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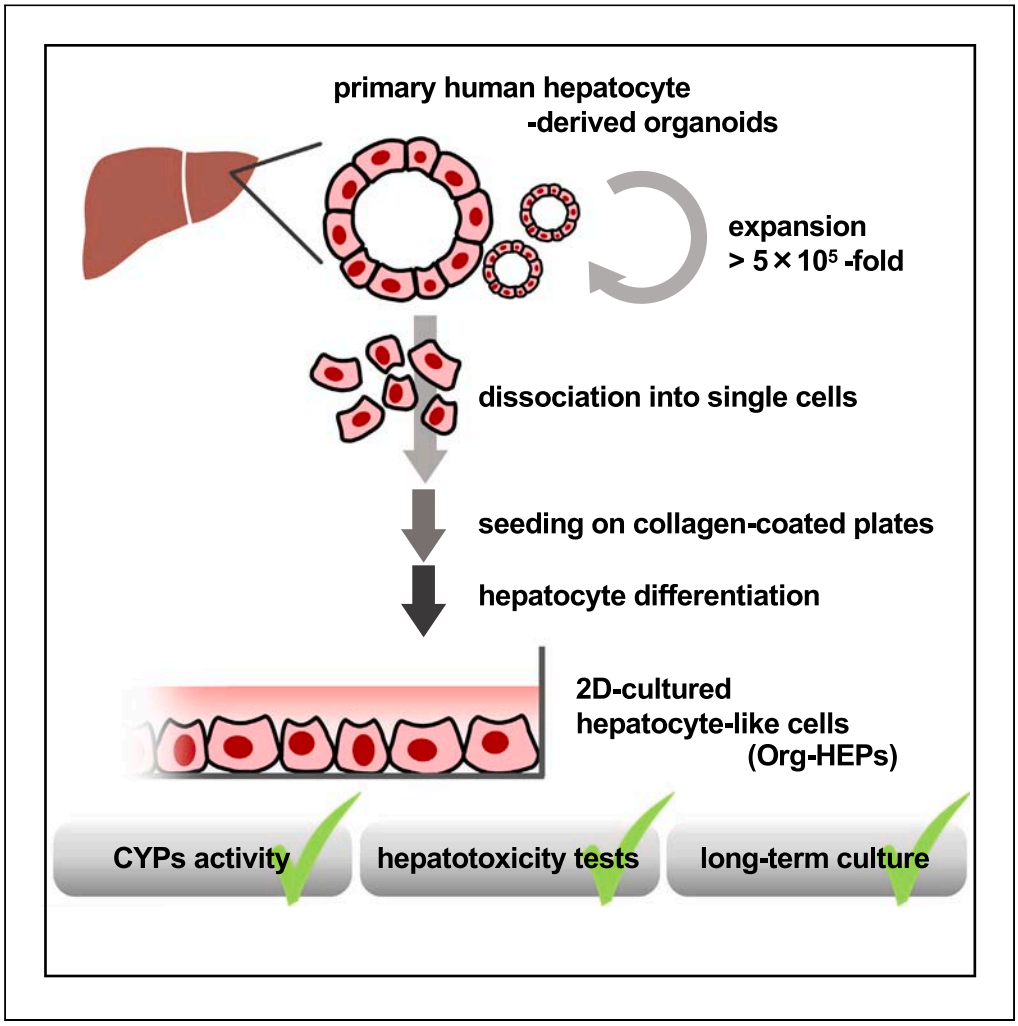

Yukiko Ueyama-Toba, Yanran Tong, Jumpei Yokota, ..., Hidetoshi Eguchi, Tetsuo Takehara, Hiroyuki Mizuguchi

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Highlights

2D-cultured hepatic differentiation method from PHH-derived organoids was developed

Org-HEPs expressed hepatocyte markers, and CYP or UGT activities comparable to PHHs

Org-HEPs were used for hepatotoxicity tests and CYP induction tests

Org-HEPs maintained part of their hepatic functions even after 30 days

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Development of a hepatic differentiation method in 2D culture from primary human hepatocyte-derived organoids for pharmaceutical research

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SUMMARY

Human liver organoids derived from primary human hepatocytes (PHHs) are expected to be a hepatocyte source for preclinical *in vitro* studies of drug metabolism and disposition. Because hepatic functions of these organoids remain low, it is necessary to enhance the hepatic functions. Here, we develop a novel method for two dimensional (2D)-cultured hepatic differentiation from PHH-derived organoids by screening several compounds, cytokines, and growth factors. Hepatic gene expressions in the hepatocyte-like cells differentiated from PHH-derived organoids (Org-HEPs) were greatly increased, compared to those in PHH-derived organoids. The metabolic activities of cytochrome P450 (CYP) 1A2, CYP2C8, CYP2C19, CYP2E1, and CYP3A4 were at levels comparable to those in PHHs. The cell viability of Org-HEPs treated with hepatotoxic drugs was almost the same as that of PHHs. Thus, PHH-derived organoids could be differentiated into highly functional hepatocytes in 2D culture. Thus, Org-HEPs will be useful for pharmaceutical research, including hepatotoxicity tests.

INTRODUCTION

Most drugs are metabolized and detoxified in the liver. Therefore, human hepatocytes are essential materials for the pharmacokinetic and drug toxicity tests in pharmaceutical research.[1,2] Because primary human hepatocytes (PHHs) retain the activities of drug metabolizing enzymes and transporters, PHHs are the main cell source used as a human liver model for preclinical *in vitro* studies of drug metabolism and toxicities.[3–6] However, PHHs have at least two problems: first, they cannot be cultured with sufficient hepatic functions for long periods of time, and second, they have supply limitations due to a lack of proliferating capacity *in vitro*. Therefore, many studies have been conducted to overcome these issues.

PHHs maintained in conventional two-dimensional (2D) monolayer cultures de-differentiate and rapidly lose their hepatic functions.[7,8] Various techniques have been developed to achieve maintenance of the hepatic functions of PHHs even after long-term culture.[9–14] Culture methods such as spheroid culture[10,11] and sandwich culture with extracellular matrix (ECM)[12–14] have proven effective for maintaining hepatic functions in PHHs, since these complex culture systems mimic the *in vivo* physiological environment. Bell et al. reported that three-dimensional (3D) PHH spheroids retained their hepatic functions for at least 5 weeks.[10] Kimoto et al. showed that a sandwich-cultured PHHs model was a reliable tool for achieving PHHs with hepatic uptake and biliary excretion.[13] These studies have contributed to significant advances in the functional maintenance of PHHs. However, since the resulting PHHs do not show proliferative ability, the supply limitation of PHHs cannot be resolved. Furthermore, when ECM substrates are used, test drugs may bind to the scaffold, ECM substrates may be difficult to handle, and batch-to-batch differences may occur, all of which affect the reproducibility of the results.[15,16] Therefore, there is a need to develop a culture system by which PHHs maintain their hepatic functions and cell proliferation ability, and as well as their versatility for different assays.

Recently, another method to maintain hepatic functions in PHHs was developed by adding compounds to the culture medium in a conventional 2D culture system. In 2019, Xiang et al. reported that treatment with five compounds (5C) enabled PHHs to maintain their hepatic functions *in vitro* for a long period of time.[17] 5C-cultured PHHs showed global gene expression profiles and hepatic functions resembling those of freshly isolated PHHs. Katsuda et al. reported that the combination of four compounds (YPAC) was useful for maintaining various

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hepatic functions in PHHs.[18] YPAC-cultured PHHs showed albumin (ALB) and urea productivity, glycogen storage, and cytochrome P450 (CYP) expression. YPAC allowed PHHs to retain the enzymatic activities of CYP1A2, CYP2B6, and CYP3A4 even after 40 days of culture. These strategies using several compounds allowed PHHs to maintain their hepatic functions and versatility in a conventional 2D culture. Moreover, some studies demonstrated that mouse or human mature hepatocytes could be made to de-differentiate into hepatic progenitor cells and expand over the long-term by treating them with a cocktail of compounds and growth factors in a conventional 2D culture system.[19–22] These chemically induced hepatic progenitor cells had a capacity for differentiation into functional hepatocytes and repopulation in injured mouse livers.

Another strategy is the use of organoids generated from leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5)—positive adult tissue stem cells,[23] which open new possibilities as a hepatocyte source, as they can be expanded in culture for over 6 months while remaining genetically stable.[24] Other studies revealed that human liver-derived cholangiocyte organoids which are like bipotent progenitor cells, can be expanded for the long-term, and can be differentiated into functional hepatocytes *in vitro*.[24–26] However, even when human liver-derived cholangiocyte organoids were differentiated into hepatocyte-like cells, their hepatic functions were not high enough in comparison to human liver tissue.[27] In 2018, Hu et al. developed a 3D organoid culture system that allowed mature hepatocytes to expand with hepatic functions.[28] Although these organoids expressed hepatocyte marker genes and CYP3A4 activity, the efficiency of establishing them was not high when adult hepatocytes were used. For application in pharmaceutical research, it is necessary to efficiently supply hepatocytes with adult-type phenotypes.

Here, we propose a novel method for providing functional hepatocyte-like cells from PHH-derived organoids. It is tedious to perform pharmacokinetic tests and hepatotoxicity tests using organoids, as organoids are cultured in droplets of Matrigel; this hampers the application of organoids to pharmaceutical research. To overcome this limitation, we seeded PHH-derived organoids on collagen-coated plates and developed a method for differentiating them into hepatocyte-like cells in a 2D culture system. To ensure versatility, PHH-derived organoids were established from commercially available PHHs. We added several factors that were selected to enable differentiation and maturation into hepatocyte-like cells from PHH-derived organoids. The hepatocyte-like cells differentiated from PHH-derived organoids (Org-HEPs) expressed many hepatocyte marker genes and proteins, and also exhibited drug-metabolizing or conjugated enzyme activities comparable to PHHs. The combination of an organoid culture system with the hepatic differentiation method developed in this study can overcome the previous described problems of PHHs by yielding PHHs with higher hepatic functions, higher cell proliferation capacity and good versatility for different assays. The Org-HEPs developed in this study could thus be a useful source of human hepatocytes for pharmaceutical research.

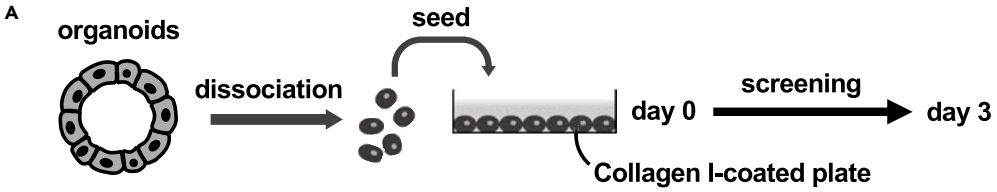
RESULTS

Establishment of PHH-derived organoids

First, we established organoids from commercially available PHHs using two different culture conditions (HEP-medium and CHOL-medium), as described in the previous studies (Figure S1A).[25,28] In the subsequent experiments, we used organoids and the parental PHHs from the same individual. Hu et al. reported that HEP-organoids, which were established from hepatocytes and maintained their hepatic functions,[28] formed a compact structure with a typical "bunch-of-grapes" appearance, while CHOL-organoids, which were established from EpCAM-positive cholangiocytes and had bipotent progenitor cell-like features,[24,25] formed a spherical structure. As shown in Figure S1B, the bunch-of grapes morphology was not observed even when PHH-derived organoids were established and cultured with HEP-medium. Both organoids cultured with HEP-medium and those cultured with CHOL-medium showed a spherical structure at passage 0 and at passage 2. It was reported that HEP-organoids maintained their hepatic functions such as ALB secretion and CYP3A4 activity.[28] However, the gene expressions of *ALB* in the organoids cultured with either HEP-medium or CHOL-medium was much lower than those in PHHs (Figure S1C). The gene expression level of a cholangiocyte marker (*EpCAM*) was similar between organoids cultured with HEP-medium, organoids cultured with CHOL-medium and PHHs (Figure S1D), and the gene expression levels of somatic stem cell marker (*LGR5*) in both organoids cultured with HEP-medium and those cultured with CHOL-medium were higher than those in PHHs (Figure S1E). The gene expression of proliferation cell marker (*Ki67*) in the organoids cultured with CHOL-medium was much higher than those in organoids cultured with HEP-medium and PHHs (Figure S1F). These results suggested that even when PHHs consisting of hepatocytes and HEP-medium were used, the resulting organoids showed CHOL-organoid-like features. Thus, it was difficult to culture HEP-organoids, and we decided to use CHOL-medium for PHH-derived organoid culture in this study. Relative number of the cells increased about 5×10^5-fold for 134 days under culture with CHOL-medium (Figure S2A). To evaluate the effect of organoid culture on hepatic functions, gene expression analysis was performed. The results showed that the gene expression levels of hepatocyte markers (*ALB* and *CYP3A4*) were decreased with organoid culture (Figure S2B). Broutier et al. developed a hepatocyte differentiation method from CHOL-organoids using differentiation medium (DM).[25] Although the *ALB* and *CYP3A4* expressions were increased in organoids cultured with DM compared to those in organoids cultured with CHOL-medium, which was called expansion medium (EM) in the report of Broutier et al.,[25] these gene expression levels were much lower than those in PHHs, and did not indicate adequate levels of hepatocytes (Figure S2C).[27] These results suggested the need for a new method of differentiation and maturation of PHH-derived organoids for application to pharmaceutical research.

Screening of compounds for 2D-hepatic differentiation of PHH-derived organoids

Since the requirement of embedding organoids in Matrigel hampers their application to pharmaceutical research, we tried to develop 2D-cultured functional hepatocytes derived from PHH-derived organoids. For this purpose, we performed compound screening to induce hepatic maturation under a 2D culture condition, as described in Figure 1A. When the single cells from PHH-derived organoids were seeded in a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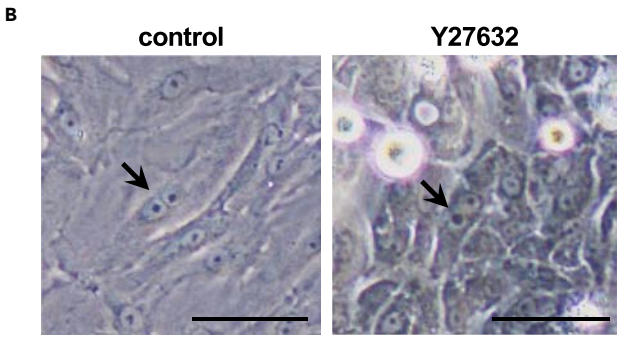
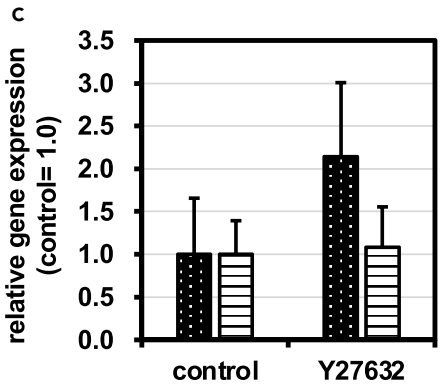
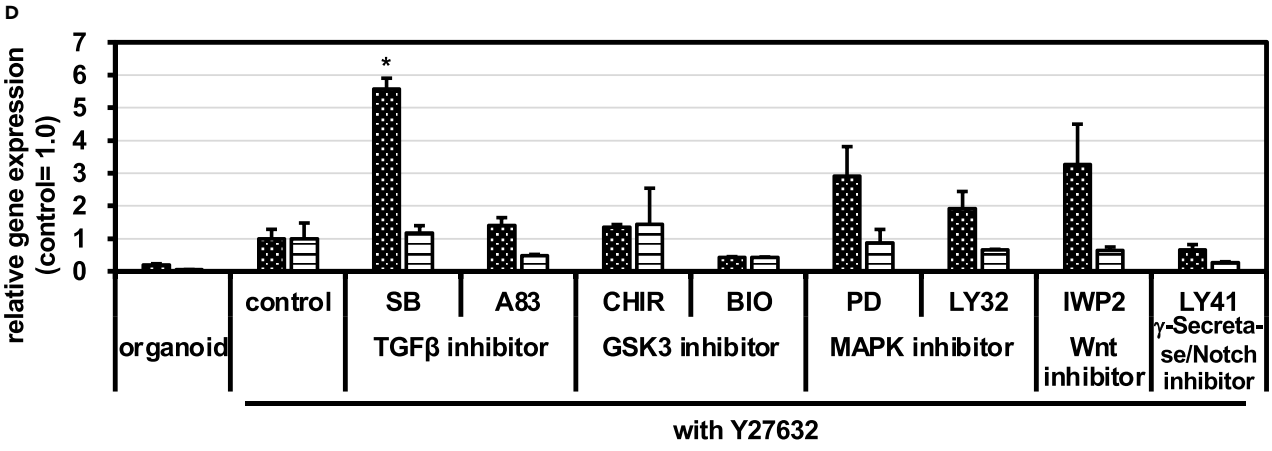
organoids
dissociation
seed
day 0
screening
day 3
Collagen I-coated plate


B
control
Y27632


C
relative gene expression
(control= 1.0)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
control
Y27632


D
relative gene expression
(control= 1.0)
7
6
5
4
3
2
1
0
*
organoid
control
SB
A83
CHIR
BIO
PD
LY32
IWP2
LY41
TGFβ inhibitor
GSK3 inhibitor
MAPK inhibitor
Wnt inhibitor
γ-Secreta-se/Notch inhibitor
with Y27632

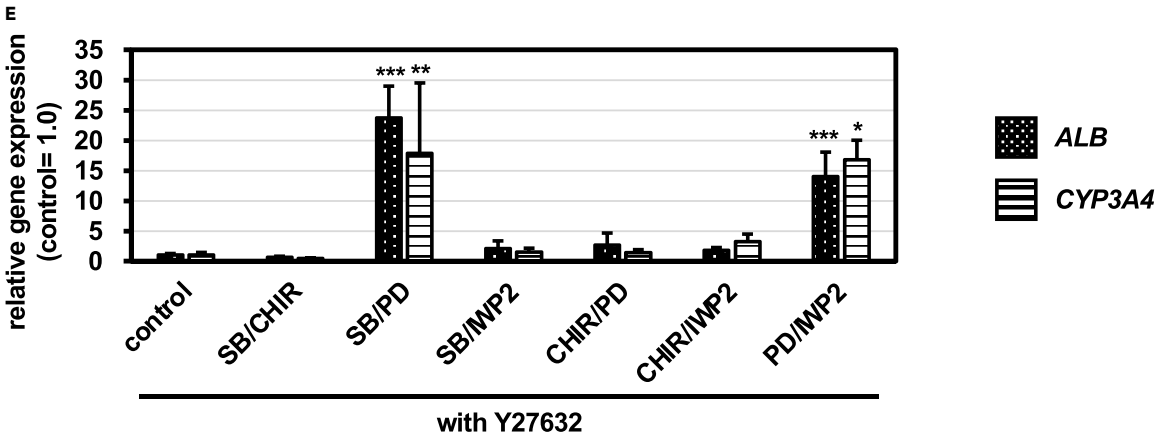

E
relative gene expression
(control= 1.0)
35
30
25
20
15
10
5
0
*** **
*** *
control
SB/CHIR
SB/PD
SB/IWP2
CHIR/PD
CHIR/IWP2
PD/IWP2
with Y27632
ALB
CYP3A4

Figure 1. Screening of compounds for 2D-hepatic differentiation of PHH-derived organoids

(A) The screening procedure is presented schematically. Details of the hepatic differentiation procedure are described in STAR Methods.

(B) Phase contrast images of the 2D-cultured cells derived from PHH-derived organoids and cultured without (control)/with Y27632 are shown. The cell with double-nuclei were indicated by black arrow. The scale bars represent 50 μm.

(C) The gene expression levels of hepatocyte markers in the human liver organoid-derived cells after 2D-culture for 3 days were examined by real-time RT-PCR.

(D) The gene expression levels of hepatocyte markers in the human liver organoid-derived cells after 3 days of 2D-culture with Y27632 and several other compounds were examined by real-time RT-PCR. The compounds used were 2 μM SB431542 (SB), 5 μM A83-01 (A83), 3 μM CHIR99021 (CHIR), 5 μM 6-Bromoindirubin (BIO), 0.5 μM PD0325901 (PD), 100 nM LY3214996 (LY32), 3 μM IWP2, and 5 μM LY411575 (LY41).

(E) The gene expression levels of hepatocyte markers in the human liver organoid-derived cells after 3 days of 2D-culture with Y27632 plus several different combinations of compounds were examined by real-time RT-PCR. PHH-derived organoids established from HC4-24 were used for analysis. The gene expression levels in cells (at day 3 of differentiation) cultured only with Y27632 (control) were taken as 1.0. Results are shown as the mean ± SD (n = 3). Statistical significance was evaluated by one-way ANOVA followed by Dunnett's post-hoc tests (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$, control vs. others).

collagen-coated plate without Y27632, a ROCK inhibitor, the cells lost epithelial morphology. In contrast, the cells showed hepatocyte-like polygonal morphology in the presence of Y27632 and the cells with double-nuclei were observed in control cells and Y27632-treated cells (Figure 1B). The cell size of Y27632-treated cells was similar to PHHs (Figure S3). The gene expression levels of hepatocyte markers (*ALB* and *CYP3A4*) were not affected by the treatment with Y27632 (Figure 1C).

To optimize the condition for differentiation into hepatocytes, we screened various compounds. Transforming growth factor β (TGFβ) inhibitors (SB431542 and A83-01), GSK3 inhibitors (CHIR99021 and BIO), MAPK inhibitors (PD0325901 and LY3214996), a Wnt inhibitor (IWP2) and a γ-secretase/Notch signaling inhibitor (LY411575) were used. When the cells were treated with SB431542, the gene expression level of *ALB* was upregulated (Figure 1D). When they were treated with PD0325901 or IWP2, the gene expression levels of *ALB* showed a tendency toward upregulation (Figure 1D). None of the treatments yielded a significant change in *CYP3A4* expression. To select the optimal combination of these compounds for hepatic differentiation, we treated the cells with different pairs of compounds. At day 3 of differentiation, the gene expression levels of *ALB* and *CYP3A4* in the cells treated with SB431542 and PD0325901 were the highest among all the treatment combinations (Figure 1E). These results suggested that when treated with Y27632, SB431542, and PD032590 (YSP), PHH-derived organoids differentiated into hepatocyte-like cells.

Selection of cytokines and compounds that promote hepatic differentiation under 2D culture

To further promote the induction of hepatic maturation from PHH-derived organoids, various cytokines and reagents that are known to induce hepatic maturation were tested in addition to YSP treatment. Stepwise screenings were performed as described in Figure 2A. At day 3 of 2D culture, several cytokines and compounds enhanced the *CYP3A4* expression level in the cells. In particular, the treatment with 50 ng/mL BMP7 increased *CYP3A4* expression in the cells up to about 75-fold as compared with the control (Figure 2B). The *ALB* expression level in the cells treated with 50 ng/mL FGF7 was about 3.3-fold higher than that in the control cells. At the next step, the cells that were differentiated with YSP and BMP7 for 3 days were treated with various recombinant proteins and compounds in the presence of YSP, and gene expression analysis was performed on day 6. The results showed that the gene expression levels of *CYP3A4* in the cells treated with 100 ng/mL FGF19 were higher than those in the other groups (Figure 2C). The *ALB* expression level was not increased. These results suggested that the treatment with 100 ng/mL FGF19 and YSP was optimal for hepatic differentiation of PHH-derived organoids from day 3 to day 6. We also performed a screening of optimal treatments for hepatic differentiation from day 6 to day 9. The gene expression levels of *ALB* and *CYP3A4* were increased in the cells treated with 10 μM DEX or 100 ng/mL FGF19 (Figure 2D). In the cells treated with both DEX and FGF19, the gene expression levels of both *ALB* and *CYP3A4* were upregulated (Figure 2E). From these results, we concluded that the protocol shown in Figure 2F was optimal for hepatic differentiation from PHH-derived organoids under a 2D-condition. The hepatocyte-like cells differentiated from PHH-derived organoids were named Org-HEPs.

Characterization of Org-heps

To analyze the gene expression profiles in PHH-derived organoids and Org-HEPs, we conducted an RNAseq analysis and detected differentially expressed genes (DEGs) by edgeR.[29,30] A total of 583 genes were observed to be differentially expressed between PHH-derived organoids and Org-HEPs. In Org-HEPs, 355 genes were upregulated, and 228 genes were downregulated compared to PHH-derived organoids. To elucidate the functions of the DEGs, we performed a gene set over-representation analysis, which is a method of enrichment analysis that measures the fraction of genes of interest belonging to a tested group of genes (Figure 3A).[31,32] We also performed a gene-concept network (cnet plot) analysis, and genes that were included in five hubs for terms of upregulated genes in Org-HEPs were shown in Figure S4. The results showed that, compared to PHH-derived organoids, genes that were enriched in terms such as "metabolism of xenobiotics by cytochrome P450", "steroid hormone biosynthesis" and "retinol metabolism" were significantly upregulated in Org-HEPs. The number of genes enriched in "metabolism of xenobiotics by cytochrome P450" was greater than the number of genes enriched in any other term. In particular, terms such as "metabolism of xenobiotics by cytochrome P450" and "retinol metabolism" contained CYP genes and UGT genes.

For further characterization of Org-HEPs, we validated the differential expression of some genes related to pharmacokinetics analysis by real-time RT-PCR. Most expression levels of the hepatocyte marker genes (Figure 3B), transporter genes (Figure 3C) and drug metabolizing and conjugating enzyme genes (Figure 3D) were similar or higher in Org-HEPs compared to those in undifferentiated PHH-derived organoids. Importantly, the gene expression level of *CYP3A4*, the most important drug-metabolizing enzyme, in Org-HEPs was even higher than that in

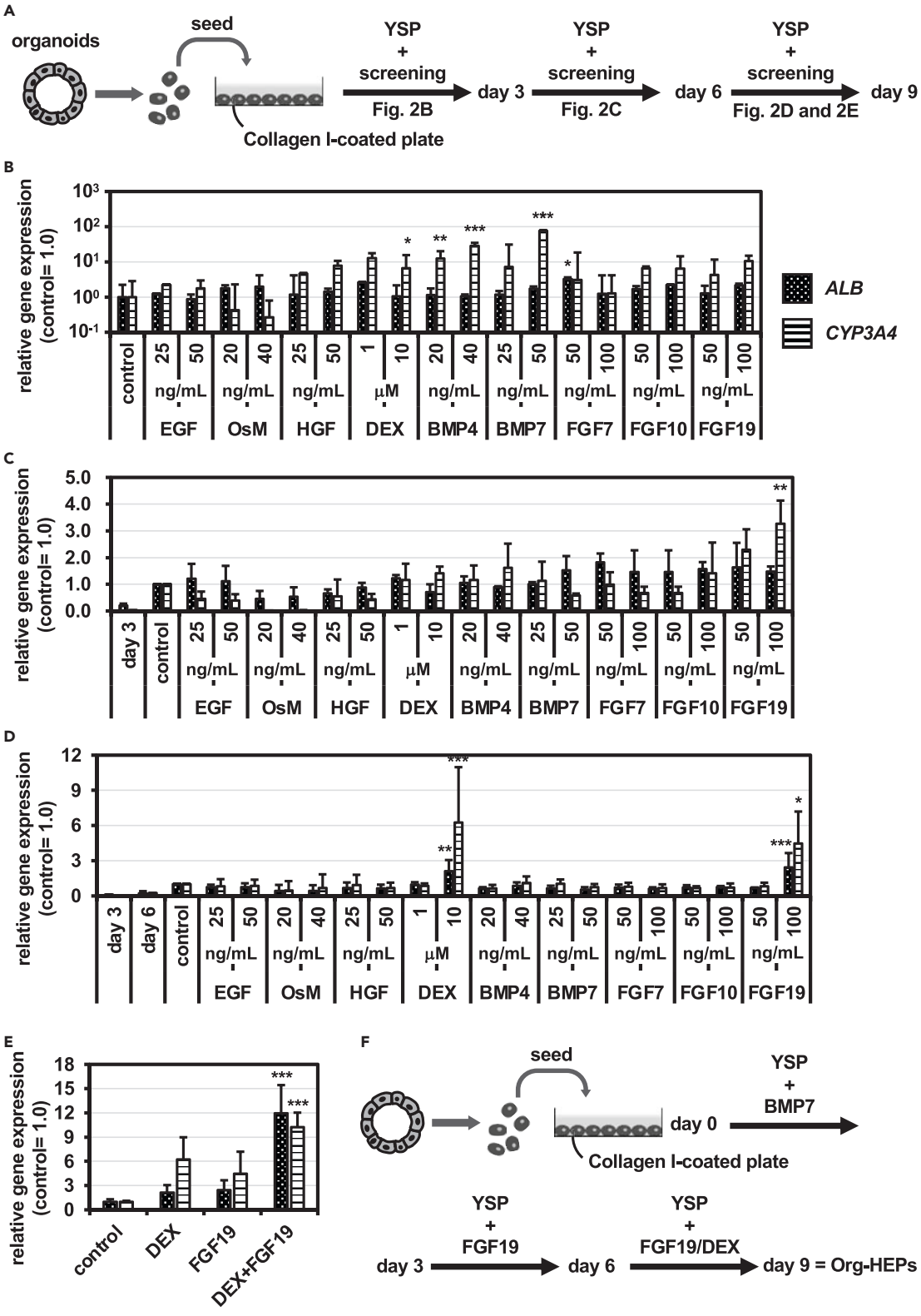

A
organoids
seed
Collagen I-coated plate
YSP + screening
Fig. 2B
day 3
YSP + screening
Fig. 2C
day 6
YSP + screening
Fig. 2D and 2E
day 9
B
relative gene expression (control= 1.0)
ALB
CYP3A4
control
EGF
OsM
HGF
DEX
BMP4
BMP7
FGF7
FGF10
FGF19
ng/mL
μM
C
relative gene expression (control= 1.0)
day 3
D
relative gene expression (control= 1.0)
day 3
day 6
E
relative gene expression (control= 1.0)
control
DEX
FGF19
DEX+FGF19
F
seed
Collagen I-coated plate
day 0
YSP + BMP7
YSP + FGF19
day 3
day 6
YSP + FGF19/DEX
day 9 = Org-HEPs

Figure 2. Screening of cytokines and compounds for 2D-hepatic differentiation of PHH-derived organoids

(A) The screening procedure for screening is presented schematically. Details of the hepatic differentiation procedure are described in STAR Methods.

(B) The gene expression levels of hepatocyte markers in the human liver organoid-derived cells after 3 days of 2D-culture with YSP and several cytokines and compounds were examined by real-time RT-PCR.

(C) The 2D-cultured cells derived from PHH-derived organoids were treated with YSP and 50 ng/mL BMP7 for 3 days. The cells were then cultured with several cytokines and compounds in addition to YSP for an additional 3 days, and then the cellular levels of *ALB* and *CYP3A4* expression were examined by real-time RT-PCR.

(D and E) The 2D-cultured cells derived from PHH-derived organoids were sequentially treated with YSP and 50 ng/mL BMP7 for 3 days, and YSP and 100 ng/mL FGF19 for an additional 3 days. After the cells were cultured with several cytokines and compounds (D) or the combination of DEX and FGF19 (E) in addition to YSP for an additional 3 days, the gene expression levels of *ALB* and *CYP3A4* in the cells were examined by real-time RT-PCR.

(F) The procedure for differentiation is presented schematically. PHH-derived organoids established from HC4-24 were used for analysis. Results are shown as the mean ± SD (n = 3). Statistical significance was evaluated by one-way ANOVA followed by Dunnett's post-hoc tests (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$, control vs. others).

PHHs. Similar results were obtained using the PHH-derived organoids derived from other lots of PHHs (DOO and HC10-10) and human resected liver tissue-derived cells (donor A and donor B) (Figure S5). Thus, it was suggested that PHH-derived organoids differentiated into much more matured hepatocyte-like cells under the determined conditions; the increase in expression was particularly pronounced for the genes related to pharmacokinetics.

Morphological evaluation was then performed. Phase contrast microscopic images showed that Org-HEPs had a polygonal-like morphology characteristic of hepatocytes (Figure 4A). Periodic acid-Schiff (PAS) staining showed that Org-HEPs had the ability to store glycogen (Figure 4B). The immunofluorescent staining showed that most cells expressed HNF4α, CYP3A4, and CK18. ALB-positive cells were also observed (Figure 4C). The percentages of cells positive for hepatocyte markers (HNF4α and CYP3A4) which were 100% positive in PHH-0 h, were determined by FACS. The percentage of HNF4α-positive cells increased from 43.6% in PHH-derived organoids to 99.6% in Org-HEPs (Figure 4D). The percentage of CYP3A4-positive cells increased from 5.1% in PHH-derived organoids to 96.7% in Org-HEPs (Figure 4D). Similar results were obtained using the PHH-derived organoids derived from other lots of PHHs (DOO and HC10-10) and human resected liver tissue-derived cells (donor A and donor B) (Figure S6). These results suggested that PHH-derived organoids were efficiently differentiated into hepatocyte-like cells.

Profiles of drug-metabolizing enzyme capacity

We next examined the profiles of CYP-mediated drug-metabolizing capacity in Org-HEPs in comparison with those in PHHs. The CYP2C8, CYP2C19, CYP2E1, and CYP3A4 (MDZ) activities in Org-HEPs were comparable to those in PHHs after 48 h of culture (PHH-48 h). In particular, CYP1A2 and CYP3A4 (TES) activities in Org-HEPs were equal to those in PHHs immediately after thawing (PHH-0 h) (Figure 5A). Similar results on CYP1A2 and CYP3A4 (MDZ and TES) activities were obtained using Org-HEPs derived from other lots of PHHs (DOO and HC10-10) and human resected liver tissue-derived cells (donor A and donor B) (Figure S7). The activity of UDP-glucuronosyltransferase 1A1 (UGT1A1), the major conjugating enzyme expressed in the liver, in Org-HEPs was comparable to those in PHH-48 h (Figure 5B). On the other hand, the activity of CYP2D6 in Org-HEPs was lower than that in PHH-0 h and PHH-48 h (Figure 5C). The protein expressions of CYP2C19, CYP2E1, CYP3A4, and UGT1A1 were detected in Org-HEPs, but not organoids, by western blotting analysis (Figure S8).

Next, we directly compared hepatocytes differentiation from PHH-derived organoids cultured with the stepwise medium developed in this study and with DM reported by Broutier et al.[25] under both 2D- and 3D-conditions (Figure S9A). In both 2D- and 3D-conditions, the gene expression levels of *ALB* and *CYP3A4* in Org-HEPs, which were differentiated with the stepwise medium, were higher than those in Org-HEPs, which were differentiated with DM (Figure S9B). The CYP1A2 and CYP3A4 (TES) activities in 2D-Org-HEPs (stepwise medium) were higher than those in 2D-Org-HEPs (DM). The CYP2D6 and CYP3A4 (MDZ) activities were similar between 2D-Org-HEPs (stepwise medium) and 2D-Org-HEPs (DM) (Figure S9C). These results suggested that the method developed in this study were able to differentiate into much more functional hepatocytes, compared with the differentiation method with DM reported by Broutier et al.[25]

Prediction of drug-induced liver toxicity and CYPs induction

Acetaminophen is metabolized by the enzymes CYP3A4, CYP2E1, and UGT1A1, and carries a risk of hepatotoxicity.[33] Troglitazone, amiodarone and clozapine are mainly metabolized by CYP3A4, and show hepatotoxicity.[34–37] When Org-HEPs and PHH-48 h were treated with these hepatotoxic drugs, there were no significant differences in cell viability between Org-HEPs and PHH-48 h (Figure 6A). These results indicated that Org-HEPs could be used for the prediction of drug-induced liver toxicity in the same way as PHH-48 h.

CYP induction-mediated drug-drug interaction is one of the major concerns in both clinical practice and pharmaceutical research. Since the gene expression levels of nuclear receptors *pregnane X receptor* (*PXR*), *constitutive androstane receptor* (*CAR*), and *farnesoid X receptor* (*FXR*), which are involved in the induction of CYPs, were comparable between Org-HEPs and PHH-48 h (Figure S10), we examined whether Org-HEPs could be applied to CYPs induction tests. Drugs that are known to induce the expression of CYPs—namely, omeprazole, phenobarbital, and rifampicin[38,39]—were applied to Org-HEPs and PHH-48 h. Compared to the levels in the DMSO-treated group, *CYP1A2* expression was increased 31.2-fold in Org-HEPs treated with omeprazole and 24.1-fold in PHH-48 h treated with omeprazole (Figure 6B). Following treatment with phenobarbital, the *CYP2B6* expression increased 5.3-fold in Org-HEPs and 4.2-fold in PHH-48 h (Figure 6B). Following treatment with rifampicin, the *CYP3A4* expression increased 28.6-fold in Org-HEPs and 8.7-fold in PHH-48 h

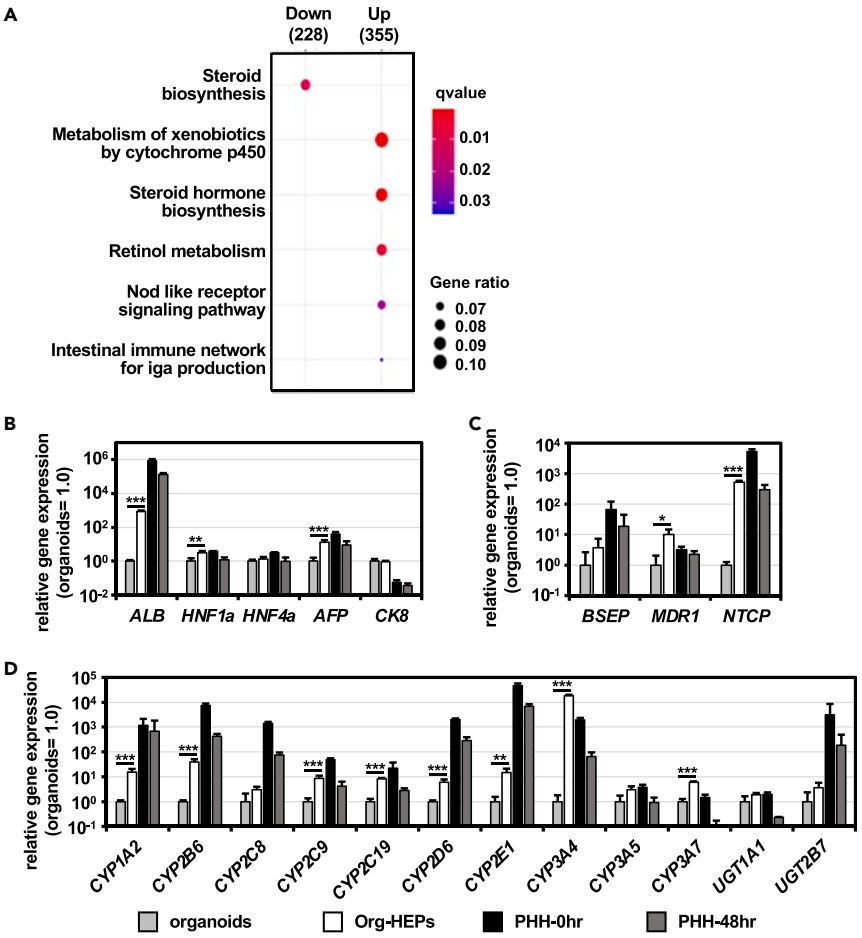


Figure 3. Gene expression profiles of Org-HEPs

(A) PHH-derived organoids and Org-HEPs were subjected to an RNA-seq analysis in two independent batches of PHH-derived organoids and Org-HEPs (HC4-24 and HC10-10). The criteria for DEG detection in this study were FDR <0.05 and a fold change >2 (PHH-derived organoids vs. Org-HEPs). The KEGG gene set was used for enrichment analysis.

(B–D) The gene expression levels of hepatocyte markers (B), transporters (C) and drug metabolic enzymes (D) in PHH-derived organoids (HC4-24), Org-HEPs (HC4-24), PHH-0 h (HC4-24) and PHH-48 h (HC4-24) were examined by real-time RT-PCR. Org-HEPs at day 9 of the differentiation were used. PHHs were used just after thawing (PHH-0 h) or cultured for 48 h after plating (PHH-48 h). Results are shown as the mean ± SD (n = 3). Statistical significance was evaluated by unpaired two-tail Student's t test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$).

(Figure 6B). Similar results on induction of CYPs were obtained using Org-HEPs derived from other lots of PHHs (DOO and HC10-10) and human resected liver tissue-derived cells (donor A and donor B) (Figure S11). These results suggest that Org-HEPs retain the capacity for induction of CYPs. Thus, Org-HEPs could be applicable to hepatotoxicity prediction and drug-metabolizing enzyme induction tests.

Maintenance of hepatic functions in Org-HEPs for long-term culture

We examined whether Org-HEPs could maintain their hepatic functions over the long-term. When the culture period was extended to day 15 of the differentiation, the gene expression levels of *ALB* and *CYP3A4* in Org-HEPs reached the peak at day 12 and were maintained until day 15 (Figure 7A). The gene expression level of the cholangiocyte marker (*EpCAM*) changed slightly in Org-HEPs (Figure 7B). The expression level of a somatic stem cell marker (*LGR5*) was decreased in Org-HEPs (Figure 7C). The CYP3A4 activity in Org-HEPs at day 15 was increased up to about 1000-fold, compared to day 0 of the differentiation (Figure 7D). Moreover, the gene expression levels of *ALB* and *CYP3A4* in day-15 Org-HEPs were maintained for an additional 15 days of differentiation (i.e., to day 30) (Figure 7E). The cells on day 30 secreted ALB (Figure 7F), and still exhibited high levels of CYP3A4 activity (Figure 7G). These results suggested that the differentiation method developed in this study not only achieved cells with higher levels of hepatic functions, but also allowed the cells to maintain part of their hepatic functions even after 30 days of 2D culture.

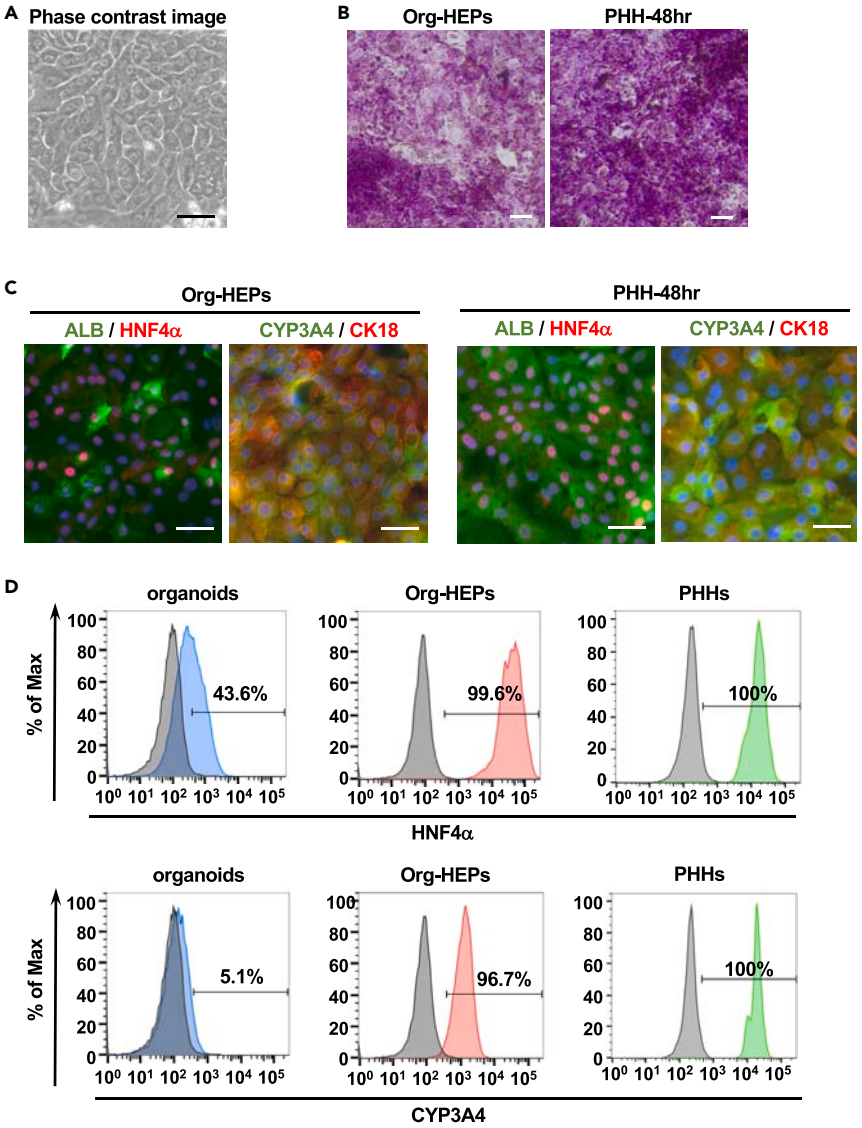


Figure 4. Characterization of Org-HEPs

(A) A phase contrast image of Org-HEPs is shown.

(B) The glycogen storage of Org-HEPs (HC4-24) and PHH-48 h (HC10-10) was assessed by Periodic acid-Schiff (PAS) staining. Glycogen storage is indicated by pink or dark red-purple cytoplasm.

(C) The protein expression levels of hepatocyte markers in Org-HEPs (HC4-24) and PHH-48 h (HC10-10) were examined by immunocytochemical analysis. Nuclei were counterstained with DAPI (blue).

(D) The percentages of hepatocyte markers-positive cells in PHH-derived organoids, Org-HEPs (HC4-24) and PHH-0 h (HC4-24) were examined by FACS analysis. Org-HEPs established from HC4-24 at day 9 of the differentiation were used. PHHs were used just after thawing (PHH-0 h) or cultured for 48 h after plating (PHH-48 h). The scale bars represent 50 μm.

DISCUSSION

In the present study, to promote the application of PHH-derived organoids to pharmaceutical research, we successfully developed a differentiation method that realized sufficient levels of hepatic functions and versatility. Because PHH-derived organoids are cultured in droplets of Matrigel, it is tedious to perform pharmacokinetic tests and hepatotoxicity tests using these organoids. To overcome this limitation of the organoid culture system, we developed a method for differentiation into hepatocyte-like cells in a 2D culture system. Matrigel was degraded to obtain single cells from PHH-derived organoids, which were then seeded onto collagen-coated plates. The cells were cultured in HCM supplemented with Y27632, SB431542, and PD0325901 (YSP) and BMP7 for 3 days. For the next 3 days, the cells were cultured in HCM with YSP and FGF19. For the last 3 days, the cells were cultured in HCM with YSP, FGF19, and DEX. We named these cells Org-HEPs. Although

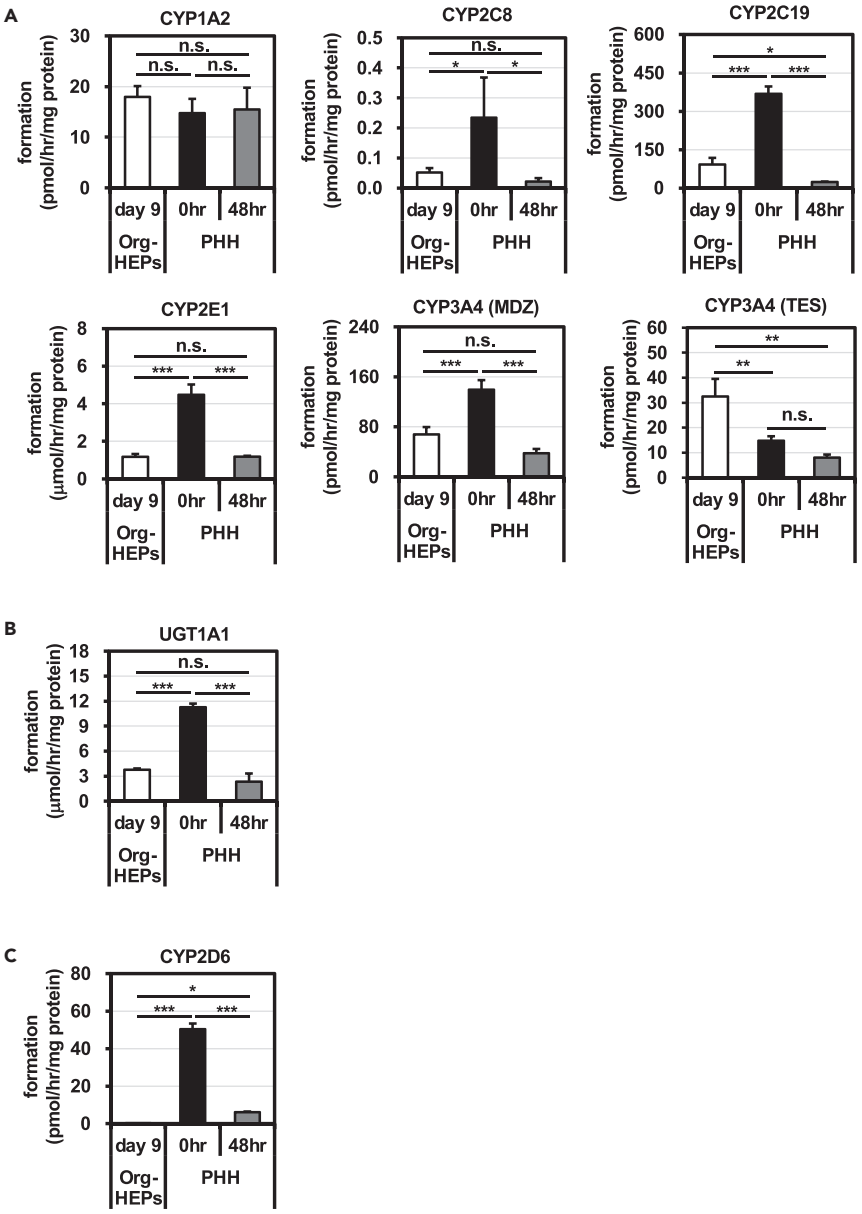


Figure 5. Drug metabolism/conjugate enzyme activities of Org-HEPs

(A–C) The CYP1A2, 2C8, 2C19, 2E1, 3A4, and 2D6, and UGT1A1 activities in PHHs (HC4-24) and Org-HEPs (HC4-24) were examined using LC-MS/MS. Substrates and concentrations for the assay of CYPs and UGT1A1 activities are shown in Table S5. Org-HEPs at day 9 of the differentiation were used. PHHs were just after thawing (PHH-0 h) or cultured for 48 h after plating (PHH-48 h). Results are shown as the mean ± SD (n = 3). Statistical significance was evaluated by one-way ANOVA followed by Tukey's post-hoc tests to compare all groups (n.s., not significant, *p < 0.05, **p < 0.01, and ***p < 0.005).

PHH-derived organoids, even when cultured with DM, lost or decreased many hepatic functions, Org-HEPs were found to retain hepatic functions such as drug-metabolizing capacity at levels comparable to those in PHHs, and thus to be suitable for pharmaceutical use.

PHHs rapidly lose hepatic function after seeding. Hepatocyte de-differentiation is one of the causes of this decline. In particular, it is known that epithelial mesenchymal transformation (EMT) has a significant effect on the deterioration of hepatocyte functions,[40] and in fact, there have been reports of successful maintenance of function by treatment of PHHs with EMT inhibitors.[17,18] Specifically, these studies showed that inhibition of TGFβ, Wnt, MAPK, and ROCK signals suppressed EMT of PHHs and maintained their hepatic functions. Overlapping with these previous reports, we recently showed that a combination we named YSP—the ROCK inhibitor Y27632, the TGFβ inhibitor SB431542 and the MAPK inhibitor PD032590—successfully suppressed EMT during the passaging of human induced pluripotent stem (iPS) cell-derived hepatocyte-like cells, and prevented functional decline.[41] Based on these reports, we speculated that YSP might be able to promote hepatic differentiation via EMT inhibition when PHH-derived organoids were cultured under a 2D condition. It is also possible

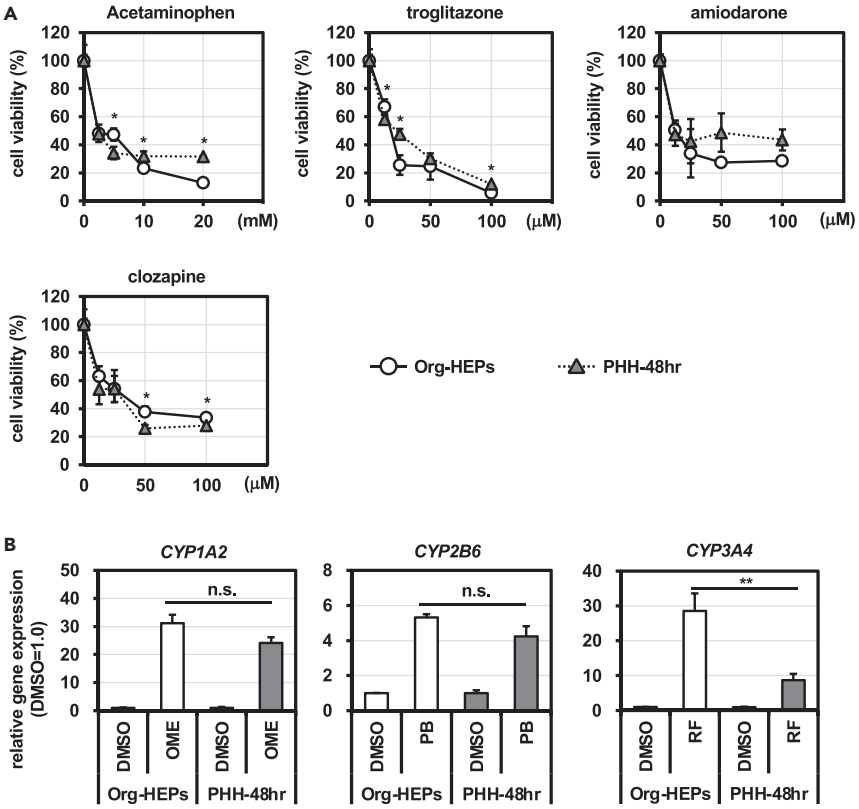


Figure 6. Application of Org-HEPs for liver toxicity prediction tests or drug metabolic enzyme induction tests

(A) Prediction of drug-induced liver injury using Org-HEPs. Org-HEPs (HC4-24) at day 9 of the differentiation and PHHs (HC4-24) cultured for 48 h after plating (PHH-48 h) were exposed to different concentrations of acetaminophen, troglitazone, amiodarone, and clozapine for 48 h. The cell viability of both cell types was examined by WST-8 assay and was calculated as a percentage of cells treated with vehicle only.

(B) The induction of CYP1A2, CYP2B6 or CYP3A4 by CYP inducers (omeprazole [OME], phenobarbital [PB], and rifampicin [RF], respectively) in Org-HEPs and PHH-48 h were examined. The gene expression levels in solvent-treated cells (DMSO) were taken as 1.0. Results are shown as the mean ± SD (n = 3). Statistical significance was evaluated by unpaired two-tail Student's t test (n.s., not significant, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$).

that YSP could promote hepatic differentiation of PHH-derived organoids via inhibition of cholangiocyte differentiation. Hepatic stem cells are the origin of hepatocytes and cholangiocytes.[42,43] During the process of hepatocyte differentiation in the liver, TGFβ signaling is known to inhibit differentiation into hepatocytes and promote differentiation into cholangiocytes.[44,45] In other words, inhibition of TGFβ signaling promotes differentiation from liver stem cells into hepatocytes. PHH-derived organoids have bi-potency toward hepatocytes and cholangiocytes.[46] We recently reported that PHH-derived organoids could be differentiated into mature cholangiocytes.[47] Therefore, SB431542, a TGFβ inhibitor, may have promoted differentiation of PHH-derived organoids into hepatocytes by inhibiting differentiation into cholangiocytes. Moreover, growth factors and maturation factors such as BMPs and FGFs have essential roles in regulating liver regeneration. In zebrafish, liver regeneration after partial hepatectomy was severely impaired when neither BMPs nor FGFs were signaling. This demonstrated that BMP signaling and FGF signaling are able to regulate both proliferation and development of hepatocytes *in vivo*.[48] BMP7 and FGF19 have functions as an endogenous regulator of adult hepatocyte proliferation and liver homeostasis or hepatoprotective activities.[49,50] Thus, BMP7 and FGF19 may have promoted the differentiation from PHH-derived organoids into hepatocytes by the same mechanism as observed *in vivo*.

Most of the drug-metabolizing or conjugated enzyme activities in Org-HEPs were comparable to those in PHHs. However, CYP2D6 activity was low (Figure 5C). This result was not due to the cells being derived from a poor metabolizer, because the parental PHHs, which were used for the generation of Org-HEPs, expressed CYP2D6 activity (Figure 5C). We would like to examine the behavior of CYPs in more detail by analyzing the regulation of such elements as the epigenome and/or microRNA from various points of view. The Org-HEPs might not be sufficiently mature to exhibit CYP2D6 activity. Spherical 3D culture of hepatocytes such as PHHs, hepatocellular carcinoma cells, and human iPS cell-derived hepatocytes has been shown to be an effective way for maintaining or improving hepatic functions because it can mimic the structure of *in vivo* tissue.[10,11,51–54] Manon et al. showed that hepatocyte-like cells derived from human intrahepatic primary hepatocyte-derived organoids have potential for use in drug responsive liver models in which the 3D structures are constructed with a bioprinting system.[55] The 3D culture of Org-HEPs may be effective for more matured hepatocytes.

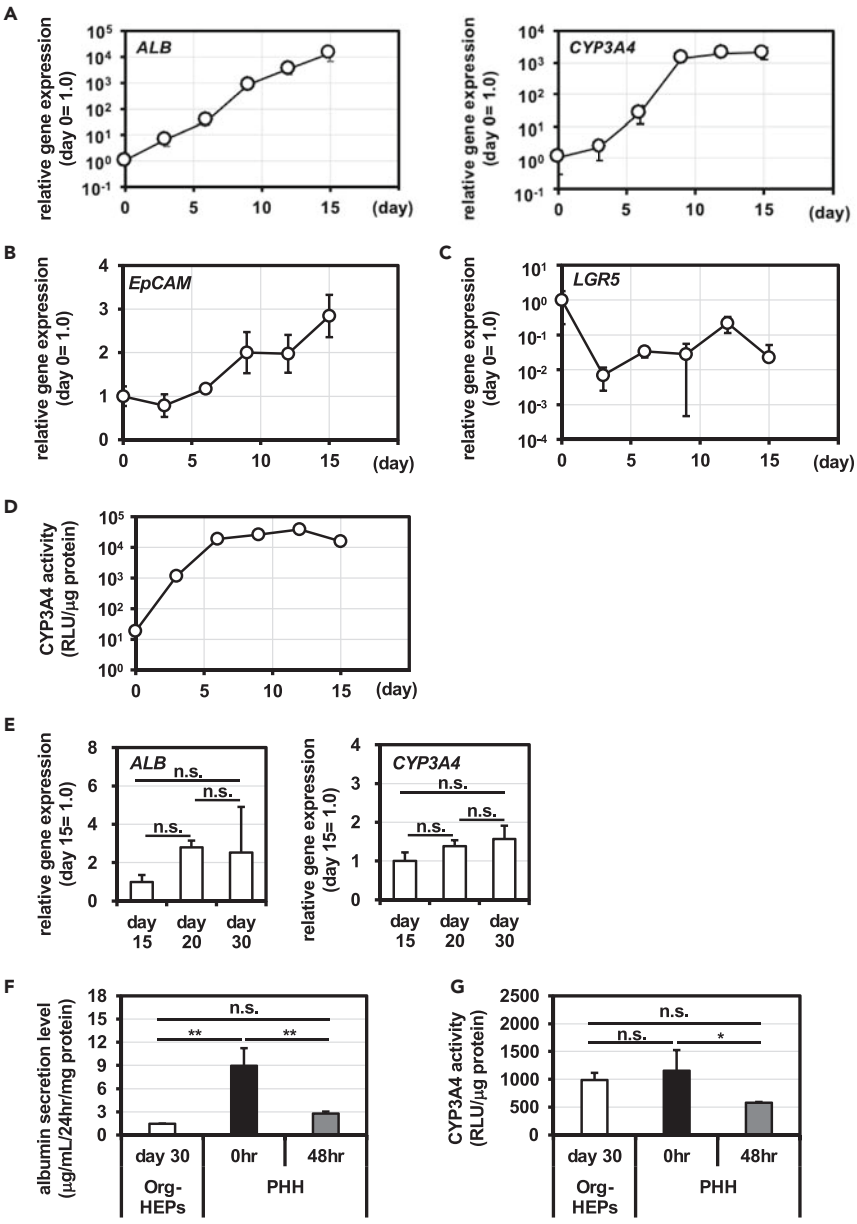


Figure 7. Long-term culture of Org-HEPs

(A–C) Temporal gene expression levels of *ALB*, *CYP3A4* (A), *EpCAM* (B) and *LGR5* (C) in Org-HEPs were examined by real-time RT-PCR from day 0 to day 15 of differentiation.

(D) Temporal activity of CYP3A4 was examined in cells from day 0 to day 15 of differentiation.

(E) The gene expressions of *ALB* and *CYP3A4* in Org-HEPs were examined by real-time RT-PCR from day 15 to day 30 of differentiation.

(F) ALB secretion capacities in Org-HEPs and PHHs were examined.

(G) CYP3A4 activities in Org-HEPs and PHHs were examined by a luminescent assay system. Org-HEPs established from HC4-24 were used for analysis. PHHs (HC4-24) were just after thawing (PHH-0 h) or cultured for 48 h after plating (PHH-48 h). Results are shown as the mean ± SD (n = 3). Statistical significance was evaluated by one-way ANOVA followed by Tukey's post-hoc tests to compare all groups (n.s., not significant, $*p < 0.05$, $**p < 0.01$, and $***p < 0.005$).

In this study, PHH-derived organoids that were established from commercially available cryopreserved PHHs could be expanded with CHOL-medium. Although more than 99% of the PHHs were ALB-positive (data not shown), it would be possible that cholangiocytes, which were contaminated a little in commercially available cryopreserved PHHs, formed organoids with CHOL-medium. Alternatively, CHOL-organoids might form from ALB-positive hepatocytes. Hu et al. reported that when hepatocyte-derived HEP-organoids were cultured with CHOL-medium, they were converted into CHOL-organoids *in vitro*.[28] Other groups reported that hepatocytes were converted into cholangiocytes *in vivo* after liver injury.[56,57] In addition, it was reported that PHHs could be serially passaged and expanded *in vitro*, and these cells exhibited

liver progenitor features.[19,20,58] These reports suggested that the conversion of hepatocytes into cholangiocyte- or progenitor-like cells was a common phenomenon *in vitro/vivo*. The reason why we were able to establish and expand organoids from EpCAM-negative hepatocytes with CHOL-medium may be that PHHs de-differentiated into hepatocyte-derived progenitor-cells or converted into cholangiocytes, and formed a CHOL-organoids-like structure. Huch et al. and Broutier et al. reported that it was difficult to achieve establishment and long-term expansion of organoids from EpCAM-negative hepatocytes using CHOL-medium, whereas organoids established from EpCAM-positive cells could be expanded over the long term (>5 months).[24,25] Since PHH-derived organoids were proliferative for only about 4 months in the present study, it is still possible that there were differences between the properties of PHH-derived organoids used in the present study and those of CHOL-organoids reported by Hu et al. and Broutier et al. Importantly, however, similar differentiation-promoting effects were observed for both PHH (EpCAM-negative cell)-derived organoids and human resected liver tissue (EpCAM-positive cell)-derived organoids (Figures S5–S8 and S11). These results suggested that, irrespective of their origin, organoids cultured with CHOL-medium can differentiate into highly functional hepatocytes under the 2D-condition developed in the present study.

The combination of organoid culture technology with the hepatic differentiation method developed in the present study is expected to solve the two problems of PHHs, i.e., decline in hepatic functions after seeding and supply limitation. We showed that gene expression levels of *ALB* and *CYP3A4* in Org-HEPs were maintained until day 30 of the differentiation, and CYP3A4 activity in Org-HEPs at day 30 was comparable to that in PHHs-0 h (Figure 7). These results suggested that Org-HEPs were superior to PHHs in long-term culture and it would enable application of these cells to long-term toxicity tests. In addition, PHH-derived organoids could be *in vitro*-expanded by least 5×10^5-fold over 4 months (Figure S2A). The cell properties, proliferative potential and differentiation potential of PHH-derived organoids were not affected by cryopreservation and thawing (data not shown). These results suggested that a large number of Org-HEPs could be derived from the same lot of PHHs. Furthermore, comprehensive gene expression analysis and enrichment analysis showed that genes associated with terms related to pharmacokinetics, such as "metabolism of xenobiotics by cytochrome P450" and "retinol metabolism", were upregulated in Org-HEPs relative to their levels in PHH-derived organoids (Figures 3A and S4). Most importantly, the metabolic activities of most drug-metabolizing enzymes in Org-HEPs were comparable to those in PHHs.

Evaluation of biliary clearance of drugs is critical for pharmaceutical research. To measure bile acid excretion capacity, Org-HEPs were overlaid with Matrigel to form bile canaliculi. However, it was not confirmed the formation of bile canaliculus in Org-HEPs (data not shown). Lot-to-lot variation in Matrigel and the compositions of basic media to culture the cells, as which HCM was used in the present study, may have an effect on the data reliability about bile canaliculi formation. The culture protocols of Matrigel-overlay, i.e., Matrigel concentration and culture period, differ among the studies.[59–61] More optimization, including the culture protocol of Matrigel-overlay, would be required for the formation of bile canaliculi in Org-HEPs.

The use of human liver organoids in drug development was reported. It was shown that primary human liver cancer-derived organoids were amenable for biomarker identification and drug-screening and led to the identification of a potential therapeutic agent for primary liver cancer.[62] In addition, human liver organoids were shown to be a platform for modeling HBV infection and related tumorigenesis, and could be utilized to study HBV pathogenesis as well as for potential anti-HBV-drug screening.[63] Human liver organoids have been shown to be applicable to the research into the pathogenesis of liver diseases and the development of drugs for them. Org-HEPs in the present study may also have potential for these studies.

In conclusion, Org-HEPs could be a very useful source of human hepatocytes for pharmaceutical research. The present method may also be useful for regenerative medicine and other fields involving human hepatocytes.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hiroyuki Mizuguchi (mizuguch@phs.osaka-u.ac.jp).

Materials availability

In this study no new materials have been generated.

Data and code availability

- The Gene Expression Omnibus (GEO) accession number for the RNA-sequencing analysis is GEO: GSE234459. RNA-seq data have been deposited at GEO and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. Original western blot images and microscopy data reported in this paper will be shared by the lead contact upon request.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Y.U.-T. and H.M. conceived the idea and designed the research. Y.U.-T. and Y.T. performed experiments and analyzed data. J.Y. and Y.U.-T. performed bulk-RNAseq data analysis. K.M., H.H., H.E., and T.T. provided human resected liver tissue-derived cells. The manuscript was primarily written by Y.U.-T. and H.M. All authors discussed the results and commented on the manuscript.

DECLARATION OF INTERESTS

There are no conflicts of interest to disclose.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Albumin	Bethyl laboratories	Cat# A80-129A
Anti-HNF4α	Santa Cruz	Cat# sc-8979
Anti-CYP3A4	Santa Cruz	Cat# sc-27639
Chemicals, peptides, and recombinant proteins		
CultureSure Y-27632	Wako	Cat# 036-24023
CultureSure SB431542		Cat# 037-24293
CultureSure A-83-01		Cat# 035-24113
CHIR99021	Tocris	Cat# 4423/50
6-Bromoindirubin	Calbio-chem	Cat# 361550
PD0325901		Cat# 162-25291
LY3214996	Selleck	Cat# S8534
LY411575	Chemscene	Cat# CS-0309
EGF, Human, Recombinant, Carrier-free	R&D Systems	Cat# 236-EG-01M
Recombinant Human Oncostatin M (OsM) Protein	R&D Systems	Cat# 295-OM-01M
Recombinant Human HGF Protein	R&D Systems	Cat# 294-HG-MTO
Dexamethasone		Cat# 041-18861
Recombinant Human BMP-4	R&D Systems	Cat# 314-BP-050
Recombinant Human BMP-7	Peprotech	Cat# 120-03P
Recombinant Human KGF (FGF-7)	Peprotech	Cat# 100-19
Animal-Free Recombinant Human FGF-10	Peprotech	Cat# AF-100-26
Recombinant Human FGF-19	Peprotech	Cat# 100-32
Critical commercial assays		
P450-Glo™ CYP3A4 Assay and Screening System	Promega	V9002
PAS staining kit	Muto pure chemicals	Cat# 15792
Albumin, Human ELISA Kit	Bethyl	E88-129
Deposited data		
RNA-Sequencing Data	This study	GEO: GSE234459
Experimental models: Cell lines		
HC4-24	Xenotech	Cat# H1500.H15B
HC10-10	Xenotech	Cat# H1500.H15B+
DOO	Celesis	Cat# M00995-P
human resected liver tissue-derived cells	This paper	
Software and algorithms		
GraphPad prism 9	GraphPad	https://www.graphpad.com/scientific-software/prism/
FlowJo Software	Becton Dickinson & Company	v10.8.1
R software program	R Development Core Team	RRID:SCR_001905 https://www.r-project.org

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Primary human hepatocytes

Three lots of PHHs (lots: HC4-24 (Xenotech), HC10-10 (Xenotech) and DOO (Celesis)) and two lots of human biopsy-derived cells (donor A and donor B) were used to generate PHH-derived organoids (Table S1).

Ethical approval

The study was approved by the Institutional Review Board (IRB) for Clinical Research at Osaka University Hospital (IRB No. 14025 and 15267) and at Graduate School of Pharmaceutical Sciences, Osaka University (no. 2019-27-1).

METHOD DETAILS

Human cholangiocyte organoid culture

PHHs or human biopsy-derived cells were washed with cold Advanced DMEM/F12 (Thermo Fisher Scientific) and spun at 400 g for 5 min. The cell pellet was mixed with Matrigel (growth factor reduced, Corning) and 1×10^4 cells were seeded per well in a 24-well plate. After Matrigel had solidified, 500 μL of PHH-derived organoids culture medium (CHOL-medium) was added to each well. PHH-derived organoids culture medium was prepared according to the previous report.[24,25] Briefly, Advanced DMEM/F12 was supplemented with 1% Antibiotic Antimycotic Solution and 1% GlutaMAX (GIBCO), 10 mM HEPES (Nacalai Tesque), 2% B27 supplement (GIBCO), 1.25 mM N-Acetylcysteine (Sigma), 10 mM Nicotinamide (Sigma), 10 nM recombinant gastrin (Merk), 50 ng/mL EGF (R&D), 10% R-Spondin1 conditioned medium (homemade), 100 ng/mL recombinant human FGF10 (PeproTech), 25 ng/mL recombinant human HGF (R&D), 5 μM A83-01 (Wako), and 10 μM Forskolin (Wako). During cultivation, medium was refreshed every three days. For the establishment of PHH-derived organoids, the medium was supplemented with 25 ng/mL recombinant Noggin (R&D), 7.5 ng/mL recombinant Wnt3a (R&D) and 10 μM Y27632 (Wako) for the first 3–4 days. Passage was performed in a 1:3 split ratio once every 10–14 days. PHH-derived organoids passaged for 3–15 times were used in all experiments.

Hepatocyte differentiation with 2D culture

PHH-derived organoids were collected and incubated in TrypLE select at 37°C for 7–10 min to dissolve the Matrigel. After incubation, the cells were pipetted up and down to dissociate organoids into single cells. The cell suspension was filtered through a 40-μm cell strainer (Corning). The suspension was centrifuged at 2000 rpm for 5 min at room temperature. The cells were seeded at $2.0\text{x}10^5$ cells/cm^2 in hepatocyte culture medium (HCM; Lonza) on collagen-coated 48-well plates (IWAKI), and cultured with HCM supplemented with 10 μM Y27632, 2 μM SB431542, 0.5 μm M PD0325901, and 50 ng/mL BMP7 for 3 days. From days 3–6, the cells were cultured in HCM containing 10 μM Y27632, 2 μM SB431542, 0.5 μM PD0325901, and 100 ng/mL FGF19. Finally, from day 6 to day 9, the cells were cultured with HCM supplemented with 10 μM Y27632, 2 μM SB431542, 0.5 μM PD0325901, 100 ng/mL FGF19, and 10 μM DEX. The resulting cells were named Org-HEPs.

Isolation of hepatocytes from human adult liver

Adult residual liver samples not used for clinical purposes were obtained from non-tumor portions of specimens obtained by hepatectomy for tumor removal of rectal cancer liver metastasis, or from donor livers for liver transplantation. The study was approved by the Institutional Review Board for Clinical Research at Osaka University Hospital (14025, 15267) and at Graduate School of Pharmaceutical Sciences, Osaka University (no. 2019-27-1). Residual liver samples were perfused with 100 mL of Liver Perfusion Medium (gibco, 17701-038) through the portal vein and deblooded. The liver samples were then perfused with 10 mg of Liberase TM Research Grade (Roche, 05401127001) through the portal vein and transferred to Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque, Inc.) supplemented with 10% fetal bovine serum (FBS; Life Technologies) to collect the hepatocyte suspension. Hepatocytes were isolated by centrifuging (4°C, 50G, 5min) and washing the hepatocyte suspension three times in fresh DMEM supplemented with 1% FBS.

Hepatocytes differentiation of PHH-derived organoids under 3D-culture condition

The differentiation of PHH-derived organoids which were cultured with CHOL-med toward hepatocytes was performed according to the previous report.[2,3] Briefly, PHH-derived organoids were cultured with the organoid culture medium (described in the Materials and Methods in the main manuscript) supplemented with 25 ng/mL BMP7 for 5 days. After 5 days, medium was changed to hepatocytes differentiation medium (DM) for 5 days. Hepatocytes differentiation medium was composed of Advanced DMEM/F12 supplemented with 1:50 B27 supplement (with vitamin A), 1:100 N2 supplement, 1 mM N-acetylcysteine, 10 nM recombinant human gastrin, 50 ng/mL recombinant human EGF, 25 ng/mL recombinant human HGF, 0.5 μM A83-01, 10 μM DAPT, 3 μM dexamethasone, 25 ng/mL BMP7 and 50 ng/mL recombinant human FGF19. The medium was refreshed every 3 days.

Real-time RT-PCR

Total RNA was isolated from PHH-derived organoids and Org-HEPs using ISOGENE (NIPPON GENE). cDNA was synthesized using 500 ng of total RNA with a Superscript VILO cDNA synthesis kit (Thermo Fisher Scientific). Real-time RT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) using a StepOnePlus real-time PCR system (Applied Biosystems). The relative quantitation of target mRNA levels

was performed by using the $2^{-\Delta\Delta CT}$ method. The values were normalized by those of the housekeeping gene, *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*). PCR primer sequences (described in Table S2) were obtained from PrimerBank (https://pga.mgh.harvard.edu/primerbank/).

Immunohistochemistry

To perform the immunohistochemistry, the cells washed with PBS were fixed with 4% paraformaldehyde for 10 min. After blocking the cells with PBS containing 2% bovine serum albumin (Nacalai Tesque) and 2% Triton X-100 (Merck) for 30 min at room temperature, the cells were incubated with the blocking buffer containing a primary antibody (described in Table S3) at 4°C overnight, and finally with the blocking buffer containing a secondary antibody (described in Table S3) at room temperature for 1 h. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Nacalai Tesque). Images were acquired with fluorescence microscope (Biozero BZ-9000; KEYENCE).

Flow cytometry

Single-cell suspensions of PHH-derived organoids and Org-HEPs were fixed with 4% paraformaldehyde at 4°C for 10 min, and then incubated with the primary antibody (described in Table S4), followed by the secondary antibody (described in Table S4). Analysis was performed on an MACSQuant Analyzer (Miltenyi Biotec) and FlowJo software (FlowJo LLC, http://www.flowjo.com/).

Periodic Acid-Schiff (PAS) assay for glycogen

Org-HEPs were fixed with 4% paraformaldehyde and stained using a Periodic Acid-Schiff staining system (Sigma) at day 9 of differentiation according to the manufacturer's instructions.

CYPs assay and LC-MS/MS analysis

Org-HEPs and PHHs were cultured with medium containing cocktail-substrates (described in Table S5). After the treatment with substrates, the supernatant was collected, and then immediately mixed with acetonitrile (FUJIFILM Wako). Samples were filtrated with AcroPrep Advance 96-Well Filter Plates (Pall Corporation) for 5 min at 1,750 *g*. Then the supernatant was analyzed by LC-MS/MS, and the concentration of each metabolite was calculated.

LC-MS/MS data were obtained by mass spectrometry (Xevo TQ-S, Waters Corp., Milford, MA, USA) connected to UPLC (ACQUITY UPLC, Waters), using BEH C18 column (1.7 μm, 2.1 × 50 mm, Waters). Mobile phase A = 0.1% formic acid/water, B = 0.1% formic acid/acetonitrile, and gradient system as follows: 0 min-2% B, 1.0 min-95% B, 1.25 min-95% B, 1.26 min-2% B, and 1.75 min-2% B. The flow rate was 1.0 mL/min.

Assessment of drug-induced cell toxicity

Org-HEPs and PHHs were exposed to different concentrations of acetaminophen (Wako), troglitazone (Wako), amiodarone (TCI Chemicals) or clozapine (Sigma) for 48 h. Cell viability was examined by a WST-8 assay, using a Cell Counting Kit-8 purchased from Dojindo Laboratories. The WST-8 assay was performed according to the manufacturer's instructions. Cell viability was calculated as a percentage of the viability of cells treated with vehicle (DMSO) only.

CYP induction test

The cells were treated with 50 μM omeprazole, 1 mM phenobarbital, or 10 μM rifampicin for 24 h (all from FUJIFILM Wako); these compounds are known to induce CYP1A2, 2B6, and 3A4, respectively.[38,39] Controls were treated with DMSO (final concentration 0.1%, Wako). To measure CYP1A2, 2B6, and 3A4 induction potencies, the gene expression levels of CYPs were measured by real-time RT-PCR.

Western blotting analysis

The cells were homogenized with lysis buffer (20 mM HEPES, 2 mM EDTA, 10% glycerol, and 1% Triton X-100) containing a protease inhibitor mixture (Sigma-Aldrich). After being frozen and thawed, the homogenates were centrifuged at 15,000 *g* at 4°C for 10 min, and the supernatants were collected. The lysates were subjected to SDS-PAGE on 7.5% polyacrylamide gel, and then transferred onto polyvinylidene fluoride membranes (Millipore). After the reaction was blocked with 1% skim milk in TBS containing 0.1% Tween 20 at room temperature for 1 h, the membranes were incubated with the primary antibodies (described in Table S6) at 4°C overnight, followed by reaction with the secondary antibody (described in Table S6) at room temperature for 1 h. The band was visualized by ECL Plus Western blotting detection reagents (GE Healthcare) and the signals were read using an LAS-3000 imaging system (Fuji Film).

ALB secretion

The culture supernatants, which were incubated for 24 h after fresh medium was added, were collected and analyzed by Enzyme-Linked Immuno Sorbent Assay (ELISA) to determine their levels of ALB secretion. A Human Albumin ELISA Quantitation Set was purchased from Bethyl Laboratories. ELISA was performed according to the manufacturer's instructions. The amount of ALB secretion was calculated according to each standard followed by normalization to the protein content per well.

Luminescent assay for CYP3A4 activity

To measure the CYP3A4 activity in the cells, we performed lytic assays by using P450-Glo CYP3A4 Assay Kits (Promega). Luciferin-IPA was used for CYP3A4 substrates. We measured the fluorescence activity with a luminometer (Lumat LB 9507, Berthold) according to the manufacturer's instructions. The CYP3A4 activity was normalized with the protein content per well.

RNA-sequencing and FASTQ file processing

According to the manufacturer's instructions, library preparation was performed using a TruSeq stranded mRNA sample prep kit (Illumina, San Diego, CA). Libraries were converted to libraries for DNBSEQ using MGIEasy Universal Library Conversion Kit (App-A). Sequencing was performed on a DNBSEQ-G400RS platform (MGI, Shenzhen, China) in 2 x100 bp paired-end mode.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical significance was evaluated using an unpaired two-tailed Student's t test or one-way analysis of ANOVA, followed by Dunnett's or Tukey's post hoc tests. Statistical analyses were performed using GraphPad Prism. Details are described in the figure legends.