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Review

Prospects for Induced Pluripotent Stem Cell-Derived Hepatocytes in Cell Therapy[1]

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Induced pluripotent stem (iPS) cells, first established in 2006, have the same characteristics of self-renewability and pluripotency as embryonic stem (ES) cells. iPS cells are inducible from patient-specific somatic cells; therefore, they hold significant advantages for overcoming immunological rejection as well as the ethical issues associated with the derivation of ES cells from embryos. Generation of patient-derived hepatocytes by iPS technology and their use in cell transplantation therapy for patients with liver disease is quite attractive. Here, we discuss recent advances and challenges in hepatocyte differentiation from iPS cells and their utility in cell therapy.

Key words: Induced pluripotent stem (iPS) cells; Hepatic differentiation; Hepatocytes

INTRODUCTION

Induced pluripotent stem (iPS) cells were first established in 2006 by Yamanaka and his coworker, as reprogrammed somatic cells with retroviral integration of pluripotency-associated transcription factors (80). The inserted genes, octamer-binding transcription factor 3/4 (Oct 3/4), sex-determining region Y-box 2 (Sox2), c-Myc, and Kruppel-like factor 4 (Klf4), enabled direct reprogramming of mouse embryo fibroblasts and were shown to be essential in reprogramming cells to a pluripotent state. Subsequently, the induction of pluripotency in human somatic cells was also demonstrated by retroviral transfer of those transcription factors (79).

Stem cells such as iPS cells are characterized by two unique features: self-renewability and pluripotency. Thus, iPS cells are able to unlimitedly proliferate in vitro, and they have a potential to differentiate into various cell lineages: iPS cells spontaneously differentiate into all three germ layers in vitro, and are able to form teratomas in vivo. Based on the above features, several attempts to generate organ-specific cells from iPS cells are now ongoing, and clinical application of these cells is anticipated as a promising new cell replacement therapy. Hanna et al. showed that a mouse model of sickle cell anemia could be successfully treated with hematopoietic progenitor cells derived from autologous iPS cells (26). Adult mouse fibroblasts were reprogrammed into iPS cells, and the genetic defect of iPS cells was repaired by homologous recombination. After differentiating iPS cells to hematopoietic progenitor cells, they transplanted those cells and rescued mice with sickle cell anemia. Wernig et al. showed that iPS cells were induced to differentiate into dopamine neurons and were able to improve behavior in a rat model of Parkinson's disease after transplantation into the brain (85). Likewise, cardiomyocytes (55,93) and insulin-producing cells (81) have been generated from iPS cells and are expected to be sources for cell therapies.

Researchers have also attempted to generate hepatocytes from iPS cells, with successful results reported in several papers. Here, we discuss the current status of

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hepatocyte differentiation from iPS cells and their potential for use in clinical therapy.

CELL THERAPY FOR LIVER DYSFUNCTION

The liver is a vital organ that has a wide range of functions, including protein synthesis, production of biochemicals, glycogen storage, and detoxification. Acute or chronic massive damage to hepatocytes causes loss of liver function and leads to a critical condition in patients, namely, liver failure. Whole-organ liver transplantation is the only curative treatment at this time for patients with liver failure (67,75). Although surgical techniques and postoperative management have improved, surgery-associated mortality is still considerable. In addition, the shortage of donor livers is still a major limitation for allotransplantation, despite the development of split-liver transplantation surgery that allows living-donor liver transplantation. Therefore, current interest has been focused on the possibility of utilizing cellular resources to sustain patients until liver transplantation or to renovate liver function.

Several clinical trials of hepatocyte transplantation have been performed to date. Hepatocyte transplantation corrected metabolic defects in patients with metabolic diseases including bilirubin metabolism (2,17,18), a urea cycle disorder (30), glycogen storage disease type 1 (53), an inborn error in fatty acid metabolism (23,71), and a clotting factor deficiency (14). For treatment of liver failure, small clinical trials have shown reduced cerebral perfusion pressure, lowered ammonia levels, and even improved overall survival using human fetal hepatocytes (20,24,40) or cryopreserved and thawed human adult hepatocytes (8,76). In marked contrast to these techniques of hepatocyte allotransplantation, chronic immunosuppression is not a prerequisite for autologous hepatocyte transplantation. In 1992, Mito et al. reported the first autologous hepatocyte transplantation in 10 patients with chronic liver disease (50). They excised a left lateral segment from each patient's cirrhotic liver and injected the isolated hepatocytes into the spleen. Despite the detection of transplanted hepatocytes in the spleen at 1–6 months, autotransplantation failed to demonstrate clinical improvement in the recipients, suggesting that the number of transplanted cells and/or the viability of the isolated cells was critical.

In recent years, various lineages of somatic stem cells have been introduced as a new approach for autotransplantation of hepatocytes. Liver stem cells, namely fetal liver stem cells (hepatoblasts) and adult liver stem cells (oval cells), have a bipotential to differentiate into either hepatocytes or cholangiocytes (29,44,62). Several candidate cells at extrahepatic sites also show potential to induce hepatocytes. Bone marrow cells differentiated into albumin-producing hepatocytes, repopulated a damaged mouse liver, reduced liver fibrosis, and improved survival in a mouse model (64). A clinical trial for nine patients with liver cirrhosis revealed that autologous bone marrow cell infusion ameliorated albumin production and improved serum protein levels in humans (82). Mesenchymal stem cells derived from bone marrow (16,43,59,94) or adipose tissues (4,89) are alternative cell sources for obtaining differentiated hepatocytes. Nevertheless, the aforementioned stem cells exist only sparsely in tissues, so that their isolation, purification, and large-scale expansion are hard to achieve (13).

iPS cells and embryonic stem (ES) cells are other spectrums of cells that can differentiate into hepatocytes and can be used for cell therapy to compensate for a failed liver. In the next section, we will review their in vitro differentiation and maturation.

HEPATOCYTE DIFFERENTIATION OF iPS CELLS

Due to their capacity of unlimited proliferation, which is a superior feature compared with the aforementioned somatic stem cell counterparts, ES cells and iPS cells are anticipated to be a source of large-scale transplantable cells. iPS cells hold noteworthy advantages over ES cells. First, they do not need immunosuppressive drugs after transplantation when patient-derived iPS cells are used. Second, iPS cells are not generated from embryos but from somatic cells; thus, they are free from ethical debates on their systematic use. It is an urgent issue to establish a method for generating patient-derived hepatocytes by iPS technology. Recent research has focused on generating hepatocytes from iPS cells, and currently several researchers have reported in vitro hepatic differentiation of mouse iPS cells and human iPS cells (19,21,31–33,35,45,46,61,65,70,72,77) (Table 1).

iPS cells show quite similar behaviors to ES cells in their differentiation (Fig. 1). We have reported that iPS cells showed almost similar mRNA expression to ES cells in each step of the embryoid body formation and subsequent definitive endoderm induction (34). The expressions of Cxcr4, a definitive endoderm-specific marker (87), and Sox17 and Foxa2, pan-endoderm-specific markers, progressively increased over the course of differentiation. Subsequently, we have successfully generated hepatocyte-like cells from mouse iPS cells by applying the differentiation protocol of human ES cells with a minor modification (33). In our protocol, basic fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) were used in addition to activin A, a member of the transforming growth factor-β superfamily that promotes endodermal fate. Likewise, other researchers have applied differentiation procedures initially used for ES cells and demonstrated that mouse iPS cells (45) and human iPS cells (21,35,46,61,70,72,77) hold a potential

Table 1. Reported Protocols to Differentiate Induced Pluripotent Stem (iPS) Cells Into Hepatocytes

Author/Reference No./Year	Cellular Origin	Differentiation Protocol	Total Days for Differentiation	Differentiation Efficacy
Song et al. (72) (2010)	Human iPS cell	Activin A: 3 days; FGF4, BMP2: 4 days; HGF, KGF: 6 days; OSM, Dex: 8 days	21 days	60%
Ghodsizadeh et al. (21) (2010)	Human iPS cell	Floating culture: 2 days; Activin A: 3 days; HGF, DMSO: 8 days; Dex: 5 days	18 days	50–57%
Huang et al. (31) (2010)	Human iPS cell	Coculture with human ES cell-derived fibroblast-like cells	15 days	70%
Inamura et al. (32) (2011)	Human iPS cell	Activin A, basic FGF: 6 days; Induction of Hex gene by adenovirus vector; BMP4, FGF4: 3 days; FGF4, HGF, OSM, Dex: 9 days	18 days	84%
Rashid (61) (2010)	Human iPS cell	Activin A, FGF2, BMP4: 3 days; Activin A: 5 days; HGF, OSM: 17 days	25 days	83%
Si-Tayeb et al. (70) (2010)	Human iPS cell	Activin A: 5 days; BMP4, FGF2: 5 days; HGF: 5 days; OSM: 5 days	20 days	81%
Sullivan et al. (77) (2010)	Human iPS cell	Activin A, Wnt 3a: 3 days; Activin A: 2 days; DMSO: 3 days; HGF, OSM: 6 days	14 days	70–90%
Gai et al. (19) (2010)	Mouse iPS cell	Hanging drop: 2 days; Floating culture: 4 days; Activin A, Wnt 3a: 3 days; basic FGF, DMSO: 4 days; HGF, DMSO: 5 days; HGF, OSM, Dex: 7 days	25 days	50–60%
Iwamuro et al. (33) (2010)	Mouse iPS cell	Floating culture: 5 days; Activin A, basic FGF: 3 days; HGF, DMSO: 8 days	16 days	ND
Li et al. (45) (2010)	Mouse iPS cell	DMSO: 4 days; NaBu: 7 days	11 days	63%
Jozefczuk et al. (35) (2011)	Human iPS cell	Activin A, NaBu: 4 days; DMSO: 7 days; HGF, OSM: 7 days	18 days	ND
Liu et al. (46) (2010)	Human iPS cell	Activin A: 5 days; FGF4, HGF: 5 days; FGF4, HGF, OSM, Dex: 10 days	20 days	ND
Sancho-Bru et al. (65) (2010)	Mouse iPS cell	Activin A, Wnt3a, Dex: 6 days; BMP4, FGF2, Dex: 4 days; acidic FGF, FGF4, FGF8b, Dex: 4 days; HGF, follistatin, Dex: 14 days	28 days	ND

Differentiation efficacy means percentages of albumin-positive cells at the final day of differentiation. FGF, fibroblast growth factor; BMP, bone morphogenetic protein; HGF, hepatocyte growth factor; KGF, keratinocyte growth factor; OSM, oncostatin M; Dex, dexamethasone; DMSO, dimethyl sulfoxide; NaBu, sodium butyrate; Hex, hematopoietically expressed homeobox.

to differentiate into functional hepatocytes. Reverse transcription polymerase chain reaction analysis has shown that iPS cell-derived hepatocytes expressed genes of the hepatic markers α-fetoprotein and albumin and a metabolic marker, cytochrome P450. Key liver functions, such as albumin secretion, cytochrome P450 induction, glycogen storage, urea production, ammonia removal, and plasma protein secretion of fibrinogen, fibronectin, transthyretin, and α-fetoprotein, have also been displayed by iPS cell-derived hepatocytes in vitro (21,32,33,35,45,46,61,65,70,72,77). These results imply that established protocols to generate ES cell-derived hepatocytes are applicable to iPS cells.

In our report, we employed an embryoid body formation strategy for generating hepatocytes from iPS cells. After removal of some factors from the medium (e.g., leukemia inhibitory factor from mouse iPS cell or mouse ES cell medium, and basic FGF from human iPS cell or human ES cell medium), the liquid suspension culture allows cells to aggregate and form spherical clusters, called embryoid bodies. A culture dish with an ultralow-attachment surface (3,15,73) or the hanging drop method (25,49) is generally employed for liquid suspension cultures. Embryoid bodies show spontaneous differentiation into all three germ layers—endoderm, methoderm, and ectoderm—thus resembling innate embryonic de-

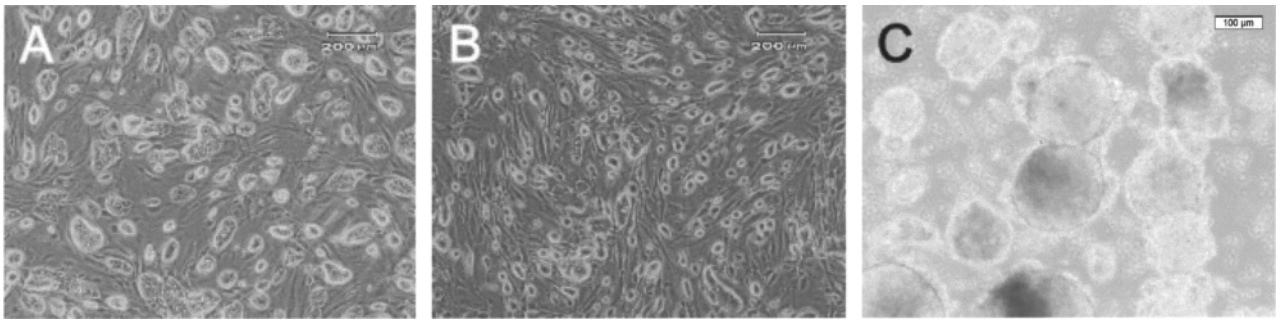

Figure 1. Phase contrast images of undifferentiated induced pluripotent stem (iPS) cells (A) and embryonic stem (ES) cells (B). iPS cells, cultured on feeder layer of SNL (STO-Neo-LIF; an immortalized murine embryonic fibroblast cell line), form round colonies, similar to ES cells. After 3 days of incubation in suspension culture, iPS cells spontaneously aggregated and formed spherical clusters, called embryoid bodies (C).

velopment. Heo et al. reported that, remarkably, a serum-free chemically defined medium supported formation of embryoid bodies and differentiation of hepatic lineage cells from ES cells without extracellular matrixes, growth factors, cytokines, or hormones (28). Interestingly, functional hepatocytes were detectable as clusters surrounded by colonies of rhythmically contracting cells, suggesting that cardiac cells play some role in promoting hepatic differentiation of some populations in embryoid bodies. In vertebrates, during the development of the embryo, endoderm-derived hepatic precursors mature into functional hepatocytes associating with the cardiac mesoderm and septum transversum. FGF signaling originating from the adjacent cardiac mesoderm initiates hepatic fate by suppressing pancreatic fate, and bone morphogenetic protein (BMP) signaling from mesenchymal cells of the neighboring septum transversum enhances hepatic differentiation (36,90,92). Therefore, embryoid body formation and subsequent cardiac mesoderm induction help spontaneous differentiation into hepatocytes. However, due to the mixed cell population from all three germ layers, the embryoid body formation strategy results in low purity of hepatic cells. Heo et al. reported that only about 30% of ES cells contained the albumin gene after 28 days of differentiation (28). In the latest report by Basma et al., 55% of human ES cells expressed albumin (7).

To attain high yields of functional hepatocytes, researchers have employed a direct differentiation strategy with various extracellular matrixes, growth factors, cytokines, and hormones. FGF, BMP, HGF, oncostatin M, and dexamethasone are typical supplementations (1,3,5–7,9,10,12,27,28,39,42,60,68,69,72–74,86,87,95). In the development of the embryo, HGF activates hepatic growth after budding of hepatic progenitor cells within the septum transversum, which holds a collagen-rich environment (1,41,66,91). Dexamethasone and oncostatin M, produced by hematopoietic cells that have migrated into the fetal liver bud, also help the maturation of hepatic progenitor cells (1,38,41,48). In the direct differentiation strategy, iPS cells do not form embryoid bodies but are exposed uniformly to these chemicals. In direct differentiation protocols, albumin-positive hepatocytes were obtained at a higher rate (60–90%) than in the embryoid body formation protocol. However, contamination of other cell lineages was still present in the direct differentiation protocol; hence, improvement of protocols or establishment of a strategy for purifying hepatocytes is required.

CURRENT TASKS FOR iPS CELL-DERIVED HEPATOCYTES

One of the procedures that improve the purity of iPS-derived hepatocytes is coculture with a combination of liver nonparenchymal cell lines or adult hepatocytes. Based on the importance of heterotopic interactions between hepatocytes and hepatic nonparenchymal cells in liver development, our group previously reported that immortalized human nonparenchymal cells including cholangiocytes, liver endothelial cells, and liver stellate cells facilitated hepatic differentiation of mouse ES cells (73). We also demonstrated that the immortalized human cholangiocytes produced interleukin-6 and tumor necrosis factor-α, liver endothelial cells produced FGF4 and vascular endothelial growth factor, and liver stellate cells produced HGF, all factors essential for liver regeneration. Other researchers have indicated that coculture with adult hepatocytes enhanced the efficacy of hepatic differentiation from ES cells (12,52). Thus, exposing iPS cells to cues from liver-populating cells is a promising strategy.

The undifferentiated cell fraction should be eliminated at the final stage of the differentiation program to avoid teratoma formation, which is obviously harmful

for the host when we utilize the differentiated product as cell therapy. Green fluorescent protein inserted in albumin promoter region (73,83) or in the α1-antitrypsin promoter region (15) is one of the tools for isolating ES cell-derived hepatocytes and excluding undifferentiated cells and poorly differentiated cells. Basma et al. introduced a new sorting approach by flow cytometry targeting the expression of asialoglycoprotein receptors, which are surface receptors of matured hepatocytes (7). An extracorporeal bioartificial liver device (47) and exchangeable implanted device (73) are other concepts to reduce the risk of teratoma implantation to hosts.

In vivo experimentation is the present challenge for iPS cell technologies. For ES cells, researchers have provided evidence of the utility of the generated cells in vivo using animal models. ES cell-derived hepatocytes could repopulate in damaged animal livers (1,9,11,22, 28), and secreted albumin (7,15,27) and α1-antitrypsin (7,9) into the recipient mice serum. Our group and other researchers have demonstrated improved survival rates of failed liver mouse models with ES cell-derived hepatocytes (12,56,73). The function of the iPS cell-derived hepatocytes should be assessed as well in the near future by transplantation into animal models of acute or chronic liver failure. Evaluation in autotransplantation models is particularly vital to demonstrate the feasibility and safety of cell therapies based on patient-derived iPS cell technology.

The safety of iPS cells itself should be evaluated. Human iPS cells were first established by retroviral transduction of adult fibroblasts with the set of four defined factors: Oct4, Klf4, Sox2, and c-Myc (78,79) or Oct4, Sox2, Nanog, and Lin28 (88). iPS cells generated by viral vectors potentially activate endogenous oncogenes within the body of the host when they are transplanted, because iPS cells carry viral vehicles that have been observed to genetically transfer during generation of iPS cells (57,63). To avoid the risk of tumor formation by insertional mutagenesis, recent studies have demonstrated that plasmids containing Oct4, Sox2, Klf4, and c-Myc could be used to generate iPS cells without integrating viruses (37,58). c-Myc, a well-known oncogene, might have tumorigenicity. c-Myc could be replaced by Lin-28, or c-Myc could be even removed (54,84). Removal of c-Myc reduces the potential risk of tumor formation. Additionally, the tissue origin of iPS cells affects the degree of risk of its forming teratomas. Miura et al. reported that secondary neurospheres derived from tail tissue fibroblast-origin iPS cells showed the highest tumorigenicity, whereas those from mouse embryo fibroblast origin iPS cells and stomach cell origin iPS cells showed the lowest tumorigenicity (51). Consequently, we have to consider which insertion method to use, what factors to insert, and which somatic cell to use before generating iPS cells for animal and clinical trials.

CONCLUSION

iPS cells have significant potential for medical use as a source of transplantable cells. Currently, it is possible to generate functional hepatocytes from iPS cells by applying established protocols initially used for ES cells. Although there are still a lot of obstacles to overcome for clinical application of iPS cells, we believe that iPS cells should enable us to produce patient-specific donor cells for cell-replacement and tissue-substitution therapy in the near future.

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