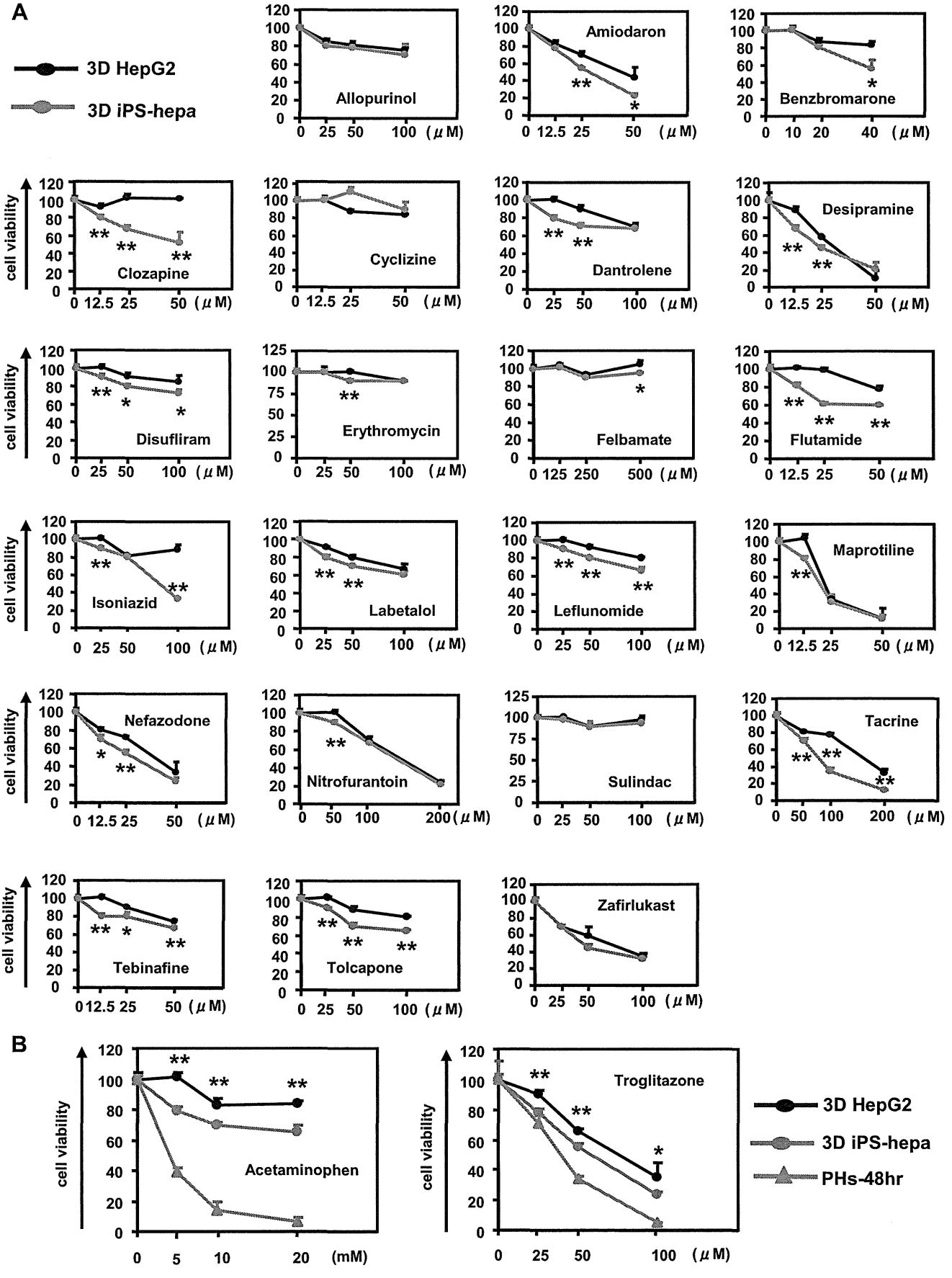
A
3D HepG2
3D iPS-hepa
cell viability
Allopurinol
Amiodaron
Benzbromarone
Clozapine
Cyclizine
Dantrolene
Desipramine
Disufliram
Erythromycin
Felbamate
Flutamide
Isoniazid
Labetalol
Leflunomide
Maprotiline
Nefazodone
Nitrofurantoin
Sulindac
Tacrine
Tebinafine
Tolcapone
Zafirlukast
B
Acetaminophen
Troglitazone
3D HepG2
3D iPS-hepa
PHs-48hr

inhibitor Cyclosporin A [22,23]. More CLF was accumulated in the 3D ES-hepa than in the mono ES-hepa (Fig. 2I *upper panel*). Moreover, CLF accumulation was inhibited by Cyclosporin A treatment only in the 3D ES-hepa (Fig. 2I *lower panel*), demonstrating that the functionality of BSEP transporter in 3D ES-hepa was greater than that in mono ES-hepa. These results suggested that hepatocyte maturation was promoted by the culture on the Nanopillar Plate. It is likely that, compared to the monolayer culture condition, the 3D spheroid-culture condition is more similar to the *in vivo* condition.

It is important to select an hESC/hiPSC line that has a strong ability to differentiate into hepatocyte-like cells in the case of medical applications such as drug screening. In this study, two hESC lines and six hiPSC lines were differentiated into the hepatocyte-like cells, and then their gene expression levels of *ALB* (Fig. 3A) and ALB secretion levels (Fig. 3B) were compared. These results suggest that the iPSC line, Dotcom, was the suitable cell line for hepatocyte maturation. Therefore, the iPSC line, Dotcom, was used to examine the possibility of the 3D iPS-hepa for drug screening. The drug metabolism capacity and the CYP induction potency of the 3D iPS-hepa were compared with those of the mono iPS-hepa. We confirmed the expression of ALB and CYP3A4 protein in the 3D ES-hepa (Fig. 4A). The activity levels of CYP enzymes in the 3D iPS-hepa were measured according to the metabolism of the CYP2C9 or CYP3A4 substrates (Fig. 4B); the levels were higher than those of the mono iPS-hepa (Fig. 4B). We further tested the induction of CYP2C9 and CYP3A4 by chemical stimulation (rifampicin was used as a CYP2C9 or CYP3A4 inducer). Compared with mono iPS-hepa, the 3D iPS-hepa produced more metabolites in response to chemical stimulation (Fig. 4C). In addition, the CYP induction was inhibited by using CYP2C9 or CYP3A4 inhibitor (Sulfaphenazole or Ketoconazole, respectively). These results indicated that drug metabolism capacity and CYP induction potency in 3D iPS-hepa were higher than those in mono iPS-hepa.

Many researchers have tried to predict the drug-induced cytotoxicity *in vitro* using hepatocarcinoma-derived cells such as HepG2 cells [24,25]. HepG2 cells are less expensive than PHs and the reproducible experiments are easier to perform than they are with PHs, although 30% of the compounds were incorrectly classified as nontoxic [24,25]. To overcome these problems, hESC/hiPSC-derived hepatocyte-like cells are expected to be used to predict drug-induced cytotoxicity. To examine its applicability to drug screening, the 3D iPS-hepa were treated with various drugs, that cause hepatotoxicity. WST-8 assay was performed to evaluate cell viability (Fig. S6). The susceptibility of the 3D iPS-hepa to most of the hepatotoxic drugs was higher than that of the mono iPS-hepa (Fig. S7). Compared to the mono iPS-hepa, the 3D iPS-hepa were more suitable tools for drug screening. Next, the susceptibility of the 3D iPS-hepa to the hepatotoxic drugs was compared with that of the 3D spheroid cultured HepG2 cells (3D HepG2; the hepatocyte functions of 3D HepG2 cells are higher than those of monolayer cultured HepG2 cells [Fig. S8]). With most of the drugs, the cell viability of the 3D iPS-hepa was lower than that of the 3D HepG2 (Fig. 5A). These results indicated that the 3D iPS-hepa are more valuable tools for drug screening than the 3D HepG2. However, the susceptibility of the 3D iPS-hepa to Acetaminophen and Troglitazone was lower than that of the PHs which were cultured for 48 h after the cells were plated (Fig. 5B). These results might be due to the lower activity levels of CYPs in 3D iPS-hepa as compared as those in PHs. Taken together, 3D iPS-hepa are more valuable tools for drug screening than the 3D HepG2, although further maturation of 3D iPS-hepa is still required for 3D iPS-hepa to be an alternative cell source of PHs in the drug screening.

To examine whether drug-induced cytotoxicity is caused by CYP metabolites in 3D iPS-hepa, Aflatoxin B1 (mainly metabolized by CYP3A4 [26]) and Benzbromarone (mainly metabolized by CYP2C9 [27]) were treated in the presence or absence of a CYP3A4 and a 2C9 inhibitor, Ketoconazole and Sulfaphenazole, respectively (Fig. 6). The cell viability of 3D iPS-hepa was partially rescued by treatment with the CYP inhibitor. These results indicated that drug-induced cytotoxicity was caused by CYP metabolites of Aflatoxin B1 and Benzbromarone.

4. Discussion

Recently, it has been expected that human pluripotent stem cells and their derivatives, including hepatocyte-like cells, will be utilized in applications for the safety assessment of drugs. We have previously reported that combinational overexpression of SOX17, HEX, and HNF4α, or combinational overexpression of FOXA2 and HNF1α could promote hepatocyte differentiation [5,6]. However, the drug metabolism capacity of the hepatocyte-like cells generated by our previous protocol was still lower than that of primary human hepatocytes [6]. To generate more matured hepatocyte-like cells as compared with our previous protocol, we established a hepatocyte differentiation method employing not only stage-specific transient overexpression of hepatocyte-related transcription factors but also a 3D culture systems using a Nanopillar Plate, was established. Although the use of hepatocyte-like cells generated from hESCs/hiPSCs in application for drug toxicity testing has begun to be focused, to the best of our knowledge, there have been few studies that have investigated whether hepatocyte-like cells could predict many kinds of drug-induced toxicity.

3D culture spheroids were generated from hESCs/hiPSCs by using a Nanopillar Plate. The diameter of the spheroids was approximately 100 μm on day 35 of differentiation (Fig. 1C). Because it is known that the no-oxygen limitation would take place in spheroids up to 100 μm in diameter [28], the size of the spheroid might be important to generate spheroids with high viability. A Nanopillar Plate has a potential to regulate the spheroid diameter simply by culturing under optimized seeding condition, on its suitably designed pillar and hole structure [11]. Therefore, a Nanopillar Plate would be a suitable environment for the generation of 3D ES/iPS-hepa that show high viability and possess high level of hepatocellular functions.

The levels of many hepatocyte functions, such as ALB secretion ability (Fig. 2B), urea secretion ability (Fig. 2C), hepatocyte-related gene expressions (Fig. 2D–H), drug metabolism capacity (Fig. 4B), and CYP induction potency (Fig. 4C), of 3D ES/iPS-hepa were higher than those of mono ES/iPS-hepa. This might have been because the structural and functional polarity, which can be seen in the naïve environment of hepatocytes, of the hepatocyte-like cells was configured by a 3D culturing condition. Previous studies have shown that a 3D culture condition is suitable to maintain the hepatic characteristics of the isolated hepatocytes because this condition mimic *in vivo* environment [29,30]. These facts indicated that the 3D culture condition is a more suitable condition for the hepatocyte-like cells than the monolayer culture condition.

Two hES cell lines and six hiPS cell lines were differentiated into the hepatocyte-like cells in this study. The hiPS cell line, Dotcom, seemed to be a suitable cell line for hepatic differentiation (Fig. 3). Because the hepatic differentiation propensity differs among the

Fig. 5. The possibility of applying 3D iPS-hepa to drug testing was examined. (A) The cell viability of the 3D HepG2 (black) and 3D iPSC-hepa (red) were assessed by WST-8 assay after 24 h exposure to different concentrations of 22 test compounds. (B) The cell viability of the 3D HepG2 (black), 3D iPSC-hepa (red), and PHs-48hr (green) were assessed by WST-8 assay after 24 h exposure to different concentrations of Acetaminophen and Troglitazone. Cell viability is expressed as a percentage of cells treated with solvent only. $^{*}P < 0.05$; $^{**}P < 0.01$.

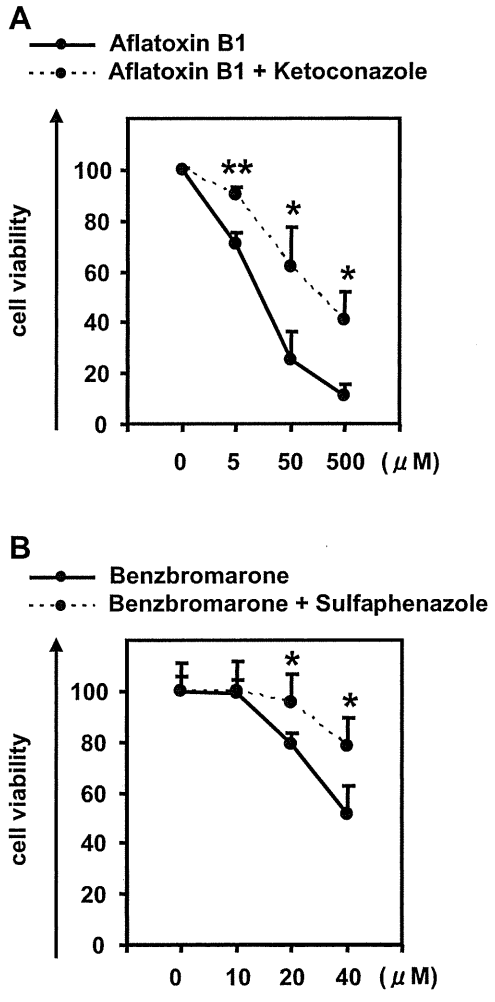

Fig. 6. Drug-induced cytotoxicity in the 3D iPS-hepa is mediated by cytochrome P450. (A, B) The cell viability of the 3D iPSC-hepa was assessed by WST-8 assay after 24 h exposure to different concentrations of (A) Aflatoxin B1 and (B) Benzbromarone in the presence or absence of the CYP3A4 or 2C9 inhibitor, Ketoconazole or Sulfaphenazole, respectively. Cell viability was expressed as the percentage of cells treated with solvent only. $^{*}P < 0.05$; $^{**}P < 0.01$.

hES/hiPS cell lines, it would be important to select an appropriate cell line for medical applications such as drug screening. However, the dominant reason for this hepatic differentiation propensity is not been well known. It would be interesting study to elucidate the mechanism of this propensity.

Although the drug metabolism capacity and CYP induction potency of 3D iPS-hepa were higher than those of mono iPS-hepa (Fig. 4B and C), they were still lower than those of primary human hepatocytes. The hepatic nuclear factors are known to be key molecules in the CYP induction of hepatocytes [30]. Therefore, overexpression of hepatic nuclear factors, which are not abundantly expressed in the hepatocyte-like cells (such as *PXR*), might upregulate the CYP induction potency of the hepatocyte-like cells.

3D iPS-hepa were more sensitive for detection of the drug-induced cytotoxicity than HepG2 cells that are widely used to predict hepatotoxicity [31,32] (Fig. 5). In addition, the decrease of cell viability, which was caused by hepatotoxic drugs, of 3D iPS-hepa was partially rescued by treatment with a CYP inhibitor (Fig. 6). These data suggest that the hepatocyte-like cells could detect the toxicity of the reactive metabolites that were generated by drug metabolizing enzymes such as CYP enzymes. Because in many cases, drug-induced hepatotoxicity is caused by the reactive metabolites produced by drug metabolizing enzymes [33], our finding that the hepatocyte-like cells could detect the toxicity of reactive metabolites should be of great potential for toxicological screening. Moreover, it might be possible to predict idiosyncratic liver toxicity by using hepatocyte-like cells generated from hiPSCs that were established from a patient with a rare CYP polymorphism. However, some compounds did not show any cytotoxicity (such as Cyclizine, Felbamate, and Sulindac) (Fig. 5). To apply the hepatocyte-like cells for wide-spread drug screening, generation of the hepatocyte-like cells are required to detect hepatotoxity in more sensitive manner. Previous studies showed that the depletion of conjugating enzymes [32] or knockdown of Nrf2 [34] expression are useful to upregulate the sensitivity to hepatotoxic drugs. Therefore, these approaches would be useful to generate more sensitive hepatocytes to toxic drugs.

5. Conclusions

In this study, we established the efficient hepatocyte differentiation method which employs not only stage-specific transient overexpression of hepatocyte-related transcription factors but also 3D spheroid culture systems by using Nanopillar Plate. To the best of our knowledge, this is the first study in which the hepatocyte-like cells, having enough hepatocyte functions, mediate drug-induced cytotoxicity against many compounds. Our hepatocyte-like cells differentiated from hESCs or hiPSCs have potential to be applied in drug toxicity testing.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2012.11.029.

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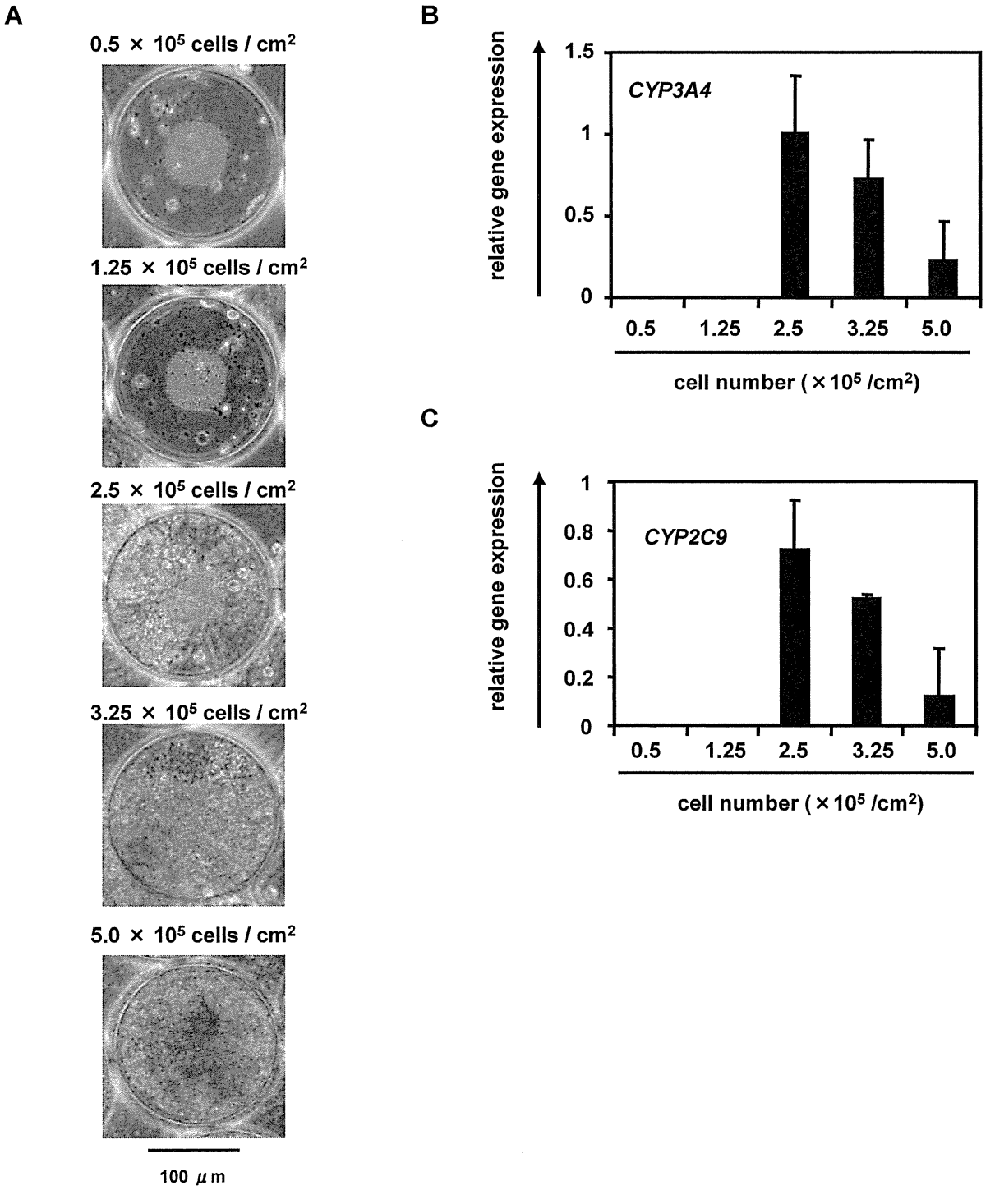
Supplemental figure 1



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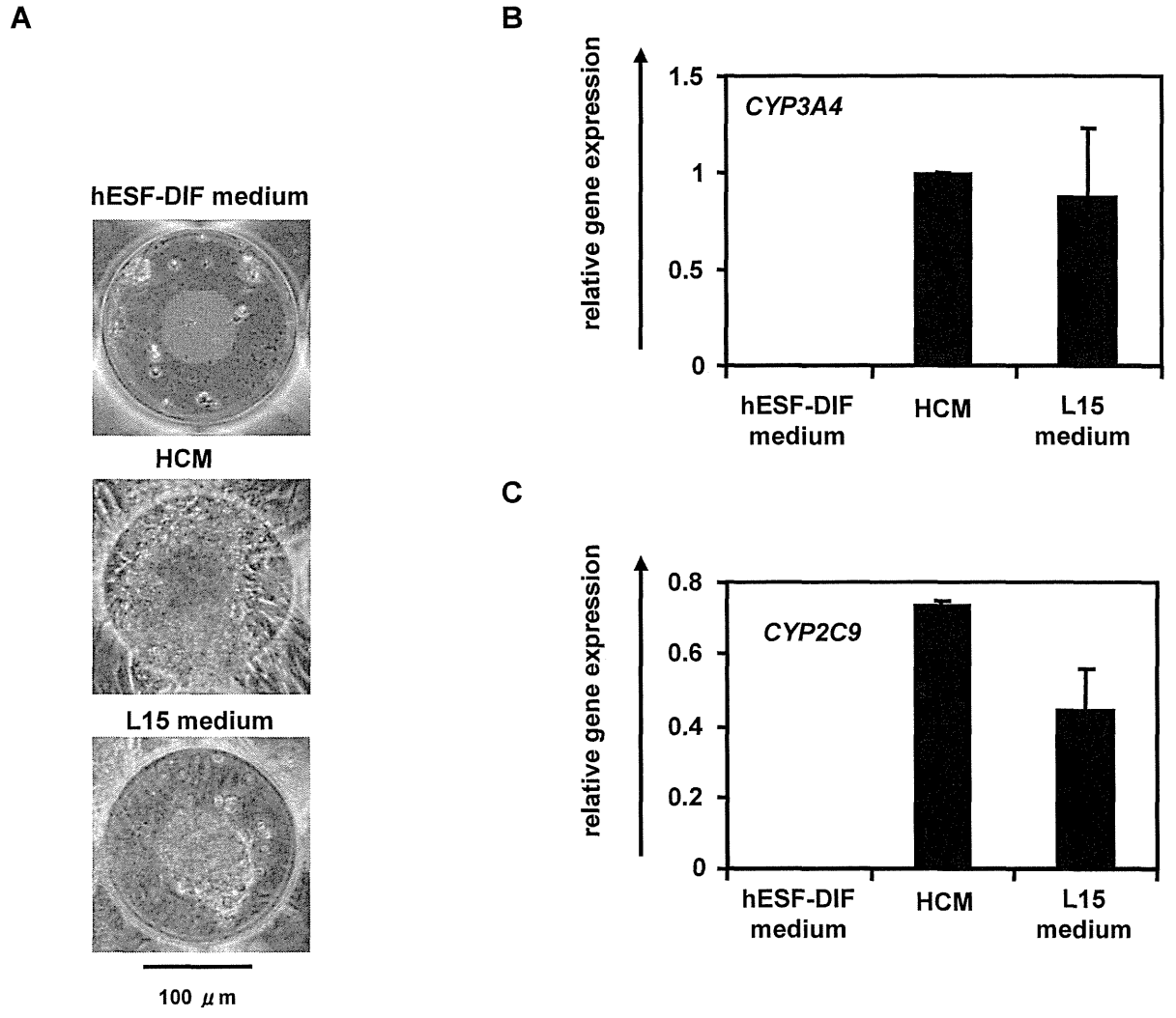
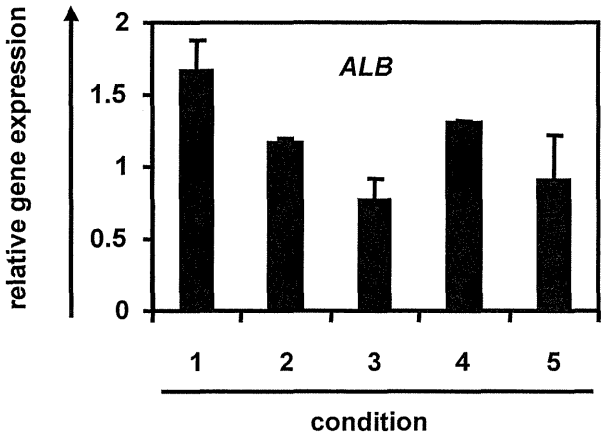


Supplemental figure 3

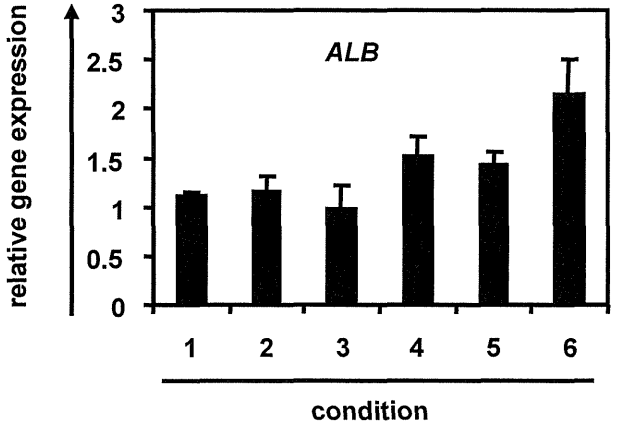
A

	day 12 – day 25
condition 1	HGF, OsM, FGF4, DEX
condition 2	OsM, FGF4, DEX
condition 3	HGF, FGF4, DEX
condition 4	HGF, OsM, DEX
condition 5	HGF, OsM, FGF4



B

	day 12 – day 25	day 25 – day 35
condition 1	HGF, OsM, FGF4, DEX	HGF, OsM, FGF4, DEX
condition 2	HGF, OsM, FGF4, DEX	OsM, FGF4, DEX
condition 3	HGF, OsM, FGF4, DEX	HGF, FGF4, DEX
condition 4	HGF, OsM, FGF4, DEX	HGF, OsM, DEX
condition 5	HGF, OsM, FGF4, DEX	HGF, OsM, FGF4
condition 6	HGF, OsM, FGF4, DEX	OsM, DEX



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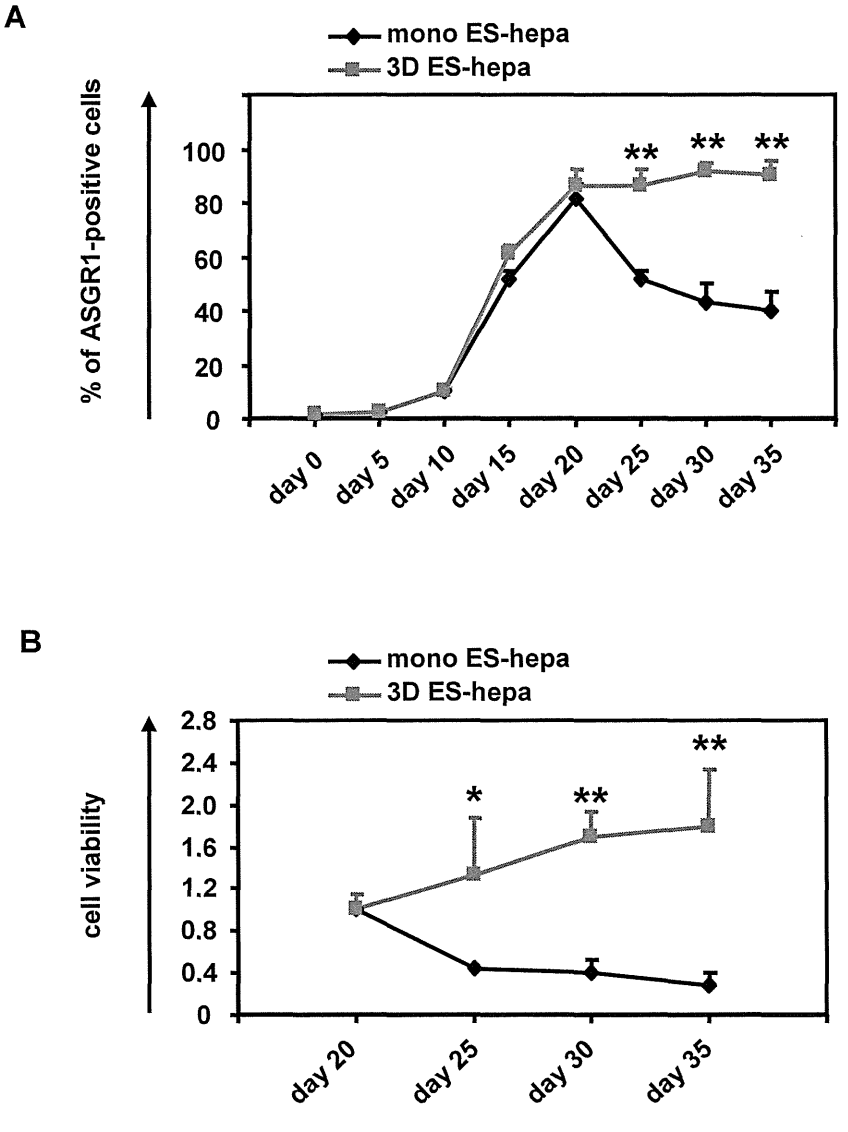

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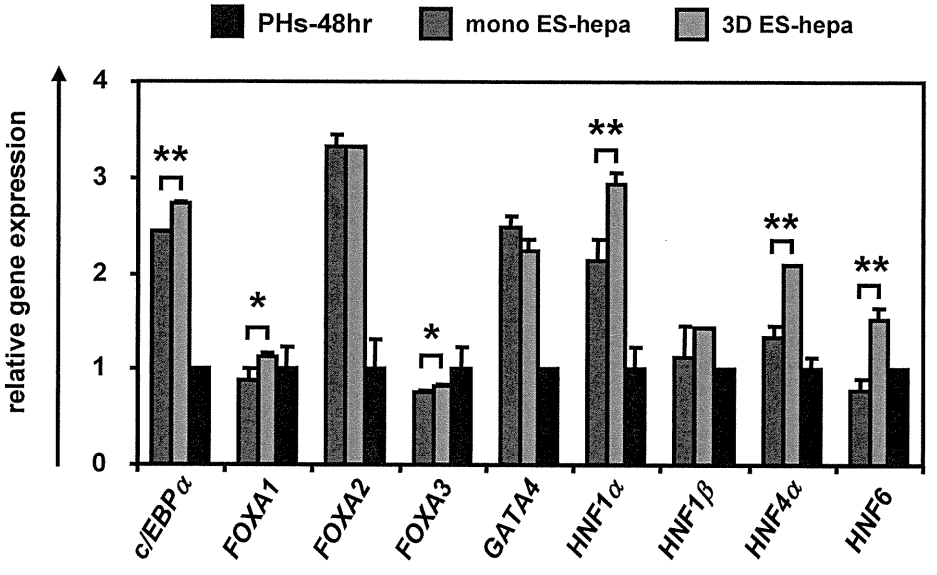

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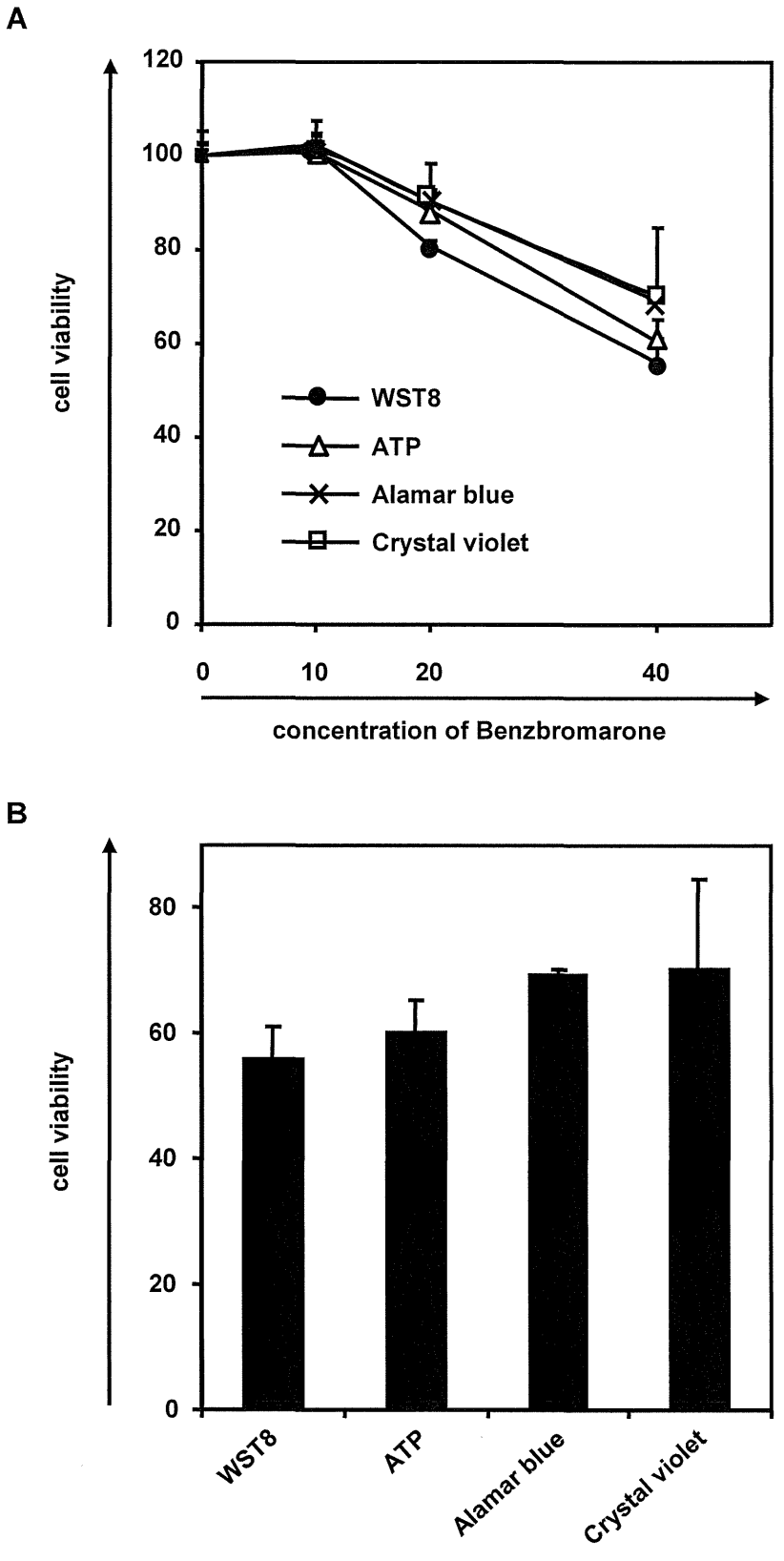
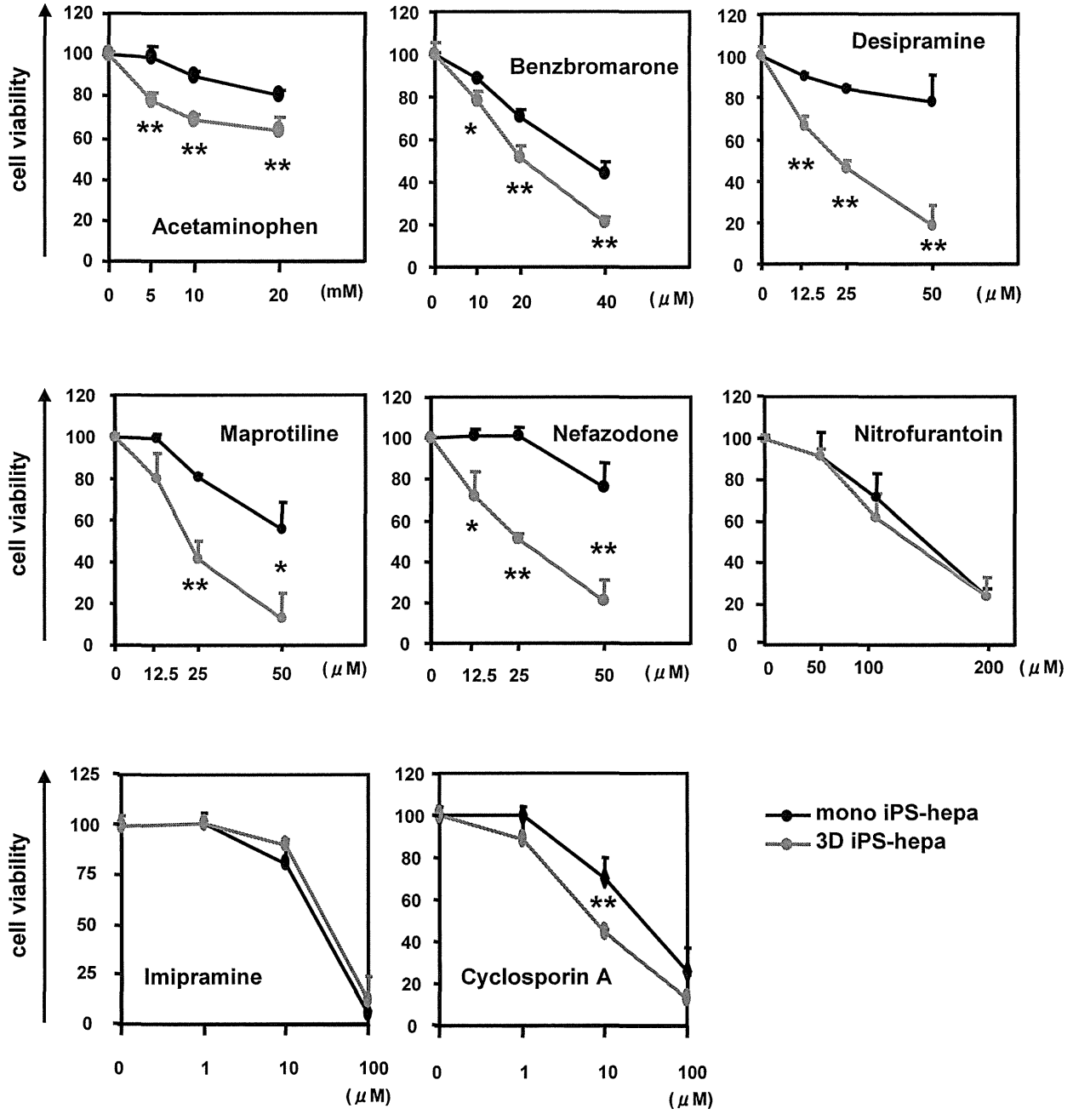
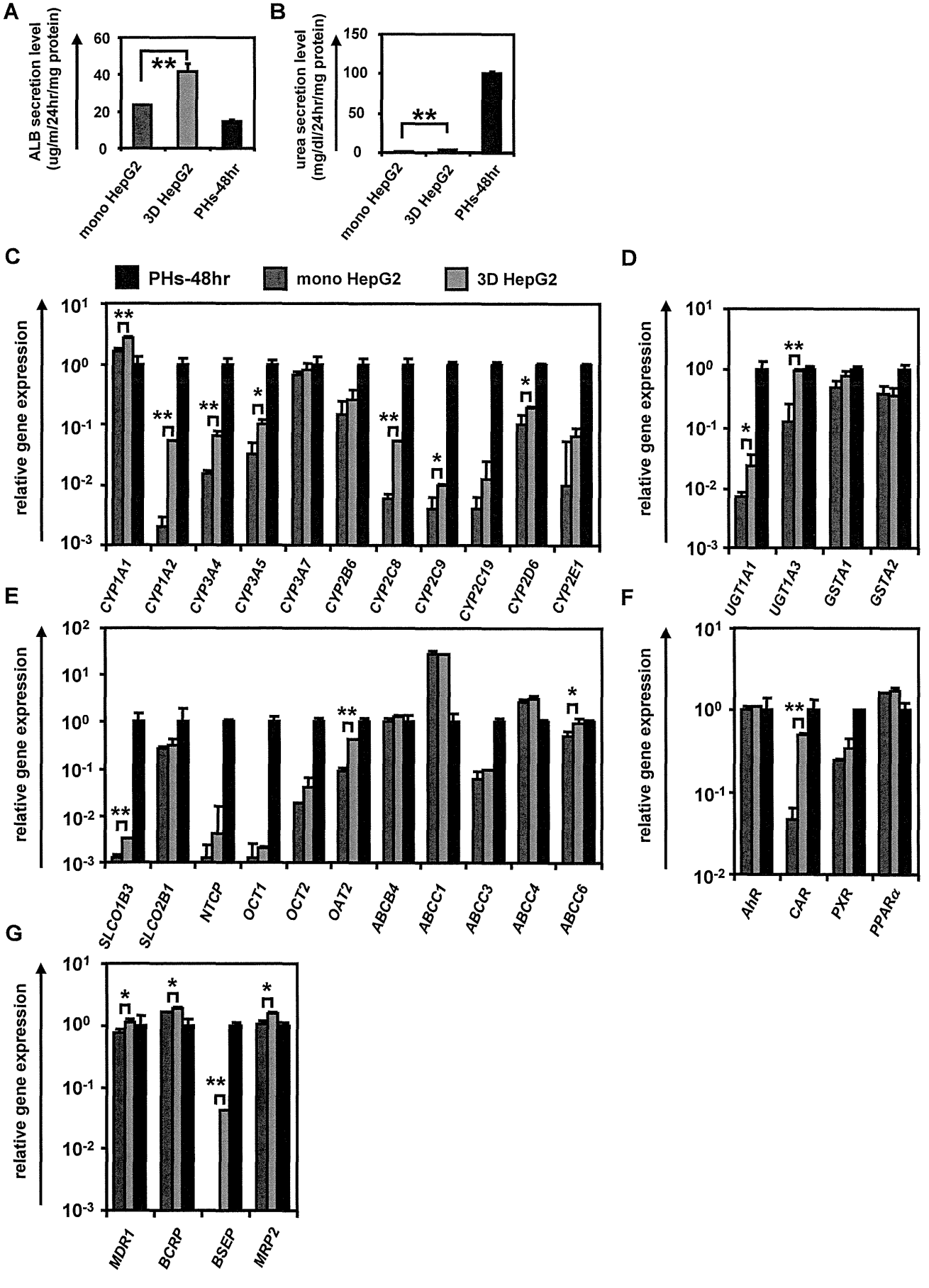


Supplemental figure 7



Supplemental figure 8



Supplemental Figure Legends

Fig. S1 Optimal cell density for the formation of the hepatocyte-like cell spheroids

On day 11, the hESC (H9)-derived cells were plated onto the Nanopillar Plate at the indicated cell density. (**A**) Phase-contrast micrographs of the 3D ES-hepa on day 35 are shown. To form the 3D ES-hepa, the cell density should be more than 2.5×10^5 cells/cm^2. Scale bar represents 100 μm. (**B, C**) Gene expression levels of *CYP3A4* (**B**) and *CYP2C9* (**C**) in the 3D ES-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. The gene expression levels of *CYP3A4* and *CYP2C9* were the highest when the cells were plated onto the Nanopillar Plate at 2.5×10^5 cells/cm^2.

Fig. S2 Optimal medium for the formation of the 3D ES-hepa

On day 11, the hESC (H9)-derived cells were plated onto the Nanopillar Plate, and then the cells were cultured in the differentiation hESF-DIF medium, HCM, or differentiation L15 medium until day 35. (**A**) Phase-contrast micrographs of the 3D ES-hepa on day 35 are shown. The 3D ES-hepa were formed by using HCM or differentiation L15 medium, although they did not form by using differentiation hESF-DIF medium. Scale bar represents 100 μm. (**B, C**) Gene expression levels of *CYP3A4* (**B**) and *CYP2C9* (**C**) in the 3D ES-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. The gene expression levels of *CYP3A4* and *CYP2C9* were the highest when the cells were cultured in HCM.

Fig. S3 Optimal growth factors for the hepatic maturation of the 3D ES-hepa

On day 11, the hESC (H9)-derived cells were plated onto the Nanopillar Plate, and then the cells were cultured in HCM containing the indicated growth factors. (**A**) The optimal growth factors for the first stage of hepatic maturation were investigated. The gene expression levels of *ALB* in the 3D ES-hepa were measured by real-time RT-PCR on day 25. (**B**) The optimal growth factors for the second stage of hepatic maturation of the 3D ES-hepa were investigated. The gene expression levels of *ALB* in the 3D ES-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.

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/TCCAGCACACACTCGTTTTC
UGT1A1	TAAGTGGCTACCCCAAAACG/GCTTTGCATTGTCCATCTGA
UGT1A3	TCAGATGGACAATGCAAAGCGC/GGCGCATGATGTTCTCCTTGTA
GSTA1	CCGTGCATTGAAGTAGTGGA/AATTCAGTTGTCGAGCCAGG
GSTA2	TGCAACAATTAAGTGCTTTACCTAAGTG/TTAACTAAGTGGGTGAATAGGAGTTGTATT
SLCO1B3	GGAAATTTTCATAATGATTCCACC/GAAAACAAGACGCTGCAATG
SLCO2B1	AGGGCTCTGCTTAGAGGGAG/GGAAATGCCCAAGGAAAAAC
NTCP	AGAAGGTGGAGCAGGTGGT/ATCTTGGTCTGTGGCTGCTC

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

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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

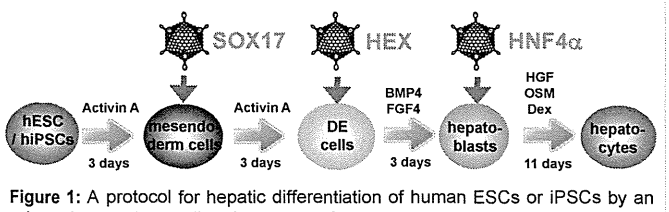

Figure 1: A protocol for hepatic differentiation of human ESCs or iPSCs by an adenovirus vector-mediated gene transfer.

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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