Fig. S4   Temporal hepatocyte differentiation efficacy was investigated in the 3D ES-hepa.

On day 11, the hESC (H9)-derived cells were plated onto the Nanopillar Plate or the flat plate, and then the cells were cultured until day 35.   (A) On day 0, 5, 10, 15, 20, 25, 30, and 35, the efficiency of hepatocyte differentiation was measured by estimating the percentage of ASGR1-positive cells using FACS analysis.   (B) The cells were counted on day 20, 25, 30, and 35 of the differentiation.   The number of cells on day 20 was taken as 1.0.   *$P<0.05$; **$P<0.01$.

Fig. S5   Upregulation of the gene expression levels of hepatic transcription factors by culturing on the Nanopillar Plate.

The gene expression levels of hepatic transcription factors of the 3D ES (H9)-hepa were measured by real-time RT-PCR on day 35.   On the y axis, the gene expression levels in PHs-48hr were taken as 1.0.   *$P<0.05$; **$P<0.01$.

Fig. S6   WST-8 assay was the most sensitive method for detecting the drug-induced cytotoxicity.

The cell viability of 3D iPSC (Dotcom)-hepa (day 35) was assessed by WST-8, ATP, Alamar blue, or Crystal violet assay after 24 hr exposure to different concentrations of Benzbromarone.   The cell viability is expressed as the percentage of cells treated with solvent only.

Fig. S7   The drug-induced cytotoxicity was more sensitively detected in the 3D iPS-hepa than in the mono iPS-hepa.

On day 11, the hiPSC (Dotcom)-derived cells were plated onto the Nanopillar Plate or the flat plate, and then the cells were cultured until day 35.   The cell viability of 3D iPSC-hepa was assessed by WST-8 assay after 24 hr exposure to different concentrations of drugs.   Cell viability is expressed as a percentage of cells treated with solvent only.

Fig. S8   Hepatocyte function was enhanced by culturing HepG2 cells on Nanopillar Plate.

HepG2 cells were cultured for 5 days on the Nanopillar Plate.   (A, B)   The amount of ALB (A) and urea (B) secretion was examined in the monolayer cultured HepG2 cells (mono HepG2), the 3D spheroid cultured HepG2 cells (3D HepG2), and PHs-48hr.   (C-G) The gene expression levels of CYP enzymes (C), conjugating

enzymes (**D**), hepatic transporters (**E**), hepatic nuclear receptors (**F**), and bile canalicular transporters (**G**) were examined by real-time RT-PCR in the mono HepG2, the 3D HepG2, and PHs-48hr. On the y axis, the expression levels of PHs-48hr were taken as 1.0. $*P<0.05$; $**P<0.01$.

Supplemental Table 1   The antibodies used in this study

Antigen	Type	Company
CYP3A4	goat	Santa Cruz Biotechnology
ALB	goat	Bethyl Laboratories
ASGR1	goat	Santa Cruz Biotechnology
goat IgG	alexa fluor 594	Molecular Probes
goat IgG	alexa fluor 488	Molecular Probes

Supplemental Table 2   The primers used for real-time RT-PCR in this study

Gene Symbol	Primers (forward/reverse; 5' to 3')
ALB	GCACAGAATCCTTGGTGAACAG/ATGGAAGGTGAATGTTTTCAGCA
CYP1A1	GAGGCCAGAAGAAACTCCGT/CCCAGCTCAGCTCAGTACCT
CYP1A2	CAATCAGGTGGTGGTGTCAG/GCTCCTGGACTGTTTTCTGC
CYP3A4	AAGTCGCCTCGAAGATACACA/AAGGAGAGAACACTGCTCGTG
CYP3A5	CGGCATCATAGGTAGGTGGT/TATGAACTGGCCACTCACCC
CYP3A7	AAGGTCGCCTCAAAGAGACA/TGCACTTTCTGCTGGACATC
CYP2B6	GTCCCAGGTGTACCGTGAAG /CCCTTTTGGGAAACCTTCTG
CYP2C8	CAGTGCCAACCAAGTTTTCA/CTCGGGACTTTATGGATTGC
CYP2C9	GGACAGAGACGACAAGCACA/CATCTGTGTAGGGCATGTGG
CYP2C19	ACTTGGAGCTGGGACAGAGA/CATCTGTGTAGGGCATGTGG
CYP2D6	CTTTCGCCCCAACGGTCTC/TTTTGGAAGCGTAGGACCTTG
CYP2E1	ACCCGAGACACCATTTTCAG/TCCAGCACACACTCGTTTC
UGT1A1	TAAGTGGCTACCCCAAAACG/GCTTTGCATTGTCCATCTGA
UGT1A3	TCAGATGGACAATGCAAAGCGC/GGCGCATGATGTTCTCCTTGTA
GSTA1	CCGTGCATTGAAGTAGTGGA/AATTCAGTTGTCGAGCCAGG
GSTA2	TGCAACAATTAAGTGCTTTACCTAAGTG/TTAACTAAGTGGGTGAATAGGAGTTGTATT
SLCO1B3	GGAAATTTTCATAATGATTCCACC/GAAAACAAGACGCTGCAATG
SLCO2B1	AGGGCTCTGCTTAGAGGGAG/GGAAATGCCCAAGGAAAAC
NTCP	AGAAGGTGGAGCAGGTGGT/ATCTTGGTCTGTGGCTGCTC

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OCT1/SLC22A1	TAATGGACCACATCGCTCAA/AGCCCCTGATAGAGCACAGA
OCT2/SLC22A2	ATACAGTTGGGCTCCTGGTG/GAGGCGGGTAGAGATTTTCC
ABCB4	AATTTATCCTGCCAATCGGA/GCATCAGCAGCAAACAAAAA
ABCC1	TGGGCAGGGATTCTCTTTTA/TCATGCTCACTTTCTGGCTG
ABCC3	GTCCGCAGAATGGACTTGAT/TCACCACTTGGGGATCATTT
ABCC4	TCTCCGTTTATGGCCAATTT/CCGTGTACCAGGAGGTGAAG
ABCC6	TGTCGCTCTTTGGAAAATCC/AGGAACACTGCGAAGCTCAT
AhR	AGTTATCCTGGCCTCCGTTT/TCAGTTCTTAGGCTCAGCGTC
CAR	AGTTGCACAGGTGTTTGCTG/GTGCTTAGATGCTGGCATGA
PXR	TCCGGAAAGATCTGTGCTCT/AGGGAGATCTGGTCCTCGAT
PPARα	AGAGTGGGCTTTCCGTGTC/GCCGCCTTCAGGTACAGTAG
MDR1	GCCAAAGCCAAAATATCAGC/TTCCAATGTGTTCGGCATTA
BCRP	TGCAACATGTACTGGCGAAGA/TCTTCCACAAGCCCCAGG
BSEP	TGATCCTGATCAAGGGAAGG/TGGTTCCTGGGAAACAATTC
MRP2	TGAGCAAGTTTGAAACGCACAT/AGCTCTTCTCCTGCCGTCTCT
c/EBPα	GACCTAGAGATCTGGCTGTG/GAGCAAAACCAAAACAAAAC
FOXA1	CTGCACCTGAAAGGGGACC/GCCTTGAAGTCCAGCTTATGC
FOXA2	GCGACCCCAAGACCTACAG/GGTTCTGCCGGTAGAAGGG
FOXA3	TCATGTAGGAGTTGAGGGGG/GAAGATGGAGGCCCATGAC
GATA4	CATCAAGACGGAGCCTGGCC/TGACTGTCGGCCAAGACCAG
HNF1α	TACACCACTCTGGCAGCCACACT/CGGTGGGTACATTGGTGACAGAAC
HNF1β	TCACAGATACCAGCAGCATCAGT/GGGCATCACCAGGCTTGTA
HNF4α	CGTCATCGTTGCCAACACAAT/GGGCCACTCACACATCTGTC
HNF6	CAAACCCTGGAGCAAACTCAA/TGTGTTGCCTCTATCCTTCCC

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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

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].
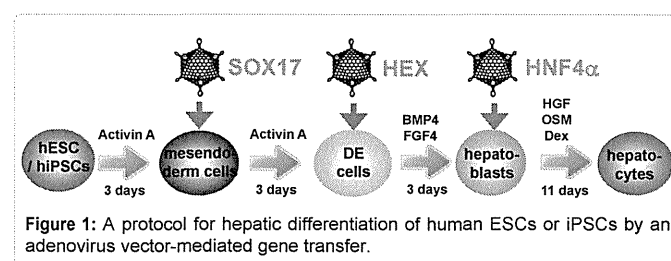
As mentioned above, growth factors that are necessary for *in vivo* hepatic development are utilized in hepatic differentiation from

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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トピックス

ヒト ES/iPS 細胞から肝細胞への
高効率分化誘導法の開発とその創薬応用

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要　旨

　薬物誘発性肝障害は，医薬品候補化合物の開発中止や医薬品の市場撤退の主要な原因であり，医薬品開発研究の初期に肝毒性を精度高く予測することができれば，医薬品開発の効率化やコスト削減に繋がる．ヒト ES 細胞やヒト iPS 細胞からヒト初代培養肝細胞に類似した機能を有した肝細胞を作製できれば，*in vitro* での毒性評価において，ヒト初代培養肝細胞の代替ソースとなりうる．本稿では，ヒト ES/iPS 細胞から肝細胞への分化誘導技術と，毒性評価系への応用に関する現状と課題について概説する．

は じ め に

　薬物によって誘発される肝障害は，医薬品候補化合物の開発中止や医薬品の市場撤退の主な原因の１つである．現在は，ヒト初代培養肝細胞（本稿では，ヒト凍結肝細胞を含めてヒト初代培養肝細胞と表記する）を用いた *in vitro* 毒性評価系で肝毒性を起こす医薬品候補化合物を創薬研究の早期段階において同定し，スクリーニングすることが試みられている．しかしながら，ヒト初代培養肝細胞は高価であり，培養後急速に薬物代謝酵素をはじめとする肝機能が減弱すること，ロット差も大きいため（高機能な肝細胞ロットの）安定供給が困難であるといった問題点を有する．そこで，ヒト ES/iPS 細胞から分化誘導した肝細胞（分化誘導肝細胞）が，ヒト初代培養肝細胞の代替ソースとして期待されている．本稿では，これまでに検討されてきたヒト ES/iPS 細胞からの肝細胞分化誘導法とその課題について紹介するとともに，分化誘導肝細胞を薬物の毒性評価に応用する試みについても紹介する．

ヒト ES/iPS 細胞から肝細胞への分化誘導

1．液性因子の作用による従来の肝分化誘導法

　ヒト ES 細胞から肝細胞への最初の分化誘導の報告では，胚様体（embryoid body：EB）を形成させた後，各種液性因子を作用させることで肝分化が試みられた[1]．しかしながら EB 形成法では細胞集団が不均一であり，分化がランダムに進行し，肝細胞への選択的な分化が制御できない．そこで効率よく肝細胞へ分化させるために，均一な分化誘導ができる平面培養で，生体内での肝発生・分化の環境を模倣してサイトカインや増殖因子などの各種液性因子を作用させることによって，中内胚葉，内胚葉，肝幹前駆細胞，肝細胞へと段階的に分化させる肝分化誘導法が開発された（図1）[2]．

　ヒト ES/iPS 細胞から内胚葉への分化誘導ス

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キーワード：ヒト ES 細胞，ヒト iPS 細胞，肝細胞，
　　　　　　毒性評価，遺伝子導入

図1　ヒト ES/iPS 細胞から肝細胞への分化

ヒト ES/iPS 細胞は，中内胚葉，内胚葉，肝幹前駆細胞を介して肝細胞へと分化する．ヒト ES/iPS 細胞から内胚葉への分化には Activin A が使用される．内胚葉から肝幹前駆細胞への分化には BMP および FGF が併用される．肝幹前駆細胞から肝細胞への分化には HGF および OsM などが使用される．
略語：巻末の「今月の略語」参照

テップでは，Activin A がほぼすべてのプロトコールで使われている[3]．また，Wnt シグナルが内胚葉分化を促進するという報告もあるため，Activin A と Wnt を併用する内胚葉分化誘導法も報告されている[4]．肝特異化の分化誘導ステップ（内胚葉から肝幹前駆細胞への分化：図1）では，肝発生を模倣するように，FGF と BMP を組み合わせる方法が広く用いられている[5]．肝成熟化の分化誘導ステップ（肝幹前駆細胞から肝細胞への分化：図1）では，胎児肝細胞の増殖を支持する HGF[6] や，胎児肝細胞の肝成熟化を促進する Oncostatin M（OsM）[7] などが使用されている．しかしながら，これらの液性因子の作用のみからなる分化誘導法では肝細胞への分化効率は不十分であり，さらなる肝分化効率の向上が要求されている．

2．肝分化関連遺伝子を導入する肝分化誘導法

　山中4因子と呼ばれる転写因子を体細胞に遺伝子導入すると細胞が初期化され iPS 細胞が樹立されるように，肝細胞への分化を含むあらゆる細胞の運命決定において遺伝子発現の制御は極めて重要なツールとなりうる．そこで筆者らは，ヒト ES/iPS 細胞から肝細胞への分化誘導の各ステップにおいても，各種液性因子の作用に加えて外来的に遺伝子発現を制御することによって肝分化の促進を試みた．ヒト ES/iPS 細胞から分化誘導された中内胚葉に，内胚葉形

成に必須である SOX17（sry-related HMG box 17）遺伝子を導入した結果，約 80% の効率で内胚葉が分化誘導された[8]．また，分化誘導された内胚葉に，肝特異化に必須である HEX（hematopoietically expressed homeobox）遺伝子を導入することによって，肝幹前駆細胞への分化が促進された[9]．さらに，分化誘導された肝幹前駆細胞に HNF4α（hepatocyte nuclear factor 4 alpha）遺伝子を導入した結果，より高い肝機能を有した肝細胞を高効率に作製できた[10]．さらに筆者らは最近，FOXA2（forkhead box A2）と HNF1α（hepatocyte nuclear factor 1 alpha）遺伝子を組み合わせて各分化ステップの細胞に導入することによって，SOX17・HEX・HNF4α 遺伝子の導入を組み合わせた方法よりも高い cytochrome P450（CYP）代謝能を有する分化誘導肝細胞を作製することに成功した[11]．

3．分化誘導肝細胞と異種細胞との共培養法

　胚発生過程では，肝幹前駆細胞は心臓中胚葉や横中隔間充織に接触しており，肝発生には隣接する中胚葉からのシグナルが重要である．そこで，ES 細胞からの肝分化過程において，胚発生を模倣するように中胚葉系の細胞との共培養が試みられた．ES 細胞を胎生中胚葉[12] や中胚葉由来の細胞株（M15）[13] と共培養することによって，肝分化が促進されることが報告され

4. 肝分化させた細胞集団からの分化誘導肝細胞の抽出

上述したような肝分化誘導技術の改良によって，肝細胞への分化効率は飛躍的に向上したが，依然として最終的に分化させた細胞集団は分化度が不均一であり，分化が不十分な細胞が混在している状態である．そこで Basma らは，未分化な細胞や内胚葉では発現せず，肝細胞に特異的に発現する表面抗原として asialoglycoprotein receptor 1（ASGR1）に着目した[14]．肝分化させた細胞集団から ASGR1 陽性細胞をソートすることで，分化誘導肝細胞のみを抽出することに成功した．また Woo らは，indocyanine green（成熟した肝細胞が特異的に取り込むことが知られている色素）を取り込んだ細胞のみをソートすることによって，分化誘導肝細胞を選択的に抽出できることを報告した[15]．肝分化させた細胞集団から分化誘導肝細胞を高純度に抽出できる技術を活用することによって，均一な機能を有し，より成熟した肝細胞集団を供給できると期待される．

分化誘導肝細胞の毒性評価系への応用

薬物が引き起こす肝毒性の多くは，薬物が薬物代謝酵素で代謝されて生じる反応性代謝物が原因であるため，反応性代謝物による毒性を検出できる評価系の開発が必要である．筆者らは，トログリタゾン，アセトアミノフェンといった肝毒性を起こす薬物を，上述の遺伝子導入を組み合わせた分化誘導法で作製した分化誘導肝細胞に作用させたところ，細胞毒性が生じることを確認した[16]．また，肝毒性を生じる 20 種類以上の薬物を分化誘導肝細胞に作用させたところ，ほぼすべての薬物について，in vitro 肝毒性評価系として汎用される HepG2 細胞（肝がん細胞由来細胞株）よりも高感度に細胞毒性を検出することが可能であった[16]．さらに，薬物代謝酵素の阻害剤を併用して細胞毒性を評価し

たところ，薬物による細胞傷害性が一部減弱し，反応性代謝物による毒性も筆者らの分化誘導肝細胞で検出できることが明らかになった[16]．分化誘導肝細胞を用いた薬物の毒性評価はいまだ研究開発段階の技術ではあるが，本細胞を毒性評価系へ応用できる可能性が示唆された．

まとめ

肝発生の基礎研究で得られた知見をもとに，ヒト ES/iPS 細胞から薬物代謝能を有した肝細胞を分化誘導する研究が活発に行われ，肝分化誘導技術は確実に進歩してきた．しかしながら現在の肝分化誘導技術では，ヒト初代培養肝細胞の完全なる代替品を作出するまでには至っていない．今後，分化誘導肝細胞の創薬応用の実現を目指して，さらなる研究の進歩が期待される．

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Efficient Generation of Hepatocyte-like Cells from Human ES/iPS Cells
for Drug Toxicity Screening

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特集

ハイスループットな創薬スクリーニングを目指して

ヒト iPS 細胞由来分化誘導肝細胞を用いた薬物毒性評価系の開発

Evaluation of Drug Toxicity by Using Hepatocytes Derived from Human iPS Cells

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ヒト iPS 細胞は再生医療だけではなく創薬への応用も強く期待されている。特に肝細胞を iPS 細胞から効率良く分化誘導できれば，ハイスループットな薬物毒性評価系や薬物代謝評価系を新規に構築できると考えられる。本稿では，筆者らが考案したヒト iPS 細胞由来肝細胞の分化誘導法とその薬物毒性評価への応用について紹介したい。

1. はじめに

現在の創薬プロセスにおいては，一つの医薬品が製品化されるまでに 10〜15 年程度の期間および 1,000 億円を超える開発費が必要であるといわれており，研究開発費のうちの 7 割強は臨床試験以前の探索研究から前臨床研究までに投入されている。その過程で数万〜100 万件の候補化合物の中から薬効，毒性などの評価を経て一つが医薬品として承認を受ける。ここでしばしば問題となるのが薬物誘発性肝障害（肝毒性）であるが，医薬品の開発プロセスの早期に肝毒性を確度良く予測することは，創薬コスト削減・期間短縮・創薬シーズのヒット率の向上をもたらし，我が国の基幹産業のひとつである製薬産業の国際競争力向上に繋がると期待される。ヒト初代培養肝細胞の利用により肝毒性評価技術の向上が見込まれるものの，我が国においては入手が困難であり，安定供給や継続性の観点からその利用には限界がある為，

より安定かつ容易に使用できる肝毒性評価系の確立が望まれている。近年，ヒト体細胞から分化多能性を有した iPS（induced pluripotent stem）細胞の樹立が報告され，iPS 細胞由来分化誘導肝細胞は上記の問題点の克服が期待できることから大きな注目を集めている。本稿では，近年目覚ましい進歩を遂げているヒト iPS 細胞から肝細胞への分化誘導法に関する知見を概説するとともに，それを利用した薬物毒性評価系への応用の可能性について筆者らの最新の結果を含めて紹介する。

2. 肝細胞の培養

肝臓は，炭水化物や脂質の代謝，グリコーゲンの貯蔵とグルコースの合成，尿素の生合成等，多くの機能を有する内胚葉由来の臓器である。肝臓を構成する細胞のうち，肝実質細胞（肝細胞）がこれらの主要な機能を担っており，*in vitro* で培養された肝細胞は，生物医学的研究だけでなく再生医療や薬物毒性評価系への応用も強く期待され

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ている。これまで肝組織の *in vitro* モデルとして初代培養肝細胞がしばしば用いられてきた。初代培養肝細胞は薬物代謝酵素や薬物トランスポーターを高発現していることから，現在でも *in vitro* での標準細胞として薬物毒性試験等で用いられている[1]。しかしながら，初代培養肝細胞は，高価であること，ドナーが制限されること，増殖しないために安定供給が難しいこと，培養後速やかにシトクロム P450 薬物代謝酵素の活性低下がみとめられること，等の問題点が指摘されている[2~4]。したがって，無限増殖能を有するヒト iPS 細胞から効率良く肝細胞が分化誘導できればこれらの問題点が解決できると期待されている。

3. ヒト iPS 細胞から肝細胞への分化誘導

ヒト iPS 細胞はヒト ES (embryonic stem) 細胞と同様に分化多能性を有し，神経や皮膚，肝臓，血液，心筋等の三胚葉へ分化することができる[5,6]。ヒト iPS 細胞の分化誘導はヒト ES 細胞の分化誘導と基本的に同等であり，いずれも共通の手法を用いて分化誘導できる。したがって，以下にヒト iPS 細胞から肝細胞への分化誘導法について紹介するが，ヒト ES 細胞から肝細胞への分化誘導法の方がより多く報告されているため，ヒト ES 細胞に関する報告も混在していることに留意されたい。

3.1　ヒト iPS 細胞から内胚葉への分化誘導

ヒト iPS 細胞の分化誘導研究において，肝細胞等の内胚葉分化に関する研究は，神経細胞等の外胚葉分化に関する研究や心筋細胞・血液細胞等の中胚葉分化に関する研究よりも遅れてきた（図1）。内胚葉分化誘導の研究が遅れてきた理由の一つとして，分化過程が複数の段階を経ることによるものと考えられる。肝細胞分化の場合，ヒト iPS 細胞は中内胚葉，内胚葉，肝幹前駆細胞を経由して成熟肝細胞へと分化し（図2），この過程で種々の液性因子が必要とされる。このうち，内胚葉への分化誘導において最も頻繁に用いられている液性因子はアクチビン A である[7,8]。アクチビン A は TGF (transforming growth factor)-β ファミリーに属する増殖因子であり，受容体に結合した後，細胞内で Smad とよばれるアダプター分子群を活性化する[9]。アクチビン A 以外では，FGF (fibroblast growth factor) 2 や Wnt3a も内胚葉分化誘導に用いられる。特に FGF2 については，アクチビン A と同時に作用させることにより，アクチビン A 単独作用時と比較し有意に内胚葉分化誘導効率が向上することが報告されている[10]。

3.2　内胚葉から肝細胞への特異化

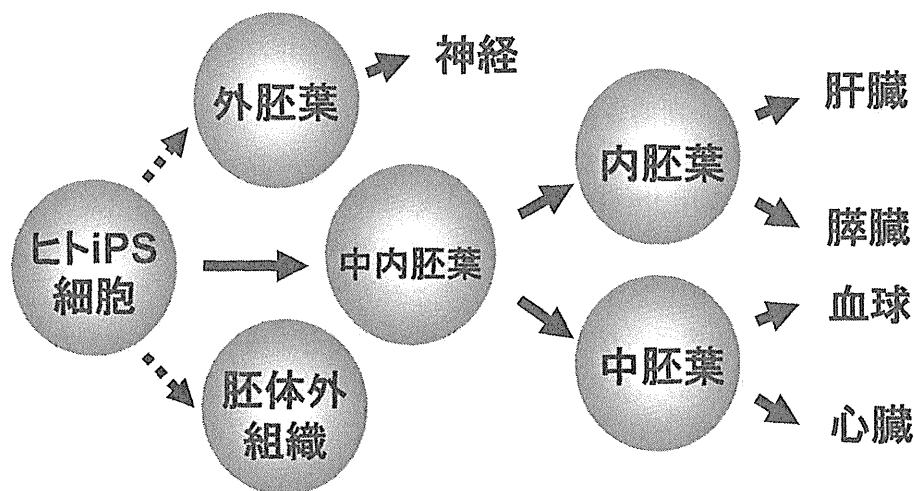内胚葉から肝細胞への分化は肝細胞特異化 (specification) と肝細胞成熟化 (maturation) の

図1　ヒト iPS 細胞から三胚葉への分化誘導
ヒト iPS 細胞はヒト ES 細胞とおなじく三胚葉に分化することができる。

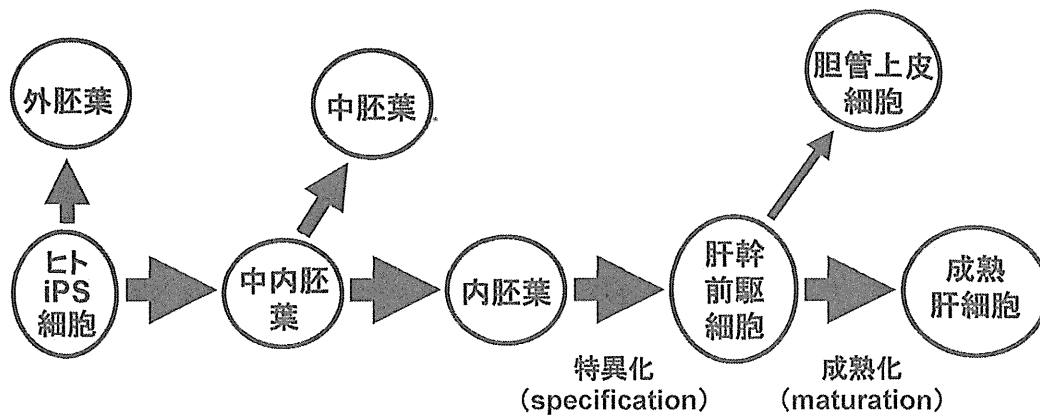図2　ヒト iPS 細胞から肝細胞への分化
ヒト iPS 細胞から成熟肝細胞への分化は複数の過程に分けることができる。

2つのステップに分かれる（図2）。このうち，肝細胞特異化の過程では肝幹前駆細胞が分化誘導され，α-フェトプロテインやトランスサイレチンを発現するようになる[11, 12]。この過程では FGF シグナルと BMP（bone morphogenetic protein）シグナルが重要であることが知られており，FGF4 と BMP2 を作用させることにより肝特異化が著明に亢進することが報告されている[13]。またその他にも，FGF1/2/4 と BMP2/4 の組み合わせによって，内胚葉から肝細胞が分化誘導できるという報告もある[10]。

3.3　肝幹前駆細胞から肝細胞への成熟化

肝幹前駆細胞は肝実質細胞と胆管上皮細胞という2種類の系列に分化することが可能である（図2）。肝幹前駆細胞から肝実質細胞へ分化するにつれて α-フェトプロテインの発現量が低下し，代わってアルブミンの発現量が上昇してくる。この過程において重要な液性因子は HGF（hepatocyte growth factor）とオンコスタチン M である[14, 15]。HGF は肝前駆細胞の増殖を促進させるとともに胆管への分化を阻害する。また，オンコスタチン M は肝前駆細胞の成熟化を促進する。

さらに各分化ステップで，培地や細胞外マトリックス（I 型コラーゲンやマトリゲルが汎用される）の種類，血清やフィーダー細胞の有無等が各プロトコールで工夫されている。ヒト iPS 細胞由来分化誘導肝細胞を再生医療に利用する場合には，血清やフィーダー細胞等の異種動物由来成分を排除し，かつ組成の明らかな培地（chemically defined medium）で分化誘導する必要がある。一方，iPS 細胞由来分化誘導肝細胞を創薬研究に応用する場合にはそのような制限は必要ではなく，むしろ創薬応用においては可能な限り成熟度が高い肝細胞を分化誘導する必要があるため，特に血清の使用は現時点では有用である。以前は，胚様体（embryoid body：EB）形成法を用いて肝細胞への分化が試みられてきたが，最近では，EB 形成を介さず，上述のように直接分化させる方法が一般的である。しかしながら，これらの増殖因子やサイトカインの添加だけからなる分化誘導法は，肝細胞への分化効率もまだまだ不十分なのが現状であり，更なる分化効率の向上が必要となっている。

3.4　遺伝子導入による肝細胞分化誘導

先述したように，iPS 細胞から肝細胞への分化誘導効率は未だ十分ではなく，薬物毒性評価系に応用するにはさらなる技術開発が必要である。筆者らや他のグループは，Sox17 とよばれる内胚葉分化に重要な転写因子の遺伝子をヒト ES 細胞や iPS 細胞に導入することにより，内胚葉への分化誘導効率が著明に向上することを明らかにした[16, 17]。また，FoxA2 とよばれる内胚葉で強く

発現している転写因子の遺伝子を導入することでも内胚葉分化は促進される[18]。肝特異化のステップでは，肝発生に重要な転写因子である Hex 遺伝子を，iPS 細胞由来内胚葉に導入することにより肝細胞分化が強く促進されることが筆者らと他のグループにより報告されている[19~21]。

　また，筆者らは複数の遺伝子を分化の適切な時期に順次導入することにより，ヒト iPS 細胞から成熟肝細胞までの分化誘導効率を向上させることを検討した。未分化 iPS 細胞からアクチビン A 処理で分化させた中内胚葉に SOX17 遺伝子を，内胚葉から肝幹前駆細胞への分化ステップでは HEX 遺伝子を，さらに肝幹前駆細胞から肝細胞への分化ステップでは HNF4α 遺伝子を導入することで，高いアルブミン産生能や薬物代謝機能を有した肝細胞を効率よく分化誘導することに成功した[17, 20, 22]。さらに最近では，ヒト iPS 細胞から肝細胞への各分化ステップにおいて 7 種類の肝関連転写因子（FoxA2，SOX17，HEX，HNF1α，HNF1β，HNF4α，HNF6）を導入し，最も効率良く肝分化を促進できる転写因子を探索した結果，FoxA2 および HNF1α 遺伝子を組み合わせて発現させることにより，さらに効率良く成熟肝細胞を分化誘導することに成功した（図3）[23]。なお，本分化誘導では，機能性に優れ，独自開発した改良型アデノウイルスベクターを用いた。iPS 細胞から肝細胞への分化のように，分化の各ステップが階層的に起こる場合には，各分化ステップでだけ導入遺伝子が機能するように（後の細胞分化に影響を与えないように）遺伝子発現期間は一過性であること，そして効率よく細胞集団を分化させるためには，100％の遺伝子発現効率で遺伝子発現させることが必須となるが，改良型アデノウイルスベクターはこのような目的に唯一叶うベクターである。本研究で用いた改良型アデノウイルスベクターは，細胞への感染に関与するウイルス表面タンパク質のファイバータンパク質の C 末端領域にポリリジン配列（KKKKKKK；リジン（K）が 7 つ続くので K7 と略称）を遺伝子工学的に付与しており，細胞表面のヘパラン硫酸を認識して多くの細胞種に効率よく遺伝子導入が可能となる（図4）。K7 型アデノウイルスベクターは，未分化ヒト iPS 細胞や，ヒト iPS 細胞から分化した細胞に対しても 100％の効率で遺伝子導入が可能であった[20]。

3.5　三次元培養技術による肝細胞の成熟化

　肝細胞をハンギングドロップ法や浮遊培養法を用いてスフェロイド培養することにより成熟化することはよく知られている。筆者らは，細胞シート工学技術を用い，シート状に回収したマウス Swiss 3T3 線維芽細胞とヒト iPS 細胞由来分化誘導肝細胞とを積層三次元共培養し肝細胞の成熟化を検討した[24]。その結果，ヒト iPS 細胞より分化誘導した単層の肝細胞と比較し，Swiss 3T3 細胞と積層三次元共培養することによりアルブミンや HNF4α，CYP1A2 などの肝細胞特異的な遺伝子発現量が上昇することが確認された。また，分化誘導した肝細胞の成熟化には Swiss 3T3 細胞の分泌する液性因子よりも，肝細胞と Swiss 3T3
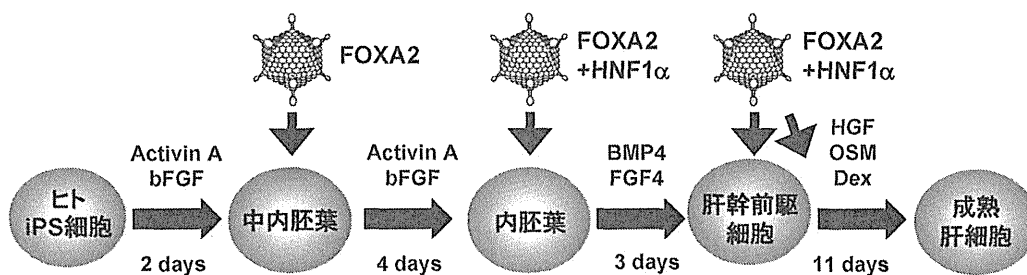

図3　遺伝子導入を用いたヒト iPS 細胞から成熟肝細胞への分化誘導
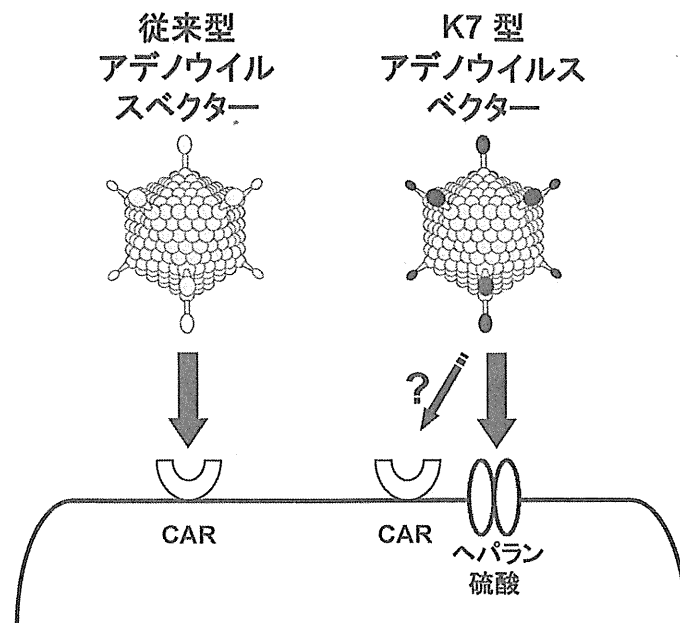分化の適切な時期に適切な遺伝子を一過性に発現させることにより，効率良く肝細胞を分化誘導できる。

図4 改良型アデノウイルスベクター
改良型（K7型）アデノウイルスベクターはアデノウイルス受容体（CAR）だけでなく，ヘパラン硫酸も認識することにより，多くの細胞種に効率よく遺伝子導入が可能となる。

細胞との直接的な接触が重要であることを見いだし，Swiss 3T3 細胞との積層三次元共培養により分化誘導した肝細胞は，成熟化がより促進されていることが明らかとなった。

4. iPS 細胞由来肝細胞を用いた薬物毒性評価系の開発

このようにしてヒト iPS 細胞から分化誘導した肝細胞は，形態学的には二核を有した成熟肝細胞の形状をしており，80〜90％以上の細胞がアルブミン，アシアロ糖タンパク質受容体，LDL（low density lipoprotein）取り込み能，インドシアニングリーン取り込み能，薬物代謝酵素（CYP3A4，CYP7A1，CYP2D6 等）陽性であり，ヒト初代培養肝細胞に匹敵する薬物代謝酵素の遺伝子発現レベルを示した。また，シトクロム P450 酵素などで代謝される9種類の薬物の代謝プロファイルを調べたところ，分化誘導肝細胞の薬物代謝能はヒト初代培養肝細胞より低いものの（シトクロム P450 酵素の種類により異なるが，分化誘導肝細胞はヒト初代培養肝細胞の1〜40％程度の活性），

いずれの薬物に対しても代謝能を有していることが確認された[23]。各シトクロム P450 酵素の遺伝子発現と代謝能との間に，iPS 細胞由来分化誘導肝細胞とヒト初代培養肝細胞で乖離が認められたが，この原因としては，そもそもシトクロム P450 酵素の活性は個人差が大きいことが知られており（数十倍〜千倍程度の個人差），低いシトクロム P450 酵素活性の個人から iPS 細胞が樹立されていた可能性や，シトクロム P450 酵素の活性発現に必要な補酵素群の発現が未だ分化誘導肝細胞では十分でないこと等が考えられた。今後，異なった個人から樹立したヒト iPS 細胞由来分化誘導肝細胞を用いて同様の検討する必要がある。また，Rashid らは α1-アンチトリプシン欠損症・家族性コレステロール血漿症・グリコーゲン貯蔵疾患症 1α の患者の皮膚細胞から iPS 細胞を作製し，肝細胞へ分化誘導させ，それぞれの病態を反映した肝細胞を作製できることを示した[25]。したがって，将来的には病態を反映した iPS 細胞由来分化誘導肝細胞を用いた薬物毒性評価や代謝評価も可能となるであろう。

筆者らは，ヒトiPS細胞由来分化誘導肝細胞を用いて，薬剤に対する毒性評価についても検討した（論文投稿中）。肝毒性を生じることが知られている多種類の薬剤について，本分化誘導肝細胞を用いて細胞毒性評価試験を行ったところ，株化細胞であるHepG2細胞を用いた場合に比べ，より感度良く毒性（細胞傷害性）を示し，かつその毒性はシトクロムP450酵素の阻害剤を加えると部分的に消失した。したがって，iPS細胞由来分化誘導肝細胞を用いることによって，シトクロムP450酵素で代謝された代謝物（反応性代謝物）によって生じた細胞傷害性を再現性良く検出できることが明らかとなった。反応性代謝物は薬物性肝障害の主な原因と考えられており，ヒトiPS細胞由来分化誘導肝細胞で反応性代謝物による細胞傷害性を検出できたことは，極めて大きな意義をもつと考えられる。以上のことから，FOXA2およびHNF1α遺伝子を導入することにより，ヒトiPS細胞から薬物代謝能を有する肝細胞を効率良く分化誘導できるだけでなく，同細胞が薬物の毒性スクリーニングに使用可能であることが示唆された。

5. おわりに

これまでのヒトiPS細胞から分化誘導させた肝細胞は，機能面において初代培養肝細胞に比べて大きく劣っており，創薬研究への応用は困難であった。しかしながら，筆者らが開発した，遺伝子導入を駆使した分化誘導法により，創薬応用に向けてようやく最低限の解析が可能なレベルにまで分化した肝細胞を得ることが可能になった。一方で，ヒトiPS細胞由来分化誘導肝細胞を幅広く創薬研究に応用するためには，実験毎に3週間に及ぶ分化誘導を行う必要があり，これは細胞供給の観点から効率が悪いと考えられる。そこで現在筆者らは，分化途中の肝幹前駆細胞の段階で，凍結融解ができないか，あるいは分化細胞を大量に増幅できないかという課題にも取り組んでいる。

今度，より一層高機能な（成熟度が高い）ヒトiPS細胞由来分化誘導肝細胞の作製法の開発を進めるとともに，本分化誘導肝細胞が創薬研究で広く活用されることを期待している。なお，本稿で紹介した分化誘導法で作製されたヒトiPS細胞由来分化誘導肝細胞は，㈱リプロセルよりRepro Hepatoとして市販されている。

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