Chapter 7
Hepatic Differentiation of Human Embryonic Stem Cells and Induced Pluripotent Stem Cells by Two- and Three-Dimensional Culture Systems In Vitro

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Abstract Hepatocytes differentiated from human embryonic stem cells (hESCs) or induced pluripotent stem cells (hiPSCs) 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 hepatic differentiation protocols. Moreover, we discuss the application of these protocols to three-dimensional culture systems in an attempt to induce hepatocyte-like cells with high hepatic functions.

Keywords Differentiation • ES cells • Hepatocytes • iPS cells • Three-dimensional culture

7.1 Hepatocytes in Cell-Based Therapy and Drug Discovery

The incidence of liver disease such as viral hepatitis, autoimmune hepatic disorders, fatty liver disease, and hepatic carcinoma is increasing worldwide [1]. Although the optimal treatment for end-stage liver disease is orthotopic liver transplantation, the

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major limitation of such treatment is the shortage of donor livers. The liver is composed of several types of cells, including hepatocytes, endothelial cells, Kupffer cells, stellate cells, and hematopoietic cells. Of these cells, hepatocytes play the most important role in major liver functions. Hepatocytes have many functions, including carbohydrate metabolism, glycogen storage, lipid metabolism, urea synthesis, drug detoxification, production of plasma proteins, and destruction of erythrocytes [2]. Therefore, the transplantation of hepatocytes has been considered an effective treatment alternative to orthotopic liver transplantation [3]. However, such a treatment requires an unlimited source of hepatocytes. Hepatocytes are useful for not only regenerative medicine but biomedical research 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. Primary human hepatocytes are the current standard in vitro model, but isolated hepatocytes lose their functions rapidly even under optimized culture conditions [4, 5]. The use of human hepatocytes is limited by the scarcity of primary tissue from healthy donors. Donor-to-donor and batch-to-batch variations are also significant problems. Moreover, human hepatocytes can no longer proliferate in in vitro culture [6]. These are crucial issues for various applications, and new and unlimited sources of human hepatocytes are urgently required to address them.

7.2 Hepatic Differentiation of hESCs/hiPSCs in Two-Dimensional Culture

hESCs and hiPSCs could be established as promising new resources for obtaining human hepatocytes. Abe et al. [7] and Levinson-Dushnik et al. [8] demonstrated that mouse ESCs (mESCs) were capable of differentiating into endodermal cells. Hamasaki et al. [9] reported that hepatocyte-like cells were induced from mESCs by using humoral factors. Rambhatla et al. demonstrated the differentiation of hESCs into hepatocyte-like cells for the first time [10]. Since then, many studies have been initiated to enhance the hepatic differentiation efficiency and the functional qualities of the hepatocyte-like cells [11–16].

Hepatic differentiation from hiPSCs has been achieved using similar protocols as for hESCs [17–20]. iPSCs were generated from somatic cells as a result of the overexpression of four reprogramming factors (Oct3/4, Sox2, Klf-4, and c-Myc) [21, 22]. Consequently, hiPSCs provide the opportunity to generate individual-specific hepatocyte-like cells. For example, drug metabolism capacity differs among individuals [23], and thus it is difficult to make a precise prediction of drug toxicity by using hepatocytes isolated from a single donor or hESC-derived hepatocytes. A hepatotoxicity screening utilizing hiPSC-derived hepatocyte-like cells would allow the investigation of individual drug metabolism capacity. Moreover, hiPSC-derived hepatocytes generated from patients suffering from a particular disease could provide a source for the disease study and disease modeling [24, 25]. These application would be expected to lead to the discovery of novel drugs.

7.2.1 *Stepwise Hepatic Differentiation from hESCs/hiPSCs*

The general strategy for hepatic differentiation from hESCs/hiPSCs is a stepwise culture with the addition of growth factors or cytokines [11, 20] (Fig. 7.1), which mimics the in vivo microenvironment during liver development [26, 27] (Fig. 7.2).

Gastrulation of the vertebrate embryo starts with the formation of three germ layers: the ectoderm, mesoderm, and endoderm. 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 [28].

In definitive endoderm (DE) differentiation, it is well known that nodal signaling, which involves members of the transforming growth factor-β super family, plays a crucial role and induces the expression of endoderm-related genes [29]. 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 [30–32]. D'Amour et al. accomplished the differentiation of hESCs to DE by using activin A [32]. Recently, protocols using the combination of activin A with other factors such as fibroblast growth factor (FGF) 2 or Wint3a have been also applied to efficiently induce the DE [33, 34, 14].

Hepatic differentiation from the DE is divided into two steps: hepatic specification and hepatic maturation. In the hepatic specification step, the DE differentiates into hepatoblasts that express alpha-fetoprotein (AFP), transthyretin, and albumin (ALB) [35–37]. At this stage, the interaction of FGFs with bone morphogenetic

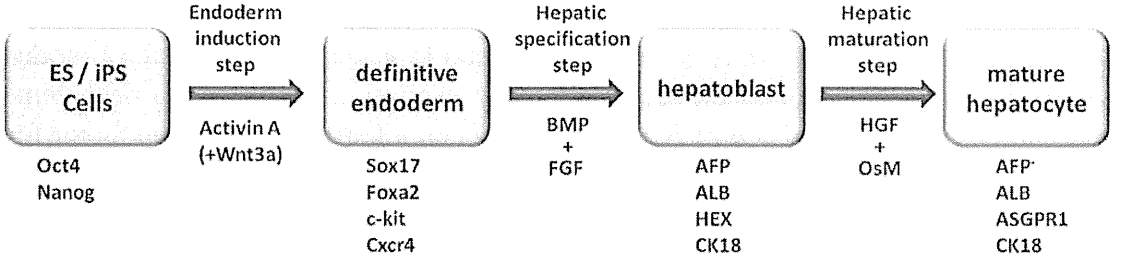

Fig. 7.1 In vitro hepatic differentiation from hESCs/hiPSCs

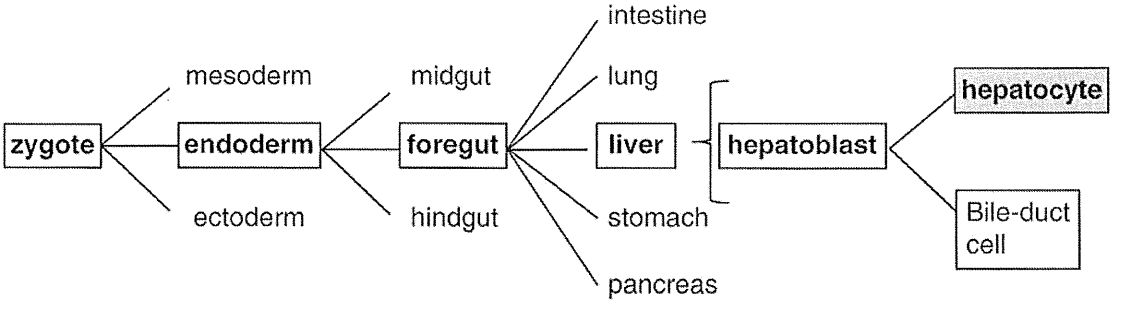

Fig. 7.2 The cell lineage steps during hepatic development

protein (BMP) 2 or BMP4 is important for the induction of hepatocyte-related genes [27, 38]. The combination of FGF4 and BMP2 promotes hepatic specification from human ESC-derived DE cells [13]. Similar results were obtained by using the combinations of aFGF and BMP4, bFGF and BMP4, or FGF4 and BMP4 [13, 33].

It is known that hepatoblasts differentiate into two distinct lineages, hepatocytes and cholangiocytes. During the fetal hepatic maturation, 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 [39]. HGF enhances hepatocyte proliferation but inhibits biliary differentiation by blocking notch signaling [40]. Although HGF is widely used for inducing hepatic phenotypes (e.g., ALB and dipeptidyl peptidase IV expression) [16, 41, 42], this is not enough to induce functional mature hepatocytes [42, 43]. OsM, which is expressed in hematopoietic cells in the fetal liver [43, 44], promotes the hepatic differentiation from hepatoblast cells [39, 40, 45]. Furthermore, supplementation of the culture medium with dexamethasone, a glucocorticoid hormone, induces the production of mature hepatocyte-specific proteins and also supports the maturation process of the hepatocytes together with OsM [14, 15, 18].

7.2.2 *Hepatic Differentiation from hESCs/hiPSCs by Transduction of Hepatic Lineage-Specific Transcription Factors*

DE differentiation methods using growth factors are useful strategies for generating a DE with the ability to differentiate into hepatic or pancreatic lineages; however, these methods are not sufficient for generation of homogenous DE populations [46, 47]. To improve the efficiency of DE differentiation, several groups have attempted the 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 an efficiency of DE differentiation of over 80 % based on the estimation of c-KIT/CXCR4 double-positive cells [47, 48]. The FOXA2 transcription factor also functions as a crucial regulator of the initial intracellular signaling pathways in DE differentiation [49, 50]. Overexpression of FOXA2 also enhances the efficiency of DE differentiation [51–53].

Several studies have demonstrated that, in the hepatic lineage specification stage, homogeneous hepatoblast populations can be generated by modulating the expression levels of hepatocyte-lineage-specific transcription factors as in the DE differentiation stage. Overexpression of HEX, which is an integral transcription factor for hepatic specification, has been shown to promote hepatic specification, resulting in enhanced expression levels of ALB and AFP in the HEX-transduced cells [54–56].

To generate functional hepatocyte-like cells which have characteristics similar to primary human hepatocytes, transduction of HNF4α genes, which are central regulators of liver development, in hESC-/hiPSC-derived hepatoblasts has been shown to successfully induce mature hepatocyte-like cells that have characteristics similar to

primary human hepatocytes [57]. Furthermore, the combination of overexpression of FOXA2 and HNF1α also could effectively induce mature hepatocyte-like cells [52]. The transduction of differentiation-related genes into hESCs/hiPSCs would be a powerful strategy to generate mature hepatocyte-like cells.

7.2.3 *Hepatic Differentiation from hESCs/hiPSCs by a Co-culture System*

In order to facilitate maturation of the hESC-/hiPSC-derived hepatocyte-like cells and to enhance the efficiency of hepatic differentiation, development of a differentiation system that more closely mimics progenitor development in vivo will be needed. The normal culture conditions of hepatocytes in vitro differ substantially from the environment in vivo. 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 [58, 59]. Moreover, it is known that cell–cell interactions between the embryonic cardiac mesoderm and definitive endoderm are essential for liver development [60]. Transcription factors that are critical for hepatic development have been identified from these cell–cell interactions [60]. ES cells co-cultured with cardiac mesoderm showed spontaneous differentiation into hepatocyte-like cells [61]. It seems that the growth factors, including FGF and BMP, secreted from the cardiac mesoderm facilitate differentiation into hepatocyte-like cells. These results suggest that the combined differentiation methods, such as addition of soluble factors into the culture medium, transduction of differentiation-related genes, or co-cultivation with other lineage cells, may further enhance the differentiation and maturation efficiency of hepatocyte-like cells.

7.3 Hepatic Differentiation of hESCs/hiPSCs in Three-Dimensional Culture

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 [62, 63] (Fig. 7.3). Spheroid culture methods allow better maintenance of the liver function of primary hepatocytes compared to two-dimensional (2D) culture [64, 65]. Moreover, various micro-patterned substrates, employing both surface engineering and synthetic polymer chemistry for utilizing spheroid culture, have been reported [66, 67] (Fig. 7.3). One of these technologies uses a nanopillar plate with an arrayed μm-scale hole structure at the bottom of each well and nanopillars that are aligned at the bottom of the respective holes. The seeded cells evenly drop into the holes,

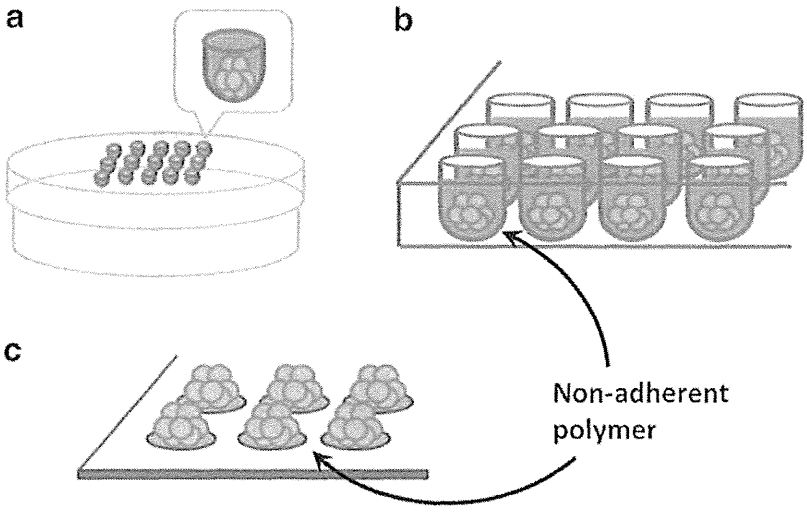

Fig. 7.3 The various spheroid culture methods: (**a**) the hanging-drop method, (**b**) the float-spheroid culture method using culture plate coated with non-adherent polymer, and (**c**) the spheroid culture method on micro-patterned plate

then migrate and aggregate on the top surface of the nanopillars, and thereby tend to form uniform spheroids in each hole. 3D spheroid culture systems using a nanopillar plate of hepatocyte-like cells have been used to promote hepatocyte maturation [68].

As a large-scale culture system of primary hepatocytes, the bioreactor methods have been used. By employing various optimized conditions, including flow conditions [69] and cell densities [70], the bioreactor method has been shown to have advantages for maintaining the functions of primary hepatocytes in vitro in comparison with 2D culture [71, 72] and also for achieving effects of spontaneous differentiation from hESCs into hepatocytes [73]. It has been reported that 3D culture using a bioreactor induces more functional hepatocyte-like cells differentiated from hESCs than in the case of 2D culture [73]. The 3D culture methods using polymer scaffold systems have also demonstrated effectiveness both in culturing primary hepatocytes [74, 75] and in differentiation from ESCs into hepatocyte-like cells in vitro [76–78]. These data showed that hepatocyte-like cells could be differentiated from hESCs on a polymer scaffold.

Furthermore, cell-sheet engineering has recently been reported [79, 80]. Cell-sheet culture was performed by using a culture dish coated with a temperature-responsive polymer, poly (*N*-isopropylacrylamide) [81–83]. Several groups have adopted culture methods with a combination of 3D culture and co-culture (3D co-culture) and showed that the liver function of primary hepatocytes could be maintained at a higher level and for longer than without the coculture conditions [84–86]. Furthermore, the hepatic maturation of hESC-/hiPSC-derived hepatocyte-like cells by stratification of a Swiss 3T3 cell sheet using cell-sheet engineering was demonstrated. The hESC-/hiPSC-derived hepatocyte-like cells in the 3D co-culture system showed significantly up-regulated ALB expression in comparison with the case of 2D culture [87]. A 3D co-culture system would be expected to enhance the degree of maturation compared with a 2D culture.

In the last decade, the hepatic differentiation from hESCs/hiPSCs has been subjected to numerous challenges. Many groups have been struggling to develop the best differentiation protocols from hESCs/hiPSCs to hepatocyte-like cells. The hepatic differentiation efficiency, which is the population of ALB-positive cells, of over 80 % has been achieved in vitro from hESCs/hiPSCs. However, several hepatic functions, including expression levels of cytochrome P450 enzyme, of hESCs-/hiPSCs-derived hepatocyte-like cells are still lower than freshly isolated hepatocytes. New approaches that generate more effective and more functional hepatocyte-like cells may be developed in the near future. The hESC-/hiPSC-derived hepatocyte-like cells are expected to be a useful source of cells not only for drug discovery but also for the treatment of liver disease in the future medicine.

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Prediction of interindividual differences in hepatic functions and drug sensitivity by using human iPS-derived hepatocytes

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Interindividual differences in hepatic metabolism, which are mainly due to genetic polymorphism in its gene, have a large influence on individual drug efficacy and adverse reaction. Hepatocyte-like cells (HLCs) differentiated from human induced pluripotent stem (iPS) cells have the potential to predict interindividual differences in drug metabolism capacity and drug response. However, it remains uncertain whether human iPSC-derived HLCs can reproduce the interindividual difference in hepatic metabolism and drug response. We found that cytochrome P450 (CYP) metabolism capacity and drug responsiveness of the primary human hepatocytes (PHH)-iPS-HLCs were highly correlated with those of PHHs, suggesting that the PHH-iPS-HLCs retained donor-specific CYP metabolism capacity and drug responsiveness. We also demonstrated that the interindividual differences, which are due to the diversity of individual SNPs in the CYP gene, could also be reproduced in PHH-iPS-HLCs. We succeeded in establishing, to our knowledge, the first PHH-iPS-HLC panel that reflects the interindividual differences of hepatic drug-metabolizing capacity and drug responsiveness.

human iPS cells | hepatocyte | CYP2D6 | personalized drug therapy | SNP

Drug-induced liver injury (DILI) is a leading cause of the withdrawal of drugs from the market. Human induced pluripotent stem cell (iPSC)-derived hepatocyte-like cells (HLCs) are expected to be useful for the prediction of DILI in the early phase of drug development. Many groups, including our own, have reported that the human iPS-HLCs have the ability to metabolize drugs, and thus these cells could be used to detect the cytotoxicity of drugs that are known to cause DILI (1, 2). However, to accurately predict DILI, it will be necessary to establish a panel of human iPS-HLCs that better represents the genetic variation of the human population because there are large interindividual differences in the drug metabolism capacity and drug responsiveness of hepatocytes (3). However, it remains unclear whether the drug metabolism capacity and drug responsiveness of human iPS-HLCs could reflect those of donor parental primary human hepatocytes (PHHs). To address this issue, we generated the HLCs differentiated from human iPSCs which had been established from PHHs (PHH-iPS-HLCs). Then, we compared the drug metabolism capacity and drug responsiveness of PHH-iPS-HLCs with those of their parental PHHs, which are genetically identical to the PHH-iPS-HLCs.

Interindividual differences of cytochrome P450 (CYP) metabolism capacity are closely related to genetic polymorphisms, especially single nucleotide polymorphisms (SNPs), in CYP genes (4). Among the various CYPs expressed in the liver, CYP2D6 is responsible for the metabolism of approximately a quarter of commercially used drugs and has the largest phenotypic variability, largely due to SNPs (5). It is known that certain alleles result in the poor metabolizer phenotype due to a decrease of CYP2D6 metabolism. Therefore, the appropriate dosage for drugs that are metabolized by CYP2D6, such as tamoxifen, varies widely among individuals (6). Indeed, in the 1980s, polymorphism in CYP2D6 appears to have contributed to the withdrawal of CYP2D6-metabolized drugs such as perhexiline from the market in many countries (7). If we could establish a panel of HLCs that better represents the diversity of genetic polymorphisms in the human population, it might be possible to determine the appropriate dosage of a drug for a particular individual. However, it is not known whether the drug metabolism capacity and drug responsiveness of HLCs reflect the genetic diversity, including SNPs, in CYP genes. Therefore, in this study we generated HLCs from several PHHs that have various SNPs on CYP2D6 and then compared the CYP2D6 metabolism capacity and responses to CYP2D6-metabolized drugs between the PHH-iPS-HLCs and parental PHHs.

Significance

We found that individual cytochrome P450 (CYP) metabolism capacity and drug sensitivity could be predicted by examining them in the primary human hepatocytes–human induced pluripotent stem cells–hepatocyte-like cells (PHH-iPS-HLCs). We also confirmed that interindividual differences of CYP metabolism capacity and drug responsiveness that are due to the diversity of individual single nucleotide polymorphisms in the CYP gene could also be reproduced in the PHH-iPS-HLCs. These findings suggest that interindividual differences in drug metabolism capacity and drug response could be predicted by using HLCs differentiated from human iPS cells. We believe that iPS-HLCs would be a powerful technology not only for accurate and efficient drug development, but also for personalized drug therapy.

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Data deposition: The DNA microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE61287).

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To this end, PHHs were reprogrammed into human iPSCs and then differentiated into the HLCs. To examine whether the HLCs could reproduce the characteristics of donor PHHs, we first compared the CYP metabolism capacity and response to a hepatotoxic drug between PHHs and genetically identical PHH-iPS-HLCs (12 donors were used in this study). Next, analyses of hepatic functions, including comparisons of the gene expression of liver-specific genes and CYPs, were performed to examine whether the hepatic characteristics of PHHs were reproduced in the HLCs. To the best of our knowledge, this is the first study to compare the functions between iPSC-derived cells from various donors and their parental cells with identical genetic backgrounds. Finally, we examined whether the PHH-iPS-HLCs exhibited a capacity for drug metabolism and drug responsiveness that reflect the genetic diversity such as SNPs on CYP genes.

Results

Reprogramming of PHHs to Human iPSCs. To examine whether the HLCs could reproduce interindividual differences in liver functions, we first tried to generate human iPSCs from the PHHs of 12 donors. PHHs were transduced with a Yamanaka 4 factor-expressing SeV (SeVdp-iPS) vector (*SI Appendix*, Fig. S1*A*) in the presence of SB431542, PD0325901, and a rock inhibitor, which could promote the somatic reprogramming (8). The reprogramming procedure is shown in *SI Appendix*, Fig. S1*B*. The human iPSCs generated from PHHs (PHH-iPSCs) were positive for alkaline phosphatase (*SI Appendix*, Fig. S1*B*, *Right*), NANOG, OCT4, SSEA4, SOX2, Tra1-81, and KLF4 (Fig. 1*A*). The gene expression levels of the pluripotent markers (*OCT3/4*, *SOX2*, and *NANOG*) in the PHH-iPSCs were approximately equal to those in human embryonic stem cells (ESCs) (*SI Appendix*, Fig. S1*C*, *Left*). The gene expression levels of the hepatic markers [*albumin (ALB)*, *CYP3A4*, and *αAT*] in the PHH-iPSCs were significantly lower than those in the parental PHHs (*SI Appendix*, Fig. S1*C*, *Right*). We also confirmed that the PHH-iPSCs have the ability to differentiate into the three embryonic germ layers in vitro by embryoid body formation and in vivo by teratoma formation (*SI Appendix*, Fig. S2 *A* and *B*, respectively). To verify that the PHH-iPSCs originated from PHHs, short tandem repeat analysis was performed in the PHH-iPSCs and parental PHHs (*SI Appendix*, Fig. S2*C*). The results showed that the PHH-iPSCs were indeed originated from PHHs. Taken together, these results indicated that the generation of human iPSCs from PHHs was successfully performed. It is known that a transient epigenetic memory of the original cells is retained in early-passage iPSCs, but not in late-passage iPSCs (9). To examine whether the hepatic differentiation capacity of PHH-iPSCs depends on their passage number, PHH-iPSCs having various passage numbers were differentiated into the hepatic lineage (Fig. 1*B*). The *tyrosine aminotransferase* (*TAT*) expression levels and albumin (ALB) secretion levels in early passage PHH-iPS-HLCs (fewer than 10 passages) were higher than those of late passage PHH-iPS-HLCs (more than 14 passages). These results suggest that the hepatic differentiation tendency is maintained in early passage PHH-iPSCs, but not in late passage PHH-iPSCs. In addition, the hepatic functions of late passage PHH-iPS-HLCs were similar to those in the HLCs derived from late passage non–PHH-derived iPS cells (such as dermal cells, blood cells, and Human Umbilical Vein Endothelial Cells (HUVEC)-derived iPS cells) (*SI Appendix*, Fig. S3). Therefore, PHH-iPSCs, which were passaged more than 20 times, were used in our study to avoid any potential effect of transient epigenetic memory retained in parental PHHs on hepatic functions.

HLCs Were Differentiated from PHH-iPSCs Independent of Their Differentiation Tendency. To compare the hepatic characteristics among the PHH-iPS-HLCs that were generated from PHHs of

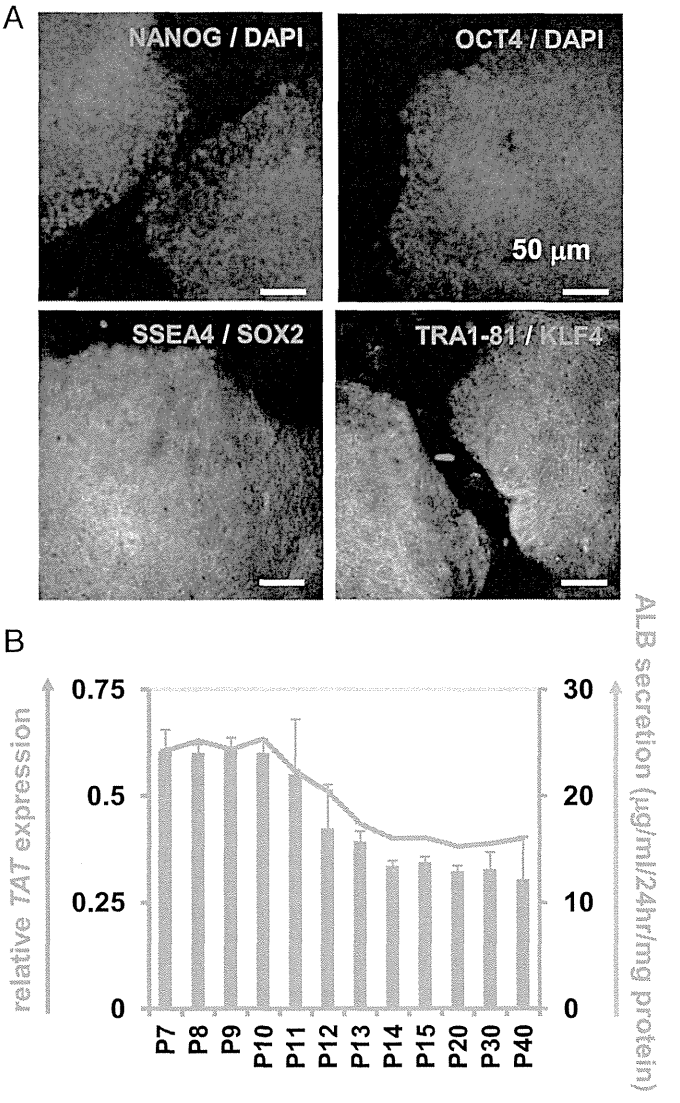

Fig. 1. Establishment and characterization of human iPSCs generated from PHHs. (*A*) The PHH-iPSCs were subjected to immunostaining with anti-NANOG (red), OCT4 (red), SSEA4 (green), SOX2 (red), TRA1-81 (green), and KLF4 (red) antibodies. Nuclei were counterstained with DAPI (blue) (*Upper*). (*B*) The *TAT* expression and ALB secretion levels in the PHH-iPS-HLCs (P7–P40) were examined. On the *y* axis, the gene expression level of *TAT* in PHHs was taken as 1.0.

the 12 donors, all of the PHH-iPSCs were differentiated into the HLCs as described in Fig. 2*A*. However, the differences in hepatic function among PHH-iPS-HLCs could not be properly compared because there were large inter-PHH-iPSC line differences in the hepatic differentiation efficiency based on ALB or asialoglycoprotein receptor 1 (ASGR1) expression analysis (Fig. 2*B*). In addition, there were also large inter-PHH-iPS-HLC line differences in ALB or urea secretion capacities (Fig. 2*C*). These results suggest that it is impossible to compare the hepatic characteristics among PHH-iPS-HLCs without compensating for the differences in the hepatic differentiation efficiency. Recently, we developed a method to maintain and proliferate the hepatoblast-like cells (HBCs) generated from human ESCs/iPSCs by using human laminin 111 (LN111) (10). To examine whether the hepatic differentiation efficiency could be made uniform by generating the HLCs following purification and proliferation of the HBCs, the PHH-iPS-HBCs were cultured on LN111 as

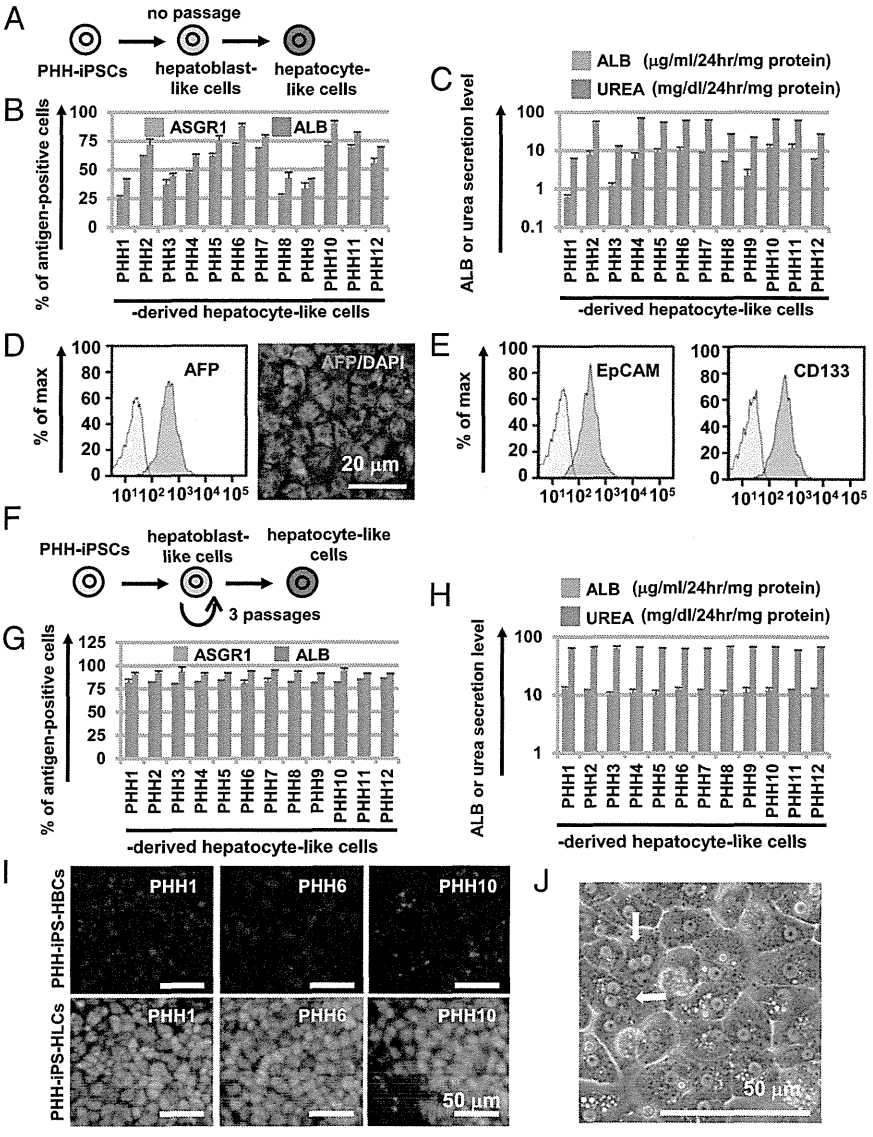

Fig. 2. Highly efficient hepatocyte differentiation from PHH-iPSCs independent of their differentiation tendency. (*A*) PHH-iPSCs were differentiated into the HLCs via the HBCs. (*B*) On day 25 of differentiation, the efficiency of hepatocyte differentiation was measured by estimating the percentage of ASGR1- or ALB-positive cells using FACS analysis. (*C*) The amount of ALB or urea secretion was examined in PHH-iPS-HLCs. (*D*) The percentage of AFP-positive cells in PHH-iPS-HBCs was examined by using FACS analysis (*Left*). The PHH-iPS-HBCs were subjected to immunostaining with anti-AFP (green) antibodies. Nuclei were counterstained with DAPI (blue) (*Right*). (*E*) The percentage of EpCAM- and CD133-positive cells in PHH-iPS-HBCs was examined by using FACS analysis (*Left*). (*F*) PHH-iPSCs were differentiated into the hepatic lineage, and then PHH-iPS-HBCs were purified and maintained for three passages on human LN111. Thereafter, expanded PHH-iPS-HBCs were differentiated into the HLCs. (*G*) The efficiency of hepatic differentiation from PHH-iPS-HBCs was measured by estimating the percentage of ASGR1- or ALB-positive cells using FACS analysis. (*H*) The amount of ALB or urea secretion in PHH-iPS-HLCs was examined. Data represent the mean ± SD from three independent differentiations. (*I*) The PHH1-, 6-, or 10-iPS-HBCs and -HLCs were subjected to immunostaining with anti-αAT (green) antibodies. Nuclei were counterstained with DAPI (blue). (*J*) A phase-contrast micrograph of PHH-iPS-HLCs.

previously described (10), and then differentiated into the HLCs. Almost all of the cells were positive for the hepatoblast marker [alpha-fetoprotein (AFP)] (Fig. 2*D*). In addition, the PHH-iPS-HBCs were positive for two other hepatoblast markers, EpCAM and CD133 (Fig. 2*E*). To examine the hepatic differentiation efficiency of the PHH-iPS-HBCs maintained on LN111-coated dishes for three passages (Fig. 2*F*), the HBCs were differentiated into the HLCs, and then the percentage of ALB- and ASGR1-positive cells was measured by FACS analysis (Fig. 2*G*). All 12 PHH-iPS-HBCs could efficiently differentiate into the HLCs, yielding more than 75% or 85% ASGR1- or ALB-positive cells, respectively. In addition, there was little difference between the PHH-iPSC lines in ALB or urea secretion capacities (Fig. 2*H*). Although there were large differences in the hepatic differentiation capacity among the PHH1/6/10 (Fig. 2*B*), PHH1/6/10-iPS-HBCs could efficiently differentiate into the HLCs that homogeneously expressed αAT (Fig. 2*I*). After the hepatic differentiation of the PHH-iPS-HBCs, the morphology of the HLCs was similar to that of the PHHs: polygonal with distinct round binuclei (Fig. 2*J*). These results indicated that the hepatic differentiation efficiency of the 12 PHH-iPSC lines could be rendered uniform by inducing hepatic maturation after the establishment of self-renewing HBCs. Therefore, we expected that differences in the hepatic characteristics among the HLCs generated from the 12 individual donor PHH-iPS-HBCs could be properly compared. In addition, the hepatic differentiation efficiency could be rendered uniform not only in the PHH-iPSC lines but also in non–PHH-iPSC lines and human ESCs by performing hepatic maturation after the establishment of self-renewing HBCs (*SI Appendix*, Fig. S4). In Figs. 3 and 4, the HLCs were differentiated after the HBC proliferation step to normalize the hepatic differentiation efficiency.

PHH-iPS-HLCs Retained Donor-Specific Drug Metabolism Capacity and Drug Responsiveness. To examine whether the hepatic functions of individual PHH-iPS-HLCs reflect those of individual PHHs, the CYP metabolism capacity and drug responsiveness of PHH-iPS-HLCs were compared with those of PHHs. PHHs are often used as a positive control to assess the hepatic functions of the HLCs, although in all of the previous reports, the donor of PHHs has been different from that of human iPSCs. Because it is generally considered that CYP activity differs widely among individuals, the hepatic functions of the HLCs should be compared with those of genetically identical PHHs to accurately evaluate the hepatic functions of the HLCs. The CYP1A2, -2C9, and -3A4 activity levels in the PHH-iPS-HLCs were ~60% of

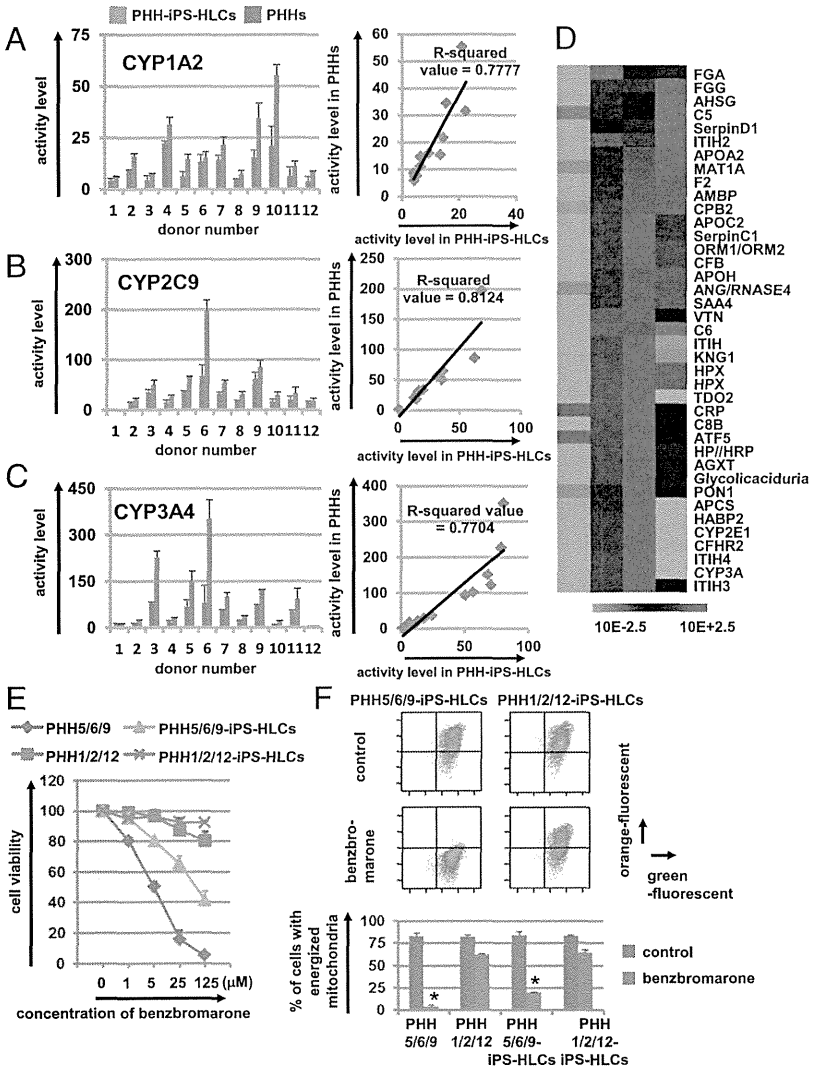

Fig. 3. The drug metabolism capacity and drug responsiveness of PHH-iPS-HLCs were highly correlated with those of their parental PHHs. (*A–C*) CYP1A2 (*A*), -2C9 (*B*), and -3A4 (*C*) activity levels in PHH-iPS-HLCs and PHHs were measured by LC-MS/MS analysis. The R-squared values are indicated in each figure. (*D*) The global gene expression analysis was performed in PHH9-iPSCs, PHH9-iPS-HLCs, PHH9s, and HepG2 (PHH-iPSCs, PHH-iPS-HLCs, and PHHs are genetically identical). Heat-map analyses of liver-specific genes are shown. (*E*) The cell viability of PHH5/6/9, PHH1/2/12, PHH5/6/9-iPS-HLCs, and PHH1/2/12-iPS-HLCs was examined after 24 h exposure to different concentrations of benzbromarone. The cell viability was expressed as a percentage of that in the cells treated only with solvent. (*F*) The percentage of cells with energized mitochondria in the DMSO-treated (control, *Upper*) or benzbromarone-treated (*Lower*) cells based on FACS analysis. Double-positive cells (green+/orange+) represent energized cells, whereas single-positive cells (green+/orange–) represent apoptotic and necrotic cells. Data represent the mean ± SD from three independent experiments (*Lower Graph*). Student *t* test indicated that the percentages in the "control" were significantly higher than those in the "benzbromarone" group ($P < 0.01$). The "PHH5/6/9" represents the average value of cell viability (*E*) or mitochondrial membrane potential (*F*) in PHH5, PHH6, and PHH9. The "PHH1/2/12" represents the average value of cell viability or mitochondrial membrane potential in PHH1, PHH2, and PHH12. PHH5, PHH6, and PHH9 were the top three with respect to CYP2C9 activity levels, whereas PHH1, PHH2, and PHH12 had the lowest CYP2C9 activity levels.

those in the PHHs (Fig. 3 *A–C* and *SI Appendix*, Fig. S5). Interestingly, the CYP1A2, -2C9, and -3A4 activity levels in the PHH-iPS-HLCs were highly correlated with those in the PHHs (the R-squared values were more than 0.77) (Fig. 3 *A*, *B*, and *C*, respectively). These results suggest that it would be possible to predict the individual CYP activity levels through analysis of the CYP activity levels of the PHH-iPS-HLCs. Because the average and variance of CYP3A4 activity levels in PHH-iPS-HLCs, non–PHH-iPS-HLCs, and human ES-HLCs were similar to each other (*SI Appendix*, Fig. S6), the drug metabolism capacity of PHH-iPS-HLCs might be similar to that of nonliver tissue-derived iPS-HLCs and human ES-HLCs. Therefore, it might be possible to predict the diversity of drug metabolism capacity among donors by using nonliver tissue-derived iPS-HLCs and human ES-HLCs as well as PHH-iPS-HLCs. On the other hand, the CYP induction capacities of PHH-iPS-HLCs were weakly correlated with those of PHHs (*SI Appendix*, Fig. S7 *A–C*). To further investigate the characteristics of the HLCs, DNA microarray analyses were performed in genetically identical undifferentiated iPSCs, PHH-iPS-HLCs, and PHHs. The gene expression patterns of liver-specific genes, CYPs, and transporters in the PHH-iPS-HLCs were similar to those in PHHs (Fig. 3*D* and *SI Appendix*, Fig. S7 *D* and *E*, respectively). Next, the hepatotoxic drug responsiveness of PHH-iPS-HLCs was compared with that of PHHs. Benzbromarone, which is known to cause hepatotoxicity by CYP2C9 metabolism (11), was treated to PHH5/6/9 and PHH5/6/9-iPS-HLCs, which have high CYP2C9 activity, or PHH1/2/12 and PHH1/2/12-iPS-HLCs which have low CYP2C9 activity (Fig. 3*E*). The susceptibility of the PHH5/6/9 and PHH5/6/9-iPS-HLCs to benzbromarone was higher than that of PHH1/2/12 and PHH1/2/12-iPS-HLCs, respectively. These results were attributed to the higher CYP2C9 activity levels in PHH5/6/9 and PHH5/6/9-iPS-HLCs compared with those in PHH1/2/12 and PHH1/2/12-iPS-HLCs. Because it is also known that benzbromarone causes mitochondrial toxicity (12), an assay of mitochondrial membrane potential was performed in benzbromarone-treated PHHs and PHH-iPS-HLCs (Fig. 3*F*). The mitochondrial toxicity observed in PHH5/6/9 and PHH5/6/9-iPS-HLCs was more severe than that in PHH1/2/12 and PHH1/2/12-iPS-HLCs, respectively. Taken together, these results suggest that the hepatic functions of the individual PHH-iPS-HLCs were highly correlated with those of individual PHHs.

Interindividual Differences in CYP2D6-Mediated Metabolism and Drug Toxicity, Which Are Caused by SNPs in CYP2D6, Are Reproduced in the PHH-iPS-HLCs. Because certain SNPs are known to have a large impact on CYP activity, the genetic variability of CYP plays an important role in interindividual differences in drug response. CYP2D6 shows the large phenotypic variability due to genetic polymorphism (13). We next examined whether the PHHs used

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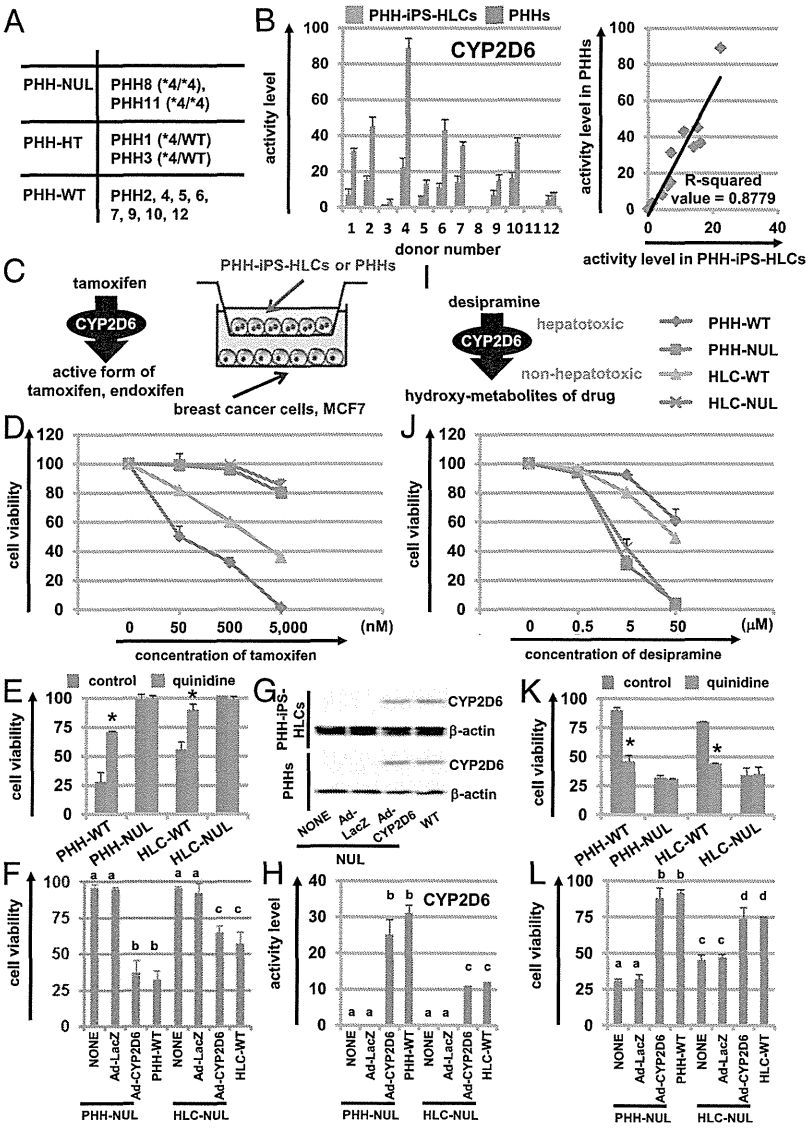

Fig. 4. The interindividual differences in CYP2D6 metabolism capacity and drug responsiveness induced by SNPs in CYP2D6 are reproduced in the PHH-iPS-HLCs. (*A*) SNPs (CYP2D6*3, *4, *5, *6, *7, *8, *16, and *21) in the CYP2D6 gene were analyzed. (*B*) The CYP2D6 activity levels in PHH-iPS-HLCs and PHHs were measured by LC-MS/MS analysis. (*C*) The pharmacological activity of tamoxifen-dependent conversion to its metabolite, endoxifen, by the CYP2D6. The coculture system of breast cancer cells (MCF-7 cells) and the PHH-iPS-HLCs are illustrated. (*D*) The cell viability of MCF-7 cells was assessed after 72-h exposure to different concentrations of tamoxifen. (*E*) The cell viability of MCF-7 cells, which were cocultured with PHH-WT, PHH-NUL, HLC-WT, and HLC-NUL, was assessed after 72-h exposure to 500 nM of tamoxifen in the presence or absence of 3 nM quinidine (a CYP2D6 inhibitor). (*F*) The cell viability of MCF-7 cells cocultured with Ad-CYP2D6-transduced PHH-NUL and HLC-NUL was examined after 72-h exposure to 500 nM of tamoxifen. (*G* and *H*) The CYP2D6 expression (*G*) and activity (*H*) levels in Ad-CYP2D6-transduced PHH-NUL and HLC-NUL were examined by Western blotting and LC-MS/MS analysis. (*I*) The detoxification of desipramine-dependent conversion to its conjugated form by the CYP2D6. (*J*) The cell viability of PHH-WT, PHH-NUL, HLC-WT, and HLC-NUL was assessed after 24-h exposure to different concentrations of desipramine. (*K*) The cell viability of the PHH-WT and HLC-WT was assessed after 24-h exposure to 5 μM of desipramine in the presence or absence of 5 μM of quinidine (a CYP2D6 inhibitor). (*L*) The cell viability of the Ad-CYP2D6-transduced PHH-NUL and HLC-NUL was examined after 24-h exposure to 5 μM of desipramine. The cell viability was expressed as a percentage of that in the cells treated with only solvent. Data represent the mean ± SD from three independent experiments. In *E* and *K*, Student *t* test indicated that the cell viability in the "control" was significantly higher than that in the "quinidine" group ($P < 0.01$). In *F*, *H*, and *L*, statistical significance was evaluated by ANOVA followed by Bonferroni post hoc tests to compare all groups. Groups that do not share the same letter are significantly different from each other ($P < 0.05$).

in this study have the CYP2D6 poor metabolizer genotypes (CYP2D6 *3, *4, *5, *6, *7, *8, *16, and *21) (5). PHH8 and -11 have CYP2D6*4 (null allele), whereas the others have a wild type (WT) or hetero allele (*SI Appendix*, Table S3 and Fig. 4*A*). Consistent with this finding, the PHH8/11-iPS-HLCs also have CYP2D6*4, whereas the others have a wild type or hetero allele. As expected, the CYP2D6 activity levels in the PHH8/11 (PHH-NUL) and PHH8/11-iPS-HLC (HLC-NUL) were significantly lower than those in the PHH-WT and HLC-WT, respectively (Fig. 4*B*). The pharmacological activity of tamoxifen, which is the most widely used agent for patients with breast cancer, is dependent on its conversion to its metabolite, endoxifen, by the CYP2D6 (Fig. 4*C*). To examine whether the pharmacological activity of tamoxifen could be predicted by using PHHs and HLCs that have either the null type CYP2D6*4 allele or wild-type CYP2D6 allele, the breast cancer cell line MCF7 was cocultured with PHHs or HLCs, and then the cells were treated with tamoxifen (Fig. 4*D*). The cell viability of MCF7 cells cocultured with PHHs-NUL or HLCs-NUL was significantly higher than that of MCF7 cells cocultured with PHHs-WT or HLCs-WT. The decrease in cell viability of MCF7 cells cocultured with PHHs-WT or HLCs-WT was rescued by treatment with a CYP2D6 inhibitor, quinidine (Fig. 4*E*). We also confirmed that the cell viability of MCF7 cells cocultured with PHHs-NUL or HLCs-NUL was decreased by CYP2D6 overexpression in the PHHs-NUL or HLCs-NUL (Fig. 4*F*). Note that the expression (Fig. 4*G*) and activity (Fig. 4*H*) levels of CYP2D6 in CYP2D6-expressing adenovirus vector (Ad-CYP2D6)-transduced PHHs-NUL or HLCs-NUL were comparable to those of PHHs-WT or HLCs-WT. These results indicated that the PHHs-WT and HLCs-WT could more efficiently metabolize tamoxifen than the PHHs-NUL and HLCs-NUL, respectively, and thereby induced higher toxicity in MCF7 cells. Similar results were obtained with the other breast cancer cell line, T-47D (*SI Appendix*, Fig. S8 *A–D*). Next, we examined whether the CYP2D6-mediated drug-induced hepatotoxicity could be predicted by using PHHs and HLCs having either a null type CYP2D6*4 allele or wild-type CYP2D6 allele. PHHs and HLCs were treated with desipramine, which is known to cause hepatotoxicity (Fig. 4*I*) (14). The cell viability of PHHs-NUL and HLCs-NUL was significantly lower than that of PHHs-WT and HLCs-WT (Fig. 4*J*). The cell viability of the PHHs-WT or HLCs-WT was decreased by treatment with a CYP2D6 inhibitor, quinidine (Fig. 4*K*). We also confirmed that the decrease in the cell viability of the PHHs-NUL or HLCs-NUL was rescued by CYP2D6 overexpression in the PHHs-NUL or HLCs-NUL (Fig. 4*L*). Similar

results were obtained with the other hepatotoxic drug, perhexiline (*SI Appendix*, Fig. S8 *E–H*). These results indicated that the PHHs-WT and HLCs-WT could more efficiently metabolize imipramine and thereby reduce toxicity compared with the PHHs-NUL and HLCs-NUL. Taken together, our findings showed that the interindividual differences in CYP metabolism capacity and drug responsiveness, which are prescribed by an SNP in genes encoding CYPs, were also reproduced in the PHH-iPS-HLCs.

Discussion

The purpose of this study was to examine whether the individual HLCs could reproduce the hepatic function of individual PHHs. A Yamanaka 4 factor-expressing SeV vector was used in this study to generate integration-free human iPSCs from PHHs. It is known that SeV vectors can express exogenous genes without chromosomal insertion, because these vectors replicate their genomes exclusively in the cytoplasm (15). To examine the different cellular phenotypes associated with SNPs in human iPSC derivatives, the use of integration-free human iPSCs is essential.

We found that the CYP activity levels of the PHH-iPS-HLCs reflected those of parent PHHs, as shown in Fig. 3 *A–C*. There were few interindividual differences in the ratio of CYP expression levels in the PHH-iPS-HLCs to those in PHHs (*SI Appendix*, Fig. S5). Together, these results suggest that it is possible to predict the individual CYP activity levels through analysis of the CYP activity levels of the PHH-iPS-HLCs. In the future, it will be necessary to confirm these results in skin or blood cell-derived iPSCs as well as PHH-iPSCs, although donor-matched PHHs and blood cells (or skin cells) are difficult to obtain. In addition, the comparison of hepatic functions between genetically identical PHHs and PHH-iPS-HLCs (Fig. 3 *A–C*) would enable us to accurately ascertain whether the HLCs exhibit sufficient hepatic function to be a suitable substitute for PHHs in the early phase of pharmaceutical development. Because the drug responsiveness of the individual HLCs reflected that of individual PHHs (Fig. 3 *E* and *F*), it might be possible to perform personalized drug therapy following drug screening using a patient's HLCs. However, the R-squared values of the individual CYP activities differed from each other (Fig. 3 *A–C*), suggesting that the activity levels of some CYPs are largely influenced not only by genetic information but also by environmental factors, such as dietary or smoking habits.

The interindividual differences of CYP2D6 metabolism capacity and drug responsiveness that were prescribed by SNP in genes encoding CYP2D6 were reproduced in the PHH-iPS-HLCs (Fig. 4). It was impossible to perform drug screening in the human hepatocytes derived from a donor with rare SNPs because these hepatocytes could not be obtained. However, because human iPSCs can be generated from such donors with rare SNPs, the CYP metabolism capacity and drug responsiveness of these donors might be possible to predict. Further, it would also be possible to identify the novel SNP responsible for an unexpected hepatotoxicity by using the HLCs in which whole genome sequences are known. We thus believe that the HLCs will be a powerful tool not only for accurate and efficient drug development but also for personalized drug therapy.

Experimental Procedures

DNA Microarray. Total RNA was prepared from the PHH9-iPSCs, PHH9-iPS-HLCs, PHH9, and human hepatocellular carcinoma cell lines by using an RNeasy Mini kit. A pool of three independent samples was used in this study. cRNA amplifying, labeling, hybridizing, and analyzing were performed at Miltenyi Biotec. The Gene Expression Omnibus (GEO) accession no. for the microarray analysis is GSE61287.

Flow Cytometry. Single-cell suspensions of human iPSC-derived cells were fixed with 2% (vol/vol) paraformaldehyde (PFA) for 20 min, and then incubated with the primary antibody (described in *SI Appendix*, Table S1), followed by the secondary antibody (described in *SI Appendix*, Table S2). In case of the intracellular staining, the Permeabilization Buffer (eBioscience) was used to create holes in the membrane thereby allowing the antibodies to enter the cell effectively. Flow cytometry analysis was performed using a FACS LSR Fortessa flow cytometer (BD Biosciences).

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Regular Article

Development of Mice Exhibiting Hepatic Microsomal Activity of Human CYP3A4 Comparable to That in Human Liver Microsomes by Intravenous Administration of an Adenovirus Vector Expressing Human CYP3A4

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Summary: Cytochrome P450 3A4 (CYP3A4) plays a crucial role in the pharmacokinetic and safety profiles of drugs. However, it is difficult to properly predict the pharmacokinetics and hepatotoxicity of drugs in humans using data from experimental animals, because the catalytic activities of CYP3A4 and other drug-metabolizing enzymes differ between human and animal organs. In order to easily generate an animal model for proper evaluation of human CYP3A4-mediated drug metabolism, we developed a human CYP3A4-expressing adenovirus (Ad) vector based on our novel Ad vector exhibiting significantly lower hepatotoxicity (Ad-E4-122aT-hCYP3A4). Intravenous administration of Ad-E4-122aT-hCYP3A4 at a dose of 2×10^{11} virus particles/mouse produced a mouse exhibiting human CYP3A4 activity at a level similar to that in the human liver, as shown in the dexamethasone metabolic experiment using liver microsomes. The area under the curve (AUC) of 6βOHD was 2.7-fold higher in the Ad-E4-122aT-hCYP3A4-administered mice, compared with the mice receiving a control Ad vector. This Ad vector-expressing human CYP3A4 would thus be a powerful tool for evaluating human CYP3A4-mediated drug metabolism in the livers of experimental animals.

Keywords: human CYP3A4; adenovirus vector; liver; drug metabolism; dexamethasone

Introduction

Foreign chemicals that enter the body are subject to metabolism by a number of xenobiotic-metabolizing enzymes, a process that functions primarily to facilitate their elimination. Cytochromes P450 (CYPs) are the most important of the enzymes responsible for the oxidative metabolism of a diverse range of foreign chemicals, including therapeutic drugs, carcinogens, toxicants, and endogenous compounds, such as steroid hormones and bile acids.[1,2] In particular, the CYP1A, CYP2C, CYP2D, CYP2E1, and CYP3A

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subfamilies, which are mainly expressed in the liver, play central roles in the metabolism and disposition of drugs.[3] Furthermore, CYP-mediated metabolism of drugs is often involved in drug-induced liver injury.[4] The evaluation of CYP-mediated metabolism of drugs is therefore crucial for the development of new drugs.

In the drug development process, the assessment of drug metabolic profiles in humans is largely based on the extrapolation of animal experiments to the human situation. Thus experimental animals, especially rodents, have been widely used as *in vivo* models to predict pharmacokinetics and toxicity in humans. However, the results obtained in *in vivo* studies using experimental animals do not always reflect the pharmacokinetics in humans.[5] It is now recognized that rodents metabolize drugs differently from humans, due in part to species differences in the expression and catalytic activities of CYPs in the organs.[6] Species differences in metabolite profiles present a challenge that may confound the nonclinical safety assessment of candidate drugs.[7,8] When metabolites are formed uniquely or disproportionately in humans, FDA guidance suggests that the major metabolite(s) should be synthesized and tested in appropriate nonclinical safety assessment studies;[9] however, the synthesis of large amounts of metabolites is often problematic.[8] This bottleneck is an important one, since encountering major human metabolites in patients or study subjects that were not previously revealed in a nonclinical model can significantly hinder drug development.

In order to overcome the problem of the differential CYP enzyme profiles between humans and experimental animals, transgenic mouse lines carrying the specific human CYPs have been developed.[10–12] Human CYP-expressing mouse models may have the ability to generate major human metabolites and eliminate or reduce the formation of mouse-specific metabolites. When compared to the current strategy for handling metabolite challenges (*i.e.*, direct administration of the metabolite), a number of advantages could be gained by the use of an appropriate human CYP-expressing animal model.[13] However, there are several problems with human CYP-expressing transgenic animals. For example, it is not possible to control the expression levels of the human CYPs. Multiple copies of human CYP expression cassettes are usually incorporated into the transgenic mouse genome, resulting in inappropriate levels of human CYP expression in the organs.[12] Unpredictable phenotypes are also found in human CYP-expressing animals. For example, human CYP3A4 transgenic mice exhibit a lactation defect caused by abnormal homeostasis of estradiol.[12]

As a different approach to generating mice possessing human CYP activities, *in vivo* transduction with an adenovirus (Ad) vector seems promising because Ad vectors possess various advantages as gene delivery vehicles.[14,15] In particular, the most attractive property of Ad vectors for use in the over-expression of human CYPs in the liver, which is the main organ for drug metabolism, is their high hepatotropism. More than 90% of the injected doses accumulate in the liver, resulting in substantially higher levels of transgene expression in the liver than in other organs.[16] However, Ad vector-mediated over-expression of human CYPs in rodent livers has not been successfully accomplished. Although Bai and Cederbaum demonstrated Ad vector-mediated over-expression of human CYP2E1 in the mouse liver, they did not evaluate the utility of the resulting strain as an animal model for analyzing drug metabolism in humans.[17] In addition, Ad vector-mediated over-expression of human CYP3A4, which is generally the most abundant hepatic and intestinal form, accounting on average for 95% of the combined liver CYP3A mRNA pool in Caucasians,[18,19] and which is the CYP isoenzyme most highly involved in drug metabolism in humans, has not been reported in rodent livers.

In addition, there is an important concern in terms of Ad vector-mediated over-expression of human CYPs in rodent livers. *In vivo* application of high doses of an Ad vector often induces severe hepatotoxicity due to Ad vector-induced innate immunity and leaky expression of Ad genes,[20] and such Ad vector-induced hepatotoxicity hampers the function analysis of the transduced genes. In order to overcome this problem, we used a novel Ad vector that exhibits extremely low levels of leaky expressions of all the Ad genes examined in the liver as well as a significantly reduced hepatotoxicity profile following intravenous administration into mice by incorporation of miR-122a-targeted sequences in the 3′-UTR of E4 genes.[21] This novel Ad vector was found to be suitable for gene function analysis in the liver.

In this study, in order to easily generate an animal model for the effective evaluation of human CYP3A4-mediated drug metabolism, we created a human CYP3A4-expressing Ad vector using a novel Ad vector that exhibits an apparent reduction in Ad vector-mediated hepatotoxicity, and administered it to mice. In the present experiments, intravenous administration of the Ad vector expressing human CYP3A4 into mice resulted in animals with hepatic microsomal expression and activity of human CYP3A4 comparable to those in human liver microsomes without apparent Ad vector-mediated liver toxicity. This novel Ad vector expressing human CYP3A4 would thus be a powerful tool for evaluating human CYP3A4-mediated drug metabolism in the livers of experimental animals.

Materials and Methods

Chemicals and enzymes: Dexamethasone and midazolam were obtained from Wako Pure Chemicals (Osaka, Japan). 6β-Hydroxydexamethazone was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). Pooled human liver microsomes (H0610) were obtained from Xenothech, LLC (Lenexa, KS). Human liver cDNA was obtained from Takara Bio (Shiga, Japan). The NADPH-regenerating system used for all NADPH-requiring oxidase assays was purchased from BD Gentest (Woburn, MA). The system consists of two solutions, solution A (26.1 mM $NADP^+$, 66 mM glucose-6-phosphate, and 66 mM $MgCl_2$ in H_2O) and solution B (40 U/mL glucose-6-phosphate dehydrogenase in 5 mM sodium acetate).

Mice and cells: Female C57BL/6J mice aged 7 weeks were obtained from Nippon SLC (Hamamatsu, Japan). HepG2 cells (a human hepatocellular liver carcinoma cell line) and 293 cells (a human embryonic kidney cell line) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and antibiotics.

Plasmids and Ad vectors: Ad vectors were prepared by an improved *in vitro* ligation method.[22] Briefly, the human CYP3A4 gene was cloned into pHMCA5,[23] creating pHMCA5-hCYP3A4. The human CYP3A4 gene was amplified by PCR using cDNA derived from human liver cDNA and the following primers: hCYP3A4-F, 5′-CGAATCGTAGGGGTACCACCATGGCTCTCATCCCAGACTTGGCCATGG-3′; hCYP3A4-F, 5′-CGAATCGTACCGCTCGAGTTATCAGGCTCCACTTACGGTGCCATCCCTTG-3′. I-*Ceu*I/PI-*Sce*I-digested pHMCA5-hCYP3A4 was ligated with the I-*Ceu*I/PI-*Sce*I-digested Ad vector plasmid pAdHM4-E4-122aT,[21] producing pAdHM4-E4-122aT-hCYP3A4.

pAdHM4-E4-122aT contains an E1/E3-deleted Ad vector genome and four copies of miR-122a-targeted sequences in the 3′-untranslated region of the E4 gene. pAdHM4-E4-122aT-hCYP3A4 was digested with *Pac*I to release the recombinant viral genome, and was transfected into 293 cells plated on 60-mm dishes. Ad vectors were propagated in 293 cells, purified by two rounds of cesium chloride-gradient ultracentrifugation, dialyzed, and stored at −80°C. The numbers of virus particles (VPs) were determined using a spectrophotometric method.[24] Ad-E4-122aT-GFP, which is a control Ad vector expressing green fluorescence protein (GFP), was similarly prepared using pHMCA5-GFP and pAdHM4-E4-122aT.

Measurement of CYP3A4 activity in HepG2 cells transduced with Ad-E4-122aT-hCYP3A4: HepG2 cells (3×10^5 cells/well) were seeded in 12-well plates. On the following day, the cells were transduced with Ad-E4-122aT-hCYP3A4 at a 100, 300, 1,000, and 3,000 VPs/cell. After a 48 h incubation, human CYP3A4 activity in the cells was measured according to the protocol included in the P450-Glo CYP3A4 Assay (Promega, Madison, WI).

Western blot analysis of human CYP3A4 in HepG2 cells and mouse liver microsomes: Total cell lysates prepared from HepG2 cells (20 μg) following Ad vector transduction were separated on 10% polyacrylamide gels. Mouse liver microsomes were prepared as described below 2 days after Ad vector administration. Mouse liver microsomal protein (2 μg) was similarly separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Human liver microsomes (2 μg) were used as a positive control. Samples were run at 20 mA for 1 h in 25 mM Tris buffer/0.2 M glycine/0.1% SDS buffer. Samples were then transferred to Immobilon-P (PVDF membrane; Millipore, Bedford, MA) for 2 h at 100 V in 25 mM Tris buffer/20% methanol. Blots were blocked with 5% skim milk for 1 h, and incubated with polyclonal goat anti-human CYP3A4 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) overnight. Subsequently, the blots were probed with the secondary antibody, mouse anti-goat IgG-horseradish peroxidase (1:5,000; Cell Signaling Technology, Beverly, MA) for 30 min. The bands were visualized by using an ECL Plus detection kit (GE Healthcare, Little Chalfont, UK).

Mouse liver microsome preparation: Ad vectors were intravenously administered into female C57BL/6J mice at a dose of 3×10^{10}, 1×10^{11}, or 2×10^{11} VPs/mouse *via* the tail vein. The livers were harvested and homogenized with three volumes of ice-cold 0.1 mM phosphate buffer (pH 7.4) 2 days after Ad vector administration. Following the centrifugation of homogenates in a refrigerated centrifuge at $9,000 \times g$ for 20 min at 4°C, the precipitate was discarded, and then 0.1 mM potassium phosphate buffer (pH 7.4) was added to the supernatant. The mixture was then centrifuged at $105,000 \times g$ for 1 h at 4°C. The pellets of microsomes were resuspended in 0.1 mM potassium phosphate buffer (pH 7.4). The protein content was measured with a Bio-Rad assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. The microsomes were stored at −80°C until use.

Real-time reverse transcription PCR analysis: Total RNA from HepG2 cells was isolated using ISOGEN (Nippon Gene, Tokyo, Japan). mRNA levels of human CYP3A4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by real-time reverse transcription PCR using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan). The protocol for thermal cycling consisted of 60 s at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The primer sequences used in this study were as follows: human CYP3A4, 5′-AAGGAAATCCACTCGGTGCTT-3′ and 5′-ATGAAAGAAAGTCGCCTCGAA-3′; human GAPDH, 5′-GGTGGTCTCCTCTGACTTCAAC-3′ and 5′-GTGGTCGTTGAGGGCAATG-3′.

Immunohistochemical analysis of human CYP3A4 expression in the mouse liver: The livers were harvested 2 days after Ad vector administration. The livers were then washed with cold phosphate-buffered saline (PBS) and embedded in optimal cutting temperature (OCT) for the frozen section (6 μm) preparation. The frozen sections were prepared using a Leica CM 1850 Cryostat (Leica Microsystems, Wetzlar, Germany) and fixed with acetone for 10 min at 4°C, followed by incubation for 30 min in 0.3% H_2O_2 in methanol to quench the endogenous peroxidase activity. Sections were then blocked for 1 h with diluted normal blocking serum and incubated overnight with a polyclonal rabbit anti-human CYP3A4 antibody (1:100; BD Gentest, Woburn, MA). Sections were washed with PBS, and treated using a peroxidase-based avidin-biotin affinity system (Vector, Burlingame, CA). The liver sections were then visualized with 3,3′-diaminobenzidine tetrachloride (DAB) (Vector) as a chromogen, counterstained with haematoxylin, and observed under a microscope.

***In vitro* determination of the human CYP 3A4 activity in mouse liver microsomes:** *In vitro* determination of human CYP3A4 activity in mouse liver microsomes was measured as previously described.[25] Briefly, a reaction mixture was prepared by mixing an NADPH-regenerating system (final concentrations: 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, and 3.3 mM $MgCl_2$) with 1 μM (final concentration) of midazolam and dexamethasone in 0.1 M potassium phosphate buffer (pH 7.4), and the mixture was pre-incubated at 37°C for 10 min. Then, 5 μL of liver microsomes (20 mg protein/mL) was added, and the mixture was further incubated at 37°C for 30 min. Fifty μL of the mixture was harvested, and the reaction was stopped by addition of 100 μL of acetonitrile/ethanol (70/30 v/v%). The samples were centrifuged at 14,000 rpm for 3 min at 4°C, and the supernatants were subjected to LC-MS/MS analysis.

***In vivo* determination of the human CYP 3A4 activity in mice (pharmacokinetic experiments):** Dexamethazone and midazolam (2 mg/10 mL) were dissolved in 8% well-solve (Celeste Corporation, Tokyo, Japan) solution. These compounds were orally administered to mice at a dose of 2 mg/kg at 48 h after Ad vector administration. At 10, 30, 60, 120, and 180 min, and 7 h after administration of the compounds, blood samples were taken from the inferior vena cava under anesthesia with isoflurane, and then the mice were sacrificed by cervical dislocation ($n = 3$ for each time point). One hundred μL of acetonitrile/ethanol (70/30 v/v%) was added to a 50 μL plasma sample, and mixed vigorously. The samples were centrifuged at 14,000 rpm for 3 min at 4°C, and the supernatants were subjected to LC-MS/MS analysis.

LC-MS/MS analyses: The LC-MS/MS system consisted of a Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan) interfaced to an AB SCIEX 5000™ LC/MS/MS System. The system was controlled by Analyst 1.45.2 software (Applied Biosystems, Foster City, CA). Separation of parent compounds and metabolites was achieved by using a Shim-pack XR-ODSII 2.0 mm i.d. × 75 mm at a flow rate of 0.5 mL/min. The mobile phase included 0.1% formic acid (Buffer A) and acetonitrile (Buffer B). The gradient cycle used for metabolite identification consisted of an isocratic elution with 10% Buffer B for 0.5 min, followed by a linear increase of Buffer B to 90% from 0.5 to