytes. Before the advent of iPS cells, ES cells had been the center of investigation of differentiation methods into hepatocytes. The topics of the investigation have been growth factors, transcription factors, extracellular matrix, and three-dimensional (3D) culture

Mouse ES cells start differentiation into the hepatocyte lineage once leukemia inhibitory factor (LIF) is deprived and embryoid bodies are formed [11-13]. Hepatocyte-like cells derived from mouse ES cells take up indocyanine green, express albumin, and form bile canaliculi [14]. The induced cells express specific liver genes such as α -1-antitrypsin and phosphoenolpyruvate carboxykinase (PEPCK). Withdrawal of LIF is not an appropriate method for inducing hiPS cell differentiation because these cells are not LIF dependent [15]. Human ES cells differentiate into mesoderm, endoderm, and ectoderm after withdrawal of the LIF and basic fibroblast growth factor (bFGF) [16], but they do not neces-

sarily differentiate into hepatocytes. Therefore, growth factors are expected to be needed for hepatocyte differentiation from human ES cells. Nerve growth factor (NGF) and hepatocyte growth factor (HGF) induce differentiation into endoderm and eventually liver cells [17]. Transcription factors also play an important role in hepatocyte differentiation. Transcription factor forkhead box protein (Fox) A2 promotes differentiation of mouse ES cell into the hepatocyte lineage [18], and these hepatocyte-like cells express phosphoenolpyruvate (PEPCK) and albumin.

To search for more efficient protocols to promote differentiation of ES cells into hepatocytes, combinations of growth factors and extracellular matrices have been investigated [19]. Shirahashi et al. reported that a mixture of Iscove's modified Dulbecco's medium with 20% fetal bovine serum, human insulin, dexamethasone, and type 1 collagen is optimum for mouse and human ES cell differentiation into the hepatocyte lineage. Bovine serum should not be used because xeno-proteins are not suitable for human application. This study suggests that extracellular matrix is important in hepatocyte differentiation.

Hepatic progenitor cells differentiate into hepatocytes in 3D structure in liver. It is expected that 3D culture is more suitable environment for ES cells to differentiate into hepatocytes. Indeed, 3D cultures of mouse ES cells have been shown to differentiate into hepatocytes [20]. Embryoid bodies (EB) were inserted into a collagen scaffold 3D culture system and stimulated with exogenous growth factors and hormones to produce hepatic differentiation.

Hepatocytes should be isolated from the other cells because ES cells could be among hepatocytes. Undifferentiated cells have been shown to form teratoma when transplanted into recipient cells mixed with hepatocytes [21]. A practical method to avoid this is to enrich the hepatocytes and eliminate the undifferentiated cells by Percoll discontinuous gradient centrifugation [22, 23].

Rambhatla et al. [24] reported that the addition of sodium butyrate leads to significant cell death and induction of hepatocyte differentiation in human ES cells. Cells cultured with sodium butyrate express albumin, α -1-antitrypsin, and cytochrome P450 and also accumulate glycogen. However, the induced cells do not express alpha-fetoprotein (AFP). Sodium butyrate is a possible candidate for a small molecule to eliminate undifferentiated cells and induce hepatic differentiation.

5. Protocols for differentiation of hiPS cells into hepatocytes

Protocols for differentiation of hiPS cells into hepatocytes follow those for mouse ES cells as mentioned above. Stepwise protocols are currently used to promote the differentiation [25-28] (Table 1). These protocols consist of sequential application of growth factors and introduction of transcription factors to mimic hepatocyte differentiation during liver development. The progression is endodermal cell, immature hepatocyte (often referred as hepatoblast), and finally mature hepatocyte.

DeLaForest [25]	D0-5	D5-10	D10-15	D15-20
	Activin A, LY294002	BMP4, FGF2	HGF	OncoM
S-Tayeb [26]	D0-5	D5-10	D10-15	D15-20
	O2: 20%	O2: 4%	O2: 4%	O2: 20%
	Activin A	BMP4, FGF2	HGF	OncoM
Song [27]	D0-3	D4-7	D8-13	D14-18
	Activin A	FGF4, BMP3	HGF, KGF	OncoM, Dex

D: day; BMP4: bone morphogenic protein 4; FGF: fibroblast growth factor 2; HGF: hepatocyte growth factor; OncoM: oncostatin M; KGF: keratinocyte growth factor; Dex: dexamethasone.

Table 1. Protocols for hepatocyte differentiation from human induced pluripotent stem cells.

6. Endodermal differentiation

All differentiation protocols apply activin A (a member of the tumor growth factor β superfamily) at a high concentration of 100-ng/mL. LY294002 (a specific inhibitor of phosphatidylinositol 3 phosphatase), B27 supplement, or bFGF are added, depending on the purpose of the research. After 3–5 days of culture, iPS cells differentiate into endodermal cells. From days 5–10, a combination of bone morphogens 2 or 4 and fibroblast growth factors 2 or 4 is applied. Takayama et al. [28] introduced sex-determining region Y box 17 to promote differentiation at this stage after incubation with activin A. Sekine et al. [29] used LY294002 in addition to 100-ng/mL activin A. In their study, FoxA2 and Sox17 expressions appeared but AFP and albumin were not analyzed. Phosphatidylinositol (PI) 3 kinase may control differentiation of iPS cells into endodermal cells, but other factors are still needed.

7. Differentiation into immature hepatocytes

Hepatocyte growth factor (HGF) or keratinocyte growth factor (KGF) is applied from days 10–14. Inamura et al. introduced hematopoietically expressed homeobox (HEX) to promote differentiation into hepatoblasts [30].

8. Differentiation into mature hepatocytes

HGF or oncostatin M is added to promote differentiation of hepatoblasts into mature hepatocytes. Takayama et al. [28] introduced hepatocyte nuclear factor-4 to provide the terminal differentiation of hepatoblasts into hepatocytes. Mature hepatocytes appeared at approximately 20 days after the initiation of the differentiation process. Si-Tayeb et al. [26] cultured cells under 4% oxygen from days 5 to 15.

In another study, Nakamura et al. [31] derived hepatocytes from human ES and iPS cells under feeder- and serum-free conditions. They succeeded in producing cholangiocytes and

proliferating progenitors. The cells produced with their protocol were confirmed to function as mature hepatocytes. Indocyanine green was taken up by 30% of the hepatocytes, and 80% stored glycogen. They also maintained the metabolic activity of CYP3A4.

Chen et al. [32] proposed another multistep protocol. They do not apply any transcription factors, but growth factors. They have succeeded in differentiation of hiPS cells into mature hepatocytes within only 12 days. The period is significantly shorter than the other researchers. With their method, activin A (100 ng/mL) and HGF (10 ng/mL) were added from days 1 to 3, and prior to that, HGF had been added at the last step of hepatocyte maturation. They also added HGF at the first step of differentiation and successfully derived hepatocyte-like cells. Sox17 and FoxA2, induced by activin A, are important markers of endodermal differentiation. HGF and activin A may have synergistic effects on the differentiating cells.

Transcription factors play an important role in liver development and hepatocyte differentiation [33]. Generally, pluripotent stem cells are hard to transfect plasmids. Adenovirus vectors provide highly efficient transduction to hiPS cells [34]. Inamura et al. [30] transduced HEX into hES and hiPS cells to efficiently produce hepatoblasts (Table 2). After differentiation into hepatoblasts, transduction of HNF4 α finally produces mature hepatocytes [28].

Inamura [34]	D0-6	D6-8	D9-18
	Activin A	BMP4, FGF4	FGF4, HGF, OncoM, Dex
	D5: passage, D6: Ad-Hex		D9: passage
Takayama [28]	D0-3	D3-6	D7-9
	Activin A	Activin A	BMP4, FGF4
	D3: Ad-Sox17	D5: passage, D6: Ad-Hex	D9: Ad-HNF4A

BMP4: bone morphogen protein 4; FGF4: fibroblast growth factor 4; HGF: hepatocyte growth factor; OncoM: oncostatin M; Dex: dexamethasone; Ad-Hex, Sox17, HNF4A: adenovirus vector transducing Hex, Sox17, and HNF4A, respectively; Hex: hematopoietically expressed homeobox; Sox17: sex determining region Y box 7; HNF4A: hepatocyte nuclear factor 4 α .

Table 2. Protocols for hepatocyte differentiation from human induced pluripotent stem cells with adenovirus vectors.

9. Current applications of hepatocytes differentiated from hiPS cells

Hepatocytes from hiPS cells are perfect for in vitro model of human diseases because human primary hepatocytes have both ethical and technical issues. Hepatitis C virus (HCV) causes liver cirrhosis and hepatocellular carcinoma (HCC). Primary human hepatocyte culture is a relevant in vitro model for HCV infection, but it presents some ethical issues. Human iPS cells are not permissive to HCV. Interestingly, hepatocyte-like cells derived from hiPS cells recapitulate permissiveness and are infected with HCV [35, 36]. Hepatocyte-like cells derived from hiPS cells exert an inflammatory response to infection [37] and may provide a suitable in vitro model to study the mechanism of HCV infection. Such a model may potentially lead to innovative methods to inhibit HCV and prevent liver cirrhosis and HCC.

Hepatocyte-like cells derived from mouse iPS cells have been shown to improve acute liver failure caused by carbon tetrachloride [38]. These cells were transplanted through peritoneal injection and significantly reduced the extent of necrotic liver. The authors concluded that the hepatoprotective effects were based on antioxidant activity.

10. Limitations of hepatocytes differentiated from hiPS cells

Cells cultured under the protocols mentioned above are referred to as hepatocyte-like cells. In these cells, detoxification activity is lower than in primary hepatocyte culture [26, 28]. Hepatocytes differentiated from hiPS cells have lower expression levels of FoxA1, FoxA2, FoxA3, and HNF1 α , and Takayama et al. [28] speculated that other factors are still needed. iPS cells retain their donor cell gene expressions. Lee et al. [35] generated mouse iPS cells from hepatoblasts and adult hepatocytes. Hepatocytes differentiated from hiPS cells express mRNA that is normally not found in fetal or adult liver [25]. An interesting finding is that hepatocytes are differentiated more efficiently from hepatoblast-derived iPS cells than from adult hepatocytes. This suggests that the efficiency of hepatocyte differentiation may depend on the origin of the iPS cells. Protocols need to be further developed given that the mentioned liver-specific genes are important for clinical and pharmacological applications.

11. Future directions

To overcome these limitations, novel approaches are under investigation. Current research efforts can be categorized into extracellular matrix, 3D culture, and cell sheet approaches.

An extracellular matrix (ECM) provides conditions suitable for cultured cells to differentiate to hepatocytes. M15, a mesonephric cell line, induces differentiation of mouse ES cells into the hepatocyte lineage [39]. Eighty percent of mouse ES cells cultured with M15 express AFP, and 9% express albumin. It is interesting that even the fixed M15 cells can promote mouse ES cell differentiation. Shiraki et al. reported a synthesized basement membrane composed of human recombinant laminin 511 [40] that induced differentiation of mouse ES cells into hepatocyte lineages.

A 3D culture system is composed of gelatin and extracellular matrix from Swiss 3T3 cells [41]. This system preserves the functions of hepatocyte-like cells differentiated from hiPS cells. The most important component of the ECM has been determined to be type 1 collagen.

Cells are 3D cultured in hollow fibers similar to embryoid bodies. Hollow fibers are useful because the efficiency of embryoid body formation is low compared with mouse ES cells, which also differentiate into hepatocytes in hollow fibers [42]. The organoid culture system efficiently allows mouse ES cells to form cellular aggregates in their lumen. Liver-specific functions of mouse ES cells are comparable with those of primary hepatocytes.

Primary rat hepatocytes have been successfully cultured for 200 days on temperature-responsive sheets [43]. These sheets attach on the bottoms of culture dishes at 37°C and detach at 25°C.

They provide easy culturing and handling of cells. Primary rat hepatocytes have preserved liver-specific functions for 28 days in hybrid sheets with endothelial cells [44]. This system enables easy manipulation of iPS cells and may promote differentiation into hepatocytes.

12. Conclusion

Human iPS cells are a promising source for hepatocytes and may be used for drug screening, for cell transplantation, and as a model for studying human diseases. Protocols have been presented for the differentiation of human iPS cells into hepatocytes; however, the differentiated cells have limited hepatocyte characteristics. In the future, as more sophisticated methods are expected to be developed, new applications of these cells will be realized.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (C) (grant No. 23591002) from the Japan Society for the Promotion of Science (JSPS).

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