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# Endodermal and Hepatic Differentiation from Human Embryonic Stem Cells and Human Induced Pluripotent Stem Cells

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## Abstract

Induced hepatocytes differentiated from human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) have a wide range of potential applications in biomedical research, drug discovery, and the treatment of liver disease. Differentiation of human ESCs and iPSCs into endodermal and hepatic cell types has been achieved by several methods, including addition of soluble factors into culture medium, transduction of differentiation-related genes, co-cultivation with other lineage cells, and a three-dimensional culture system. Each of these methods has an advantage from various points of view, such as the degree of maturation of differentiated hepatocytes, differentiation efficiency, clinical safety, and ease of handling. Currently, it is possible to select or combine the differentiation protocols to obtain ideal hepatocytes. The aim of this review is to describe the recent progress in endodermal and hepatic differentiation protocols from human ESCs and iPSCs in order to foster the suitable choice of induced hepatocytes on clinical and industrial applications.

**Keywords:** Embryonic stem cells; Induced pluripotent stem cells; Liver; Definitive endoderm; Differentiation

## Introduction

The liver has many functions, including carbohydrate metabolism, glycogen storage, lipid metabolism, urea synthesis, drug detoxification, production of plasma proteins, and destruction of erythrocytes. The liver is composed of several types of cells, including epithelial, endothelial, and hematopoietic cells. Of these cells, hepatocytes play the most important role in major hepatic functions. Hepatocytes are thus useful cells for biomedical research, regenerative medicine, and drug discovery. They are particularly useful for drug screenings, such as for the determination of metabolic and toxicological properties of drug compounds in *in vitro* models. For these applications, however, it is necessary to prepare a large number of the functional hepatocytes, which can no longer proliferate in *in vitro* culture. Isolated primary hepatocytes are the current standard *in vitro* model, because they express large amounts of drug-metabolizing enzymes and transporters [1]. However, isolated hepatocytes lose their differentiated properties, such as some cytochrome P450 activities that are induced by reference compounds, even under the optimized culture conditions [2,3]. Moreover, it can be difficult to set up long-term cultures with primary hepatocytes, because they can no longer proliferate in *in vitro* culture [4].

Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are able to replicate indefinitely and differentiate into most cell types of the body, and have the potential to provide an unlimited source of cells for a variety of applications [5-8]. Among the differentiated cells from ESCs and iPSCs, induced hepatocytes have a wide range of potential applications in biomedical research, drug discovery, and the treatment of liver disease. In this review, we provide an up-to-date overview of the wide variety of endodermal and hepatic differentiation protocols. These protocols were designed to reconstruct the *in vivo* environment in a variety of ways, including by addition of soluble factors into culture medium, transduction of differentiation-related genes, co-cultivation with other lineage cells, and use of a three-dimensional culture system.

## Definitive Endoderm Differentiation from ESCs

Gastrulation of the vertebrate embryo starts with the formation of three germ layers: the ectoderm, mesoderm, and endoderm. The endoderm contributes to the digestive and respiratory tracts and their associated organs [9]. The endoderm differentiates into various organs, including the liver, pancreas, lungs, intestine, and stomach. To examine the molecular mechanisms of endoderm specification during early embryogenesis, endoderm differentiation from ESCs has been widely investigated as an *in vitro* model [10]. It has been reported that mouse ESCs have the ability to differentiate into definitive endoderm (DE) cells [11-13]. In recent studies, specific growth factors are used to generate DE cells from ESCs. In DE differentiation, it is well known that nodal signaling plays a crucial role and induces the expression of endoderm-related genes [14]. Activin A, a member of the nodal family, is a ligand of the type II activin receptor and can transmit a downstream signal by using Smad adaptor proteins [15-18]. Therefore, activin A is widely used to generate DE from ESCs. Although embryoid body (EB) formation is also used in the differentiation of ESCs, activin A could generate DE more efficiently than the EB formation [19]. In addition, using activin A with other factors such as fibroblast growth factor (FGF) 2 or Wnt3a proved to be more effective. Simultaneous addition of activin A and FGF2 could synergistically promote more efficient DE

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differentiation in comparison with using activin A alone [20,21]. It has also been found that DE differentiation was promoted by using activin A plus Wnt3a in comparison with activin A plus sodium butyrate [22].

Although DE differentiation methods using growth factors are useful strategies for generating DE with the ability to differentiate into hepatic or pancreatic lineages, they are not efficient enough for generation of homogenous DE populations [23,24]. To improve the DE differentiation efficacy, several groups have attempted a modulation of expression levels in endoderm-related transcription factors. It has been demonstrated that overexpression of SOX17, which is an integral transcription factor for DE formation, promotes DE differentiation, resulting in a DE differentiation efficacy of over 80% based on the estimation of c-kit/CXCR4 double-positive cells [24,25]. The FOXA2 transcription factor as well as SOX17 also functions as a crucial regulator of the initial intracellular signaling pathways in DE differentiation [26]. Overexpression of FOXA2 in ESCs enhances the efficacy of DE differentiation [27,28].

## Hepatic Specification from ESC-derived DE cells

Hepatic differentiation is divided into two steps: hepatic specification and hepatic maturation. In hepatic specification, DE differentiates into hepatoblasts that express α-fetoprotein (AFP), transthyretin, and albumin (ALB) [29-31]. At this stage, repression of Wnt signaling and FGF 4 is necessary for hepatic specification [32,33]. Also, interaction of FGFs with bone morphogenetic protein (BMP) 2 and BMP 4 is important for the induction of hepatocyte-related genes [34-36]. The combination of FGF4 and BMP2 promotes hepatic specification from human ESC-derived DE cells [37]. Similar results were obtained by using the combinations of aFGF and BMP4, bFGF and BMP4, or FGF4 and BMP4 [37]. It has been reported that heterogeneous hepatoblast populations could be differentiated from DE cells by using the combination of BMP2/4 and FGF1/2/4 [20]. With respect to the generation of homogeneous hepatoblast populations, several studies have demonstrated that this can be accomplished by modulating the expression levels of hepatocyte-related transcription factors as well as DE differentiation stage. Overexpression of HEX, which is an integral transcription factor for hepatic specification, has been shown to promote hepatic specification, with the result that the expression levels of ALB and AFP are up-regulated in HEX-transduced cells [38-40]. Conditioned medium from human hepatocellular carcinoma cell line, HepG2, could also promote the hepatic differentiation from human ES cells [41].

## Hepatic Maturation from ESC-derived Hepatoblasts

Hepatoblasts differentiate into two distinct lineages, hepatocytes and cholangiocytes. During the fetal hepatic maturation, the number of hepatoblasts decreases, and in turn, the number of mature hepatocytes increases [42]. In this process, AFP is highly expressed in the fetal liver, and then the number of AFP-positive cells decreases in a later maturation step and almost disappears in the adult liver [43,44]. Growth factors that are secreted by surrounding non-parenchymal liver cells, such as hepatocyte growth factor (HGF) and Oncostatin M (OsM), are essential for hepatic maturation [42]. HGF enhances hepatocyte proliferation but it inhibits biliary differentiation by blocking notch signaling [43]. OsM, which is expressed in hematopoietic cells in the fetal liver [45], promotes the hepatic differentiation from liver progenitor cells [42,43,46].

As mentioned above, growth factors that are necessary for *in vivo* hepatic development are utilized in hepatic differentiation from ESC-derived hepatoblasts. Measurement of urea synthesis [47], ALB production [47], glycogen storage [37], uptake low-density lipoprotein (LDL) [48], uptake and secrete Indocyanine Green [48], coagulation factor VII activity [49], have been used to verify if ESC-derived hepatocyte-like cells function adequately as hepatocytes. Measurement of the ability of human immunodeficiency virus (HIV)-hepatitis C virus (HCV) pseudotype viruses to enter into human ESC-derived hepatocyte-like cells, has also been used to estimate hepatic maturation [37]. Although HGF is widely used for inducing hepatic phenotypes (e.g., ALB and dipeptidyl peptidase IV expression) [50,51], this is not enough to induce functional maturation [51,52]. To generate functional hepatocytes, combinations of FGF, HGF, and a mixture of insulin-transferrin-sodium selenite (ITS), dexamethasone, and OsM are often used [53-55]. Combination of HGF, activin A, and Wnt3a promoted the differentiation of human iPSCs into mature hepatocyte-like cells [56]. Minor modifications to this strategy resulted in 70% to –80% purity (based on estimating ALB-positive cells) of ESC-derived hepatocytes [57,58].

Because drug discovery is one of the most anticipated applications of ESC-derived hepatocyte-like cells, it is important to generate ESC-derived hepatocyte-like cells that have the same characteristics as primary human hepatocytes. Even when the various hepatic functions described above are observed in ESC-derived hepatocytes, expression level of hepatocyte-related genes in ESC-derived hepatocytes is often lower than that of human hepatocytes [59]. To generate functional hepatocytes which have characteristics similar to primary human hepatocytes, exogenous transduction of transcription factor genes that can control the expression of hepatocyte-related genes is suitable for efficient differentiation of hepatocyte-like cells from ESCs. Sequential transduction of the SOX17, HEX, and HNF4α genes, which are central regulators of liver development, in ESC-derived hepatoblasts has been shown to successfully induce mature hepatocyte-like cells that have the same features as primary human hepatocytes [60] (Figure 1). Furthermore, these hepatocyte-like cells could catalyze the toxication of several compounds, suggesting that the ESC-derived hepatocytes have potential for use in drug-screening applications. Overexpression of the Foxa2, Hnf4α, and c/EBPα genes into expandable liver-derived progenitor cells resulted in mature hepatocyte phenotypes [61]. Many other studies have shown the effect of the transduction of differentiation-related genes to promote hepatic differentiation from various origins (summarized in Table 1) [24,25,27,28,38,39,60,61,62-67], demonstrating that transduction of differentiation-related genes into ESCs would be a powerful strategy to generate mature hepatocyte-like cells.

## Hepatic Differentiation from iPSCs

The iPSC technology raises the possibility of generating patient-specific cell types of all lineages [68,69]. Because drug metabolism capacity differs among individuals [70], it is difficult to make a precise

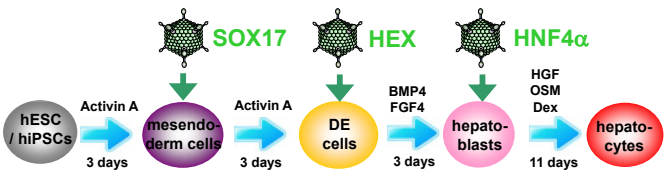


**Figure 1:** A protocol for hepatic differentiation of human ESCs or iPSCs by an adenovirus vector-mediated gene transfer.

| Origin | Species | hepatic transcription factor genes | ref |
|---|---|---|---|
| ESCs | mouse | FOXA2 | [27] |
| ESCs | mouse | FOXA2 | [28] |
| ESCs | mouse | E-cadherin | [62] |
| ESCs | mouse | HEX | [38] |
| ESCs | human | SOX17 | [24] |
| ESCs/iPSCs | human | SOX17 | [25] |
| ESCs/iPSCs | human | HNF4α | [60] |
| ESCs/iPSCs | human | HEX | [39] |
| hepatic progenitor cells isolated from E14 fetal mouse | mouse | HNF4α | [63] |
| lineage-depleted OsM receptor β expressing bone marrow cells | mouse | HNF4α | [64] |
| human unbilical cord mesenchymal stem cells | human | hTERT | [64] |
| human mesenchymal stem cells | human | HNF4α | [65] |
| adult liver derived progenitor cells | mouse | FOXA2, HNF4α, c/EBPα | [61] |
| fibroblasts | mouse | HNF4α, FOXA1-3 | [66] |
| fibroblasts | mouse | GATA4, HNF1α, FOXA3 (+ inactivation of p19Arf) | [67] |

**Table 1:** Strategies for in vitro hepatic differentiation by using hepatic transcription factor genes.

prediction of drug toxicity by using primary human hepatocytes isolated from a single donor. A hepatotoxicity screening utilizing iPSC-derived hepatocyte-like cells would allow the investigation of individual drug metabolism capacity [71-77]. A study has shown the generation of hepatocyte-like cells from patient-specific human iPSCs [78-80]. In the same study, it was demonstrated that patient-specific iPSC-derived hepatocytes are a potential source for modeling diseases whose phenotypes are caused by protein dysregulation within adult cells. A novel drug discovery that reflects the individual genetic information would be possible by using an iPSC library representing different ethnic groups, sexes, and disease phenotypes.

## Hepatic Differentiation by Co-culture and Three-dimensional Culture

In order to facilitate maturation of the ESC- or iPSC-induced hepatocyte-like cells and to enhance the differentiation efficiency of those cells, development of a differentiation system that more closely mimics progenitor development *in vivo* will be needed. Such culture system is also relevant to the culture of primary hepatocytes. Normal culture condition of hepatocytes *in vitro* differs substantially from the environment *in vivo*. Thus, it is difficult to maintain the physiological function of the hepatocytes. To overcome this difficulty, development of a culture system for highly functional hepatocytes is required. So far, co-culture methods with other lineage cells and three-dimensional culture methods have been used to support these challenges.

Co-culture methods have been attempted with primary hepatocytes and other kinds of cells [81-85], because cell-cell interactions are important in embryogenesis and organogenesis. In particular, heterotypic cell-cell interactions in the liver, such as interactions of parenchymal cells with non-parenchymal cells, play a fundamental role in liver function [86]. It has been reported that small hepatocytes could be induced to differentiate into mature hepatocytes by co-culturing with non-parenchymal cells *in vitro* [87]. Cell-cell interactions between embryonic cardiac mesoderm and definitive endoderm have been shown to be essential for liver development [88]. Transcription factors that are critical for hepatic development have been identified from these cell-cell interactions [88]. ES cells co-cultured with cardiac mesoderm showed spontaneous differentiation into hepatocytes [89]. These results suggest that the combined differentiation methods, such as addition of soluble factors into culture medium, transduction of differentiation-related genes or co-cultivation with other lineage cells, may further enhance the differentiation and maturation efficiency of hepatocytes.

Recently, numerous three-dimensional (3D) culture methods have been reported. Among these, the spheroid culture methods, which include the hanging-drop method and the float-culture method using culture dishes coated with non-adherent polymer, have been widely used to culture primary hepatocytes *in vitro*. As various micro-patterning technologies have been developed, various micro-patterned substrates, employing both surface engineering and synthetic polymer chemistry for utilizing spheroid culture, have been reported [90,91]. Spheroid culture methods permit the maintenance of liver-function of primary hepatocytes in comparison with the two-dimensional (2D)-culture.

The bioreactor method is also used for culturing primary hepatocytes. By studying various optimized conditions, flow conditions [92] and cell densities [93], this system has not only shown advantages in terms of maintaining the functions of primary hepatocytes *in vitro* in comparison with 2D-culture [94,95], but also has shown effects of spontaneous differentiation from ESCs into hepatocytes [96,97]. It has been reported that 3D culture using a bioreactor induces more functional maturation in hepatocytes differentiated from ESCs than 2D-culture [97]. The 3D culture methods using polymer scaffold systems have also demonstrated effectiveness both in culturing primary hepatocytes [98,99] and in differentiation from ESCs into hepatocytes *in vitro* [100-102]. These data showed that hepatocytes could be induced from ESCs on a polymer scaffold. ALB expression was detected earlier and the mRNA expression level of ALB was higher than in 2D culture. Furthermore, cell-sheet engineering has recently been reported [103,104]. Cell-sheet 3D culture was performed by using a culture dish coated with a temperature-responsive polymer, poly (N-isopropylacrylamide) [105-107]. Some groups have adopted culture methods with a combination of 3D culture and co-culture and showed that the liver function of primary hepatocytes could be maintained more strongly and longer than without co-culture conditions [108-110]. These combined methods will likely be a more effective differentiation condition to gain mature hepatocytes from ESCs and iPSCs.

## Transplantation of Human ESC- or iPSC-derived Hepatocyte-like Cells

Because of the species differences between humans and other animals, it is difficult to apply biological phenomena of animals to humans in the early phase of drug screening [111]. It is known that chimera mice with human hepatocytes would be a powerful tool to predict drug toxicity and drug metabolism *in vivo* [112-115]. In addition, chimera mice are useful to investigate the molecular mechanisms involved in infection with human hepatitis B virus (HBV) and HCV, because there is no suitable small animal model for such study [116-118]. However, large amounts of human hepatocytes must

be prepared for these technologies, thus requiring large numbers of chimera mice. If it becomes possible to generate a robust chimera mouse model with hepatocyte-like cells differentiated from human ESCs or iPSCs, then chimera mice with humanized livers could be widely used in pharmaceutical development. To this end, several groups have reported the generation of chimera mice with hepatocyte-like cells differentiated from human ESCs and iPSCs. Cai et al. reported that human ESC-derived hepatocyte-like cells were transplanted into the carbon tetrachloride ($CCl_4$)-injured liver of severe combined immunodeficiency (SCID) mice and human alpha-1-antitrypsin (AAT) expression was detected in the liver [37]. Touboul et al. [119] showed that human ESC-derived hepatocyte-like cells can engraft and express human ALB and AAT in the liver of urokinase-type plasminogen activator-transgenic Rag2IL-2Rg$^{-/-}$ (uPA-Rag2IL-2Rg$^{-/-}$) mice. Duan et al. [120] reported that human ESC-derived hepatocyte-like cells were transplanted into the liver of NOD.CB17-Prkdc$^{scid}$/NcrCrl (NOD/SCID) mice and a significant level of human ALB was detected in the recipient mouse serum. Basma et al. [49] generated chimera mice and rats that secreted higher levels of human ALB than previously reported chimera mice. They sorted human ESC-derived hepatocyte-like cells based on surface asialoglycoprotein-receptor 1 (ASGPR1) expression and injected them into the spleen of uPA-SCID mice. Thereafter, they detected a much higher level of human ALB and human AAT in the mouse serum on day 75 after transplantation. They also performed transplantation into Nagase analbuminemic rats treated with both retrorsine, which can prevent proliferation of rat hepatocytes, and FK506, which can suppress immune response, after partial hepatectomy, demonstrating that large clusters of engrafted cells were observed in these rats and human ALB levels were reached at 20,000 ng/ml [49].

The growth speed of hepatocyte-like cells is slower than that of DE cells and hepatoblasts, both of which are immature stage cells as compared with hepatocyte-like cells [60]. It is likely that immature cells can proliferate better than mature cells in the mouse liver. Therefore, several groups have attempted to transplant DE cells or hepatoblasts. In one such attempt, human ESC-derived DE cells were successfully engrafted into the livers of NOD/SCID mice, which were treated with $CCl_4$ and retrorsine, and these mice expressed human AAT in the liver [57]. Recently, Liu et al. [121] compared the engraft efficiency of human ESC-derived multi-stage hepatic cells. They transplanted human DE, hepatoblasts and hepatocyte-like cells differentiated from human ESCs into the dimethylnitrosamine-injured liver of NOD/Lt-SCID/IL-2Rg$^{-/-}$ (NSG) mice, demonstrating that at low cell dosages, the engraftment efficiency of DE cells was slightly higher than that of hepatoblasts and hepatocyte-like cells differentiated from human ESCs. These results suggest that DE cells, which have proliferative capability, can regenerate liver better than hepatocyte-like cells, which have lower proliferative capability.

These technologies, which use ESC-derived cells, can be applied to iPSC-derived hepatocyte-like cells. Si-Tayeb et al. [59] injected human ESC- and iPSC-derived hepatocyte-like cells into the liver of neonatal mice and they detected human ALB expression clusters. Liu et al. [121] also transplanted human ESC- and iPSC-derived hepatocyte-like cells into mice, and achieved similar results. These findings indicate that human iPSC-derived hepatocyte-like cells can engraft into the rodent liver in a manner similar to human ESC-derived hepatocyte-like cells.

Although human ESC- or iPSC-derived hepatocyte-like cells can engraft in the mouse liver, the human ALB levels in chimera mice engrafted with human ESC- or iPSC-derived hepatocyte-like cells are much lower than those in chimera mice engrafted with human primary hepatocytes [49,112,117,121], suggesting that the efficiency of replacement in chimera mice generated with human ESC- or iPSC-derived hepatocyte-like cells would be low. Therefore, the chimerism of mice with human ESC or iPSC-derived hepatocyte-like cells should be improved to apply this technology to industrial applications.

## Conclusions

In this review, we have described several protocols that could promote the differentiation of human ESCs or iPSCs into endodermal and hepatic cells. These methods are all based on the *in vivo* developmental process of embryos. In the future, by using a combination of these protocols or through the discovery of molecular findings about liver development, more efficient protocols for hepatic differentiation could be developed for regenerative medicine and drug development.

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