

Human chemically-derived hepatic progenitors (hCdHs) as a source of liver organoid generation: Application in regenerative medicine, disease modeling, and toxicology testing

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ABSTRACT

Background & aims: Several types of human stem cells from embryonic (ESCs) and induced pluripotent (iPSCs) to adult tissue-specific stem cells are commonly used to generate 3D liver organoids for modeling tissue physiology and disease. We have recently established a protocol for direct conversion of primary human hepatocytes (hPHs) from healthy donor livers into bipotent progenitor cells (hCdHs). Here we extended this culture system to generate hCdH-derived liver organoids for diverse biomedical applications.

Methods: To obtain hCdHs, hPHs were cultured in reprogramming medium containing A83-01 and CHIR99021 for 7 days. Liver organoids were established from hCdHs (hCdHOs) and human liver cells (hLOs) using the same donor livers for direct comparison, as well as from hiPSCs. Organoid properties were analyzed by standard *in vitro* assays. Molecular changes were determined by RT-qPCR and RNA-seq. Clinical relevance was evaluated by transplantation into FRG mice, modeling of alcohol-related liver disease (ARLD), and *in vitro* drug-toxicity tests. **Results:** hCdHs were clonally expanded as organoid cultures with low variability between starting hCdH lines. Similar to the hLOs, hCdHOs stably maintained stem cell phenotype based on accepted criteria. However, hCdHOs had an advantage over hLOs in terms of EpCAM expression, efficiency of organoid generation and capacity for directed hepatic differentiation as judged by molecular profiling, albumin secretion, glycogen accumulation, and CYP450 activities. Accordingly, FRG mice transplanted with hCdHOs survived longer than mice injected with hLOs. When exposed to ethanol, hCdHOs developed stronger ARLD phenotype than hLOs as evidenced by transcriptional profiling, lipid accumulation and mitochondrial dysfunction. In drug-induced injury assays *in vitro*, hCdHOs showed a similar or higher sensitivity response than hPHs.

Conclusion: hCdHOs provide a novel patient-specific stem cell-based platform for regenerative medicine, toxicology testing and modeling liver diseases.

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Lay summary

The growing demand for *in vitro* models that recapitulate the pathophysiological processes of human liver diseases has led us to generate organoids (hCdHOs) from the chemically derived hepatic progenitors (hCdHs). This approach provided a robust practical novel tool for the efficient long-term generation of the patient-derived liver organoids with improved properties for addressing diverse research objectives from modeling liver disorders to drug testing and cell therapy applications.

1. Introduction

During the past decade, there has been remarkable progress in the field of organoid culture technology. Organoid systems enable to recapitulate physiological, biochemical, and biomechanical environment of *in vivo* tissue and thus represent more relevant models to study *in vivo* organ development and human pathologies as compared to two-dimensional (2D) cell cultures [1–4]. Due to the unlimited self-renewal and differentiation potential, organoids can potentially serve as an alternative source of functionally mature cells for regenerative cell therapy [5,6]. The most recent application for liver organoids is the creation of biobanks as screening platforms for toxicology testing and evaluating drug efficiency against various liver diseases in pre-clinical studies [7–9].

Liver organoids (LOs) have been established from various pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) in addition to tissue-resident stem/progenitor cells isolated from embryonic or adult tissues [2,8,10,11]. Previous studies have demonstrated that LOs can develop from a single EpCAM⁺ ductular cell isolated from an adult human liver [12]. Similarly, in animal liver damage models, biliary epithelial cells have been shown to acquire bi-potential characteristics and function as a source of hepatic generation [13].

Despite the promise of organoid technology for medical applications, there remain several demanding issues, such as modeling of the micro-environment and improved maturation, standardization of outcomes and quality control [14,15], as well as exploration of novel cell sources which can afford high efficiency and large scale production, while reducing time and high cost of LO generation.

Recently, we have generated chemically-derived hepatic progenitor cells (hCdHs) that satisfy all criteria of the progenitor/stem cell model [16]. Liver specimens from healthy donors were dissociated and directly reprogrammed into bipotent progenitor cells by a combined treatment with two small molecules A83-01 and CHIR99021 in the presence of hepatocyte growth factor (HGF) [16–18]. These cells preserved chromosomal stability during long-term expansion in culture and could be generated from both fresh and frozen human hepatocytes. Starting from the early passages, hCdHs were readily converted into functional hepatocytes and cholangiocytes both *in vitro* and *in vivo* as evidenced by gene expression profiling and biochemical and functional assessment. In several models of therapeutic liver repopulation including Alb-TRECK/SCID mice [19], NGS (NOD.Cg-Prkdc^{skid}Il2^{rgtm}tlm^{Wjl}/SzJ [20] and FRGN [16], undifferentiated hCdHs successfully integrated into diseased parenchyma and acquired properties characteristic for mature hepatocytes and cholangiocytes implying great hepatocyte/cholangiocyte-forming potential of hCdHs in adequate hepatic microenvironment [16]. In a proof-of-principle study, hCdHs microtopographically differentiated toward the biliary epithelial lineage and successfully populated the implantable bioengineered bile duct

constructs used for reconstructive surgery in a rabbit model of acute common bile duct injury [17] providing an efficient stem cell-based tool for a wide range of *in vivo* applications.

In this study, we aimed to extend these results to generate and validate the hCdH-derived liver organoids as a promising novel tool for human liver disease modeling, regenerative medicine, and toxicological testing.

2. Materials and methods

2.1. Generation of human chemically derived hepatic progenitors (hCdHs)

The study was conducted according to the methods allowed by the Institutional Review Board of Hanyang University, Seoul, Korea (HYUH2017-11-012-014). Human liver tissues were obtained from twenty-one donors operated on in Hanyang University Medical Center with informed patient consent. Liver cells were isolated by a two-step collagenase perfusion method and purified by Percoll (Sigma-Aldrich). Crude liver cells were seeded on collagen-coated plate at 5000 cells/cm² in Williams medium (Gibco). After overnight incubation, the Williams medium was changed to the reprogramming medium based on DMEM/F-12 medium (Gibco) supplemented with 10 % FBS (Gibco), 1 % insulin-transferrin-selenium (Gibco), 10 mM nicotinamide (Sigma-Aldrich), 0.1 μM dexamethasone (Sigma-Aldrich), 50 μM β-mercaptoethanol (Sigma-Aldrich), 20 ng/mL epidermal growth factor (EGF, Peprotech), 20 ng/mL hepatocyte growth factor (HGF, Peprotech), 4 μM A83-01 (Gibco) and 3 μM CHIR99021 (STEMCELL Technologies) known as HAC medium [16]. The reprogramming media was changed every two days. hCdHs were passaged with 1X TrypLE Express Enzyme (Gibco) at a ratio of 1:3–1:5 every 4–5 days.

2.2. Generation and culture of liver organoids

Liver organoids were generated from liver cells (hLOs, with ductal cells origin) and hCdHs (hCdHOs). Isolated liver cells and trypsinized hCdHs at passages 1–3 were washed with Advanced DMEM/F-12 media (Gibco) and centrifuged at 300 g for 5 min. After removing the supernatant, the cell pellet was resolved with Matrigel (Gibco) and 50 μL of the cell-Matrigel mixture containing 10,000–20,000 cells were seeded per well in 24-well plate. After incubating at 37°C for 5–10 min, organoid expansion medium (EM) based on Advanced DMEM/F-12 media (Gibco) containing 1 % N2, 1 % B27, 1.25 mM N-Acetylcysteine, 10 nM gastrin, 50 ng/mL EGF, 10 % RSP01 conditioned media, 100 ng/mL FGF10, 25 ng/mL HGF, 10 mM Nicotinamide, 5 μM A83.01, and 10 μM FSK, was supplemented with 25 ng/mL Noggin, 100 ng/mL Wnt3a and 10 μM Y-27632 [12]. After 3 days, Noggin, Wnt3a and Y-27632 were removed from the medium. The organoid culture media was changed every 2–3 days, and cells were passaged at a ratio of 1:4–1:8 every 7–9 days.

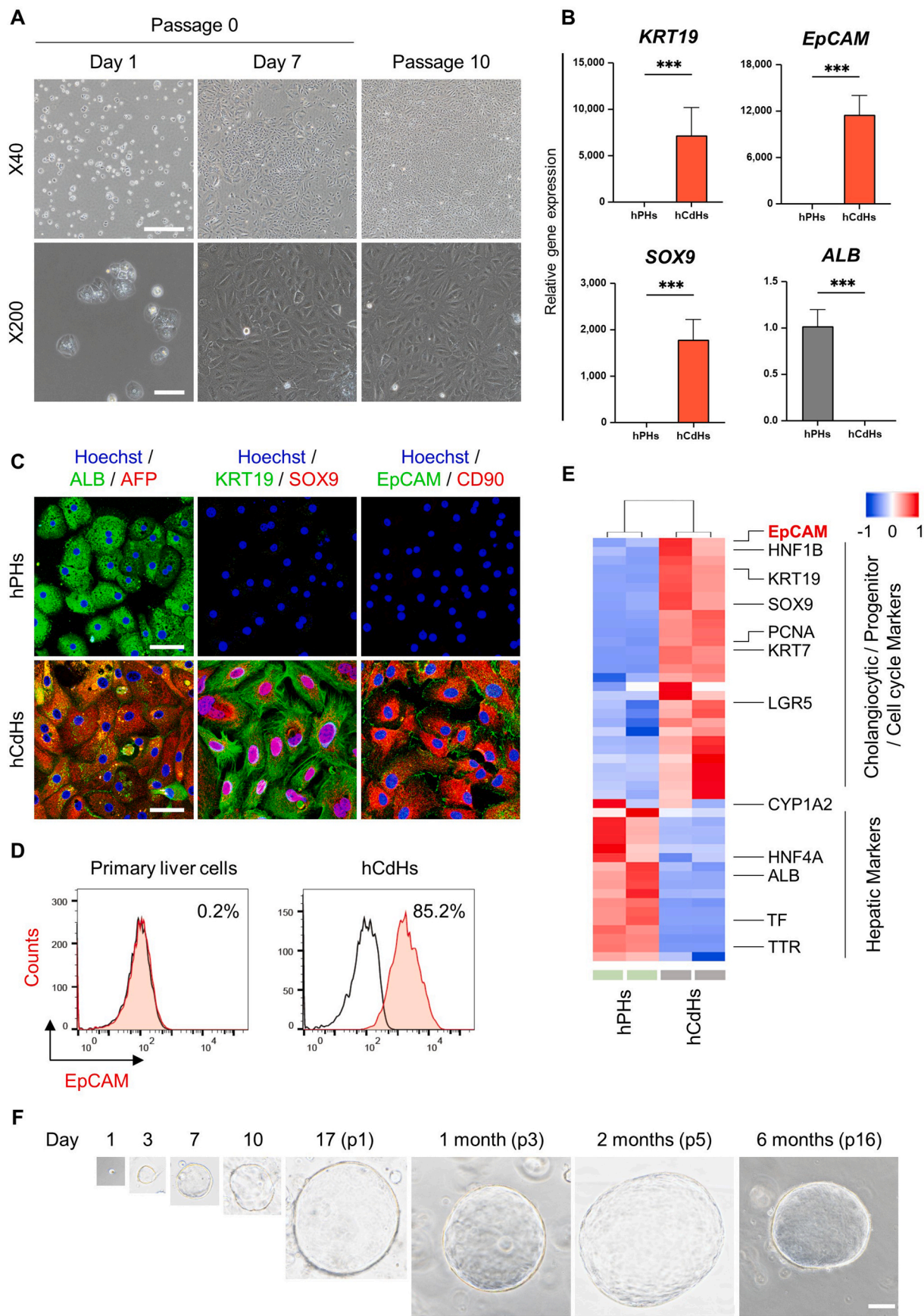
2.3. Hepatic differentiation

To induce hepatic differentiation in 2D system, hCdHs were seeded on collagen-coated plate at 1000 cells/cm². After overnight incubation, the culture media was changed to hepatic induction media consisting of HAC medium with 20 ng/mL Oncostatin M (R&D system) and 10 μM dexamethasone (Sigma). The hepatic induction media was changed every two days. After 6 days, cells were covered with 12.5 % Matrigel solution for 2 days.

Table 1
Clinical characteristics of human liver donors.

Sample	Donor number	Ethnicity	Sex	Age (years)	Diagnosis	Operation	Total bilirubin (mg/dL)	ALP (U/L)	AST (U/L)	ALT (U/L)	Albumin (g/dL)	Organoid generation	Experiments
1	N10	Asian	Female	28	Liver metastasis from rectal cancer	Wedge resection (Seg IV)	0.4	46	21	16	3.9	Yes	Fig. 1A, B, D Fig. S1C
2	N13	Asian	Female	66	Liver metastasis from ovarian cancer	Excisional biopsy of metastatic mass	1	48	29	34	3.6	Yes	Fig. 1B Fig. S1C/Fig. S2
3	N24	Asian	Male	59	Left intrahepatic bile duct stricture	Left hemihepatectomy	0.32	79	21	10	4	Yes	Fig. 1B, C, F Fig. 2C, E/Fig. 3A and B/Fig. 4 Fig. S1C/Fig. S2
4	N40	Asian	Female	49	Liver metastasis from colon cancer	Wedge resection (segment II, IVA, IVB)	0.46	424	37	45	4	Yes	Fig. 2C Fig. S1C/Fig. S2
5	N27	Asian	Male	70	Cholangiocarcinoma	Right posterior sectionectomy	0.45	99	27	14	4.4	Yes	Fig. 2C Fig. S1C
6	N46	Asian	Female	75	Intra hepatic duct stone (IHD)	Left lateral sectionectomy and caudate lobectomy and IHD exploration and cholecystectomy	0.39	139	32	9	3.7	Yes	Fig. 2B, C
7	N51	Asian	Male	57	Hepatocellular carcinoma (segment VIII)	Wedge resection of liver, segment VIII	0.88	63	30	50	4	Yes	Fig. 2C
8	N29	Asian	Male	61	Liver metastasis from colon cancer	Right hemihepatectomy	0.8	70	19	11	3.9	Yes	Fig. 2C Fig. 3B
9	N52	Asian	Male	58	Intra hepatic duct stone	Right hemihepatectomy	0.69	410	34	23	3.2	Yes	Fig. 2C
10	N53	Asian	Female	52	Liver metastasis from colon cancer	Right hemihepatectomy and intraoperative RFA(segment II)	1.21	94	19	10	3.7	Yes	Fig. 2C, E Fig. 3C
11	N5	Asian	Female	31	Gallbladder polyp	Laparoscopic cholecystectomy and liver biopsy	0.62	50	19	10	4.6	Yes	Fig. 1E/Fig. 2C, E Fig. 3B/Fig. 4
12	N67	Asian	Female	29	Liver metastasis from colon cancer	Right hemihepatectomy & Hepaticojejunostomy	0.57	387	30	32	3.8	Yes	Fig. 1E Fig. 2C, F, G/ Fig. 3E, F, G Fig. 6/Fig. 7 Fig. S5
13	N6	Asian	Female	58	Hemangioma of liver	Left lateral sectionectomy and cholecystectomy	0.52	84	20	18	4.6	Yes	Fig. 2C,F, G/Fig. 3F, G Fig. 4 Fig. 6A, B, D, E, F, G, H Fig. 7 Fig. S5
14	N153	Asian	Female	54	Intra hepatic duct stone	Left Hemihepatectomy	0.48	81	30	37	3.5	No	
15	N159	Asian	Male	66	Gallbladder cancer	Radical cholecystectomy	0.44	60	39	45	3.4	Yes	Fig. 5/Fig. S6
16	N165	Asian	Male	35	Hemangioma of liver	Laparoscopic Right hemihepatectomy	0.3	65	15	11	4.8	Yes	Fig. 5/Figs. S1A and B/Fig. S3/Fig. S4/ Fig. S6
17	N168	Asian	Female	69	Intra hepatic duct stone	Left lateral sectionectomy of liver (S2,3), Open cholecystectomy	0.63	66	22	18	4.3	Yes	Fig. 2D/Fig. 5/ Fig. S3/Fig. S4/ Fig. S6
18	N174	Asian	Male	71	Intra hepatic duct stone	Left hemihepatectomy	0.54	114	25	13	4.4	Yes	Fig. 2D/Fig. S1A,B
19	N176	Asian	Female	67	Hepatic cyst	Laparoscopic Right hemihepatectomy	0.5	83	17	9	4.4	No	
20	N177	Asian	Female	29	Hepatocellular carcinoma	Laparoscopic S6 wedge resection	0.9	50	22	16	4.4	Yes	Fig. S3/Fig. S4/ Fig. 5/Fig. S6
21	N156	Asian	Female	51	Hepatocellular carcinoma/ Intrahepatic cholangiocellular carcinoma (segment VI)	Right hemihepatectomy	0.38	64	22	15	4.4	Yes	Fig. 2D/Fig. 5/ Fig. S6/Fig. S7

ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.



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Fig. 1. Establishment of organoid cultures from hCdHs. (A) Human primary hepatocytes (hPHs) were isolated from donors operated on in Hanyang University Medical Center and reprogrammed into chemically derived hepatic progenitors (hCdHs) in medium containing A83-01, CHIR99021, and HGF for 7 days. Cells were maintained in reprogramming medium and split at a ratio of 1:3–1:5 every 5 days for 10 passages. Bright field microscopy images. Original magnifications shown on the left. Scale bars, 500 μ m (top) and 100 μ m (bottom). (B) Induction of hepatic progenitor marker genes analyzed by RT-qPCR at passage 3. GAPDH was used as an internal control. Data are mean $\pm$ SD. Experiments were performed in triplicate in three independent donor-derived cultures and analyzed by unpaired *t*-test (two-tailed), ****p* < 0.001. (C) Immunofluorescence images of staining for hepatic progenitor marker proteins. Nuclei were counterstained with Hoechst 33342. Scale bars, 50 μ m. (D) Percentage of EpCAM⁺ cells determined by flow cytometry in freshly isolated human liver cells and hCdHs at passage 3. (E) Heatmap of differentially expressed genes determined by mRNA sequencing in human primary hepatocytes (hPHs), and hCdHs (passage 3) derived from the same donor liver tissue. Representative genes are shown on the right. Red and blue represent higher and lower gene expression levels, respectively. Two different donors were analyzed. (F) Brightfield microscopy images of clonal organoid cultures were obtained by seeding one cell per well in a 96-well plate and cultured in organoid expansion medium for over 6 months. Scale bars, 100 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

For organoid hepatic differentiation, hLOs and hCdHOs were cultured in EM medium supplemented with 25 ng/mL BMP7 for 5 days. After 5 days, the media was changed to hepatic differentiation media (DM): Advanced DMEM/F-12 media containing B27 (1:50) supplement, N2 (1:100) supplement, 50 ng/mL EGF, 25 ng/mL HGF, 0.5 μ M A83-01, 10 μ M DAPT, 3 μ M dexamethasone, 25 ng/mL BMP7 and 100 ng/mL FGF19. DM was changed every 2–3 days for 15 days as Broutier reported [21].

2.4. Functional analyses

To detect glycogen accumulation, cells were stained with PAS reagent using a PAS staining kit as recommended by the supplier. Human albumin and α -1-Antitrypsin (A1AT) were measured by human albumin enzyme-linked immunosorbent assay (ELISA) kit (Abcam) and Human A1AT ELISA Kit (Bethyl Laboratories) following the manufacturer recommendations. To measure CYP1A2, CYP3A4 and, CYP2C9 activity, cells were treated with 100 μ M omeprazole and 25 μ M rifampicin, respectively, for 72 h. Cytochrome P450 activities were measured by P450-Glo Assay Kit (Promega, USA) as recommended by the supplier.

2.5. RT-qPCR

Total RNAs were isolated using Trizol Reagent. One μ g of RNA was reverse transcribed using a Transcriptor First Strand cDNA Synthesis Kit (Roche). RT-qPCR was performed using a CFX Connect Real-Time PCR Detection system (Bio-Rad). The primer sequences are listed in Table S1.

2.6. Immunostaining

Cells were fixed in 4 % paraformaldehyde overnight at 4 °C, washed in PBS and permeabilized. To inhibit unspecific staining, cells were incubated in PBS containing 1 % bovine serum albumin, 22.52 ng/mL glycine, and 0.1 % Tween 20 for 1 h at room temperature. Primary antibodies were incubated overnight at 4 °C. Using standard techniques, the organoids were fixed in 10 % formalin and stained with antibodies. The primary antibodies were detected using Alexa Fluor 488-, 546- or 633- conjugated secondary antibodies. Nuclei were counterstained with Hoechst 33342. Primary antibodies used in this study are listed in Table S2. Stained samples were visualized under a TCS SP5 confocal microscope (Leica).

2.7. Flow cytometry analysis

Isolated human adult liver cells and hCdHs were fixed with 4 % paraformaldehyde overnight at 4 °C. Cells were incubated with EpCAM antibody for 1 h at room temperature. EpCAM-positive cell populations were analyzed by FACS Canto II (BD Bioscience).

2.8. Alcoholic disease model

For alcoholic liver disease modeling, hLOs and hCdHOs were grown in hepatic differentiation medium supplemented with 100 mM EtOH for 15 days. To characterize the induction of ARLD phenotype, liver organoids were stained with 1 μ M Nile Red fluorescence probe C to detect lipid content or with 15 μ M JC-1 probe for 30 min at 37 °C to measure mitochondrial membrane potential. After incubation, organoids were washed three times with PBS. Nile Red and JC-1 immunofluorescence was visualized under a TCS SP5 confocal microscope and analyzed by Image J.

2.9. Organoid transplantation

Fah^(-/-)/*Rag2*^(-/-)/*Il2rg*^(-/-) (FRG) mice were a kind gift by Dr. Markus Grompe, (Oregon Health Sciences University, Portland, OR, USA). Six to 8 weeks-old female mice were housed and cared for under specific pathogen-free conditions in accordance with the Principles of Laboratory Animal Care and the Guide for the Use of HYU Industry-University Cooperation Foundation regulations (2020-0025A). Three days before transplantation, NTBC (2-(2-nitro-4-trifluoro-methylbenzyl)-1,3-cyclohexanedione) was withdrawn from the drinking water to induce acute liver damage. Liver organoids were washed with cold PBS (1 mL). Colonies of liver organoids and hCdHOs were resuspended as single cells, and 100,000 cells in 150 μ L of PBS were injected into spleen as described in Ref. [22]. Mice were maintained cycle NTBC-containing water.

2.10. Hepatotoxicity screening

hPHs, hCdHs and hCdHO_DM were exposed to different drugs defined as cytotoxic, withdrawn, and non-toxic (*n* = 5 for each group). For 2D cultures, 10,000 hPHs and hCdHs were seeded per well in 96-well plates in Williams and HAC medium supplemented with HGF, A83-01 and CHIR99021 medium, respectively. Three to four independent experiments for each cell type (hPHs, and hCdHs) were carried out in triplicate. hCdHs were used between passages 1–3. Drugs were administered at 1, 5, 10 and 20 μ M for 24–72 h at 37 °C. DMSO was used as control. In 3D cultures, the Matrigel was removed with Cell Recovery Solution (Corning 354253) from hCdHO_DM before treating them with the same drug concentrations diluted in the DM medium as described for 2D cultured cells. Cell viability was assayed using Cell counting kit 8 (DOJINDO Laboratories) for 1 h. hCdHO_DM were disaggregated into a single cell suspension before the test. Absorbance was measured at 450 nm. Half maximal inhibitory concentrations (IC₅₀) were calculated at 24 h of treatment using a sigmoidal log vs response and variable slope equation [23]. The data were analyzed by one-way ANOVA followed by Holm-Sidak test [24].

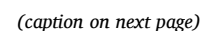


Fig. 2. Characterization of hCdH-derived organoids. (A) Schematic diagram of protocol for hLOs and hCdHOs generation from the same donor liver tissue ($n = 18$). All organoids were cultured in the expansion medium before analyses (passage 3–10). (B) Representative brightfield microscopy images. Original magnification on the left. Scale bars, 500 μm (top), 100 μm (bottom). (C) Efficiency of organoid formation. Data are mean $\pm$ SD for twelve independent hLOs and hCdHOs organoid cultures and analyzed by paired t -test (two-tailed), $***p < 0.001$. (D) Double Immunofluorescence staining of hepatic (ALB) and cholangiocyte/progenitor (AFP, KRT7, EpCAM, KRT19 and SOX9) marker proteins. Nuclei were counterstained with Hoechst 33342. Scale bars, 100 μm . (E) Gene expression levels of hepatic (ALB) and cholangiocyte/progenitor (EpCAM, KRT19, SOX9, CFTR, AE2, AQP1, and AFP) markers were measured by RT-qPCR. GAPDH was used as internal control. Data are mean $\pm$ SD. Experiments were performed in triplicate in three independent donor-derived cultures and analyzed by one-way ANOVA followed by Tukey test, $***p < 0.001$. (F) Hierarchical cluster analysis of global gene expression profiles in hCdHs, hCdHOs, hLOs, hPHs and fetal liver. Red and blue represent higher and lower gene expression levels, respectively. We conducted Correlation (uncentered) using complete linkage method. (G) Gene set enrichment plot showing strong enrichment of indicated gene signature in transcriptome of hCdHOs. NES, normalized enrichment score. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.11. Library preparation and transcriptome sequencing

A library was prepared with 1 μg of total RNA for each sample using an Illumina TruSeq Stranded mRNA Sample Prep Kit. The initial step of library preparation involved purifying poly-A-containing mRNA molecules with poly-T-attached magnetic beads. Thereafter, the purified mRNA was fragmented into small pieces using divalent cations under elevated temperature. The cleaved mRNA fragments were copied into the first strand cDNA using SuperScript II reverse transcriptase and random primers, and the reverse complement strand of cDNA was synthesized using DNA polymerase I, RNase H, and dUTP. The cDNA fragments then underwent an end repair process, which included addition of a single 'A' base and adapter ligation. After these steps, the final cDNA libraries were created, purified, and enriched with PCR. The libraries were quantified using KAPA Library Quantification kits for Illumina Sequencing platforms according to the qPCR Quantification Protocol Guide (Kapa Biosystems, USA) and qualified by the TapeStation D1000 ScreenTape (Agilent Technologies). Indexed libraries were then paired-end sequenced with Illumina HiSeq 2500 (Illumina, Inc.) at Macrogen, Inc. (Korea).

2.12. Bioinformatics analysis

The standard Illumina pipeline and real-time analysis tools were employed for processing of raw images, base calling, and generation of FASTQ data from the paired-end RNA-sequencing data. The 100 bp $\times$ 2 read sequences were preprocessed using Sickle (V1.33, <https://github.com/najoshi/sickle>) to trim sequences with poor quality, and then aligned to the hg19 human reference genome (UCSC data from Illumina iGenomes, https://support.illumina.com/sequencing/sequencing_software/igenome.html) using RSEM (v1.2.31) with STAR (v2.5.2b) alignment software. The expression level of transcripts was measured as the score of transcripts per kilobases million for fair comparison of the proportion of reads that mapped to a gene in each sample. Clustering analysis was performed using Cluster3.0 (<http://eisenlab.org/>) and heatmap (v3.3.2, <https://www.r-project.org>).

Gene set enrichment analysis (GSEA) scores were generated for gene sets in the C1, C5 and C8. Normalized enrichment scores and p -values were calculated using GSEA software (<https://www.gsea-msigdb.org/gsea/index.jsp>).

Correlation coefficient values and principal component analysis (PCA) were evaluated by using R (4.0.2) with ggcorrplot (0.1.3) and factoextra (1.0.7), respectively [25,26].

2.13. Statistical analysis

Quantitative data are presented as means $\pm$ standard deviations (SD). Statistical significance was evaluated by one-way ANOVA

followed by multiple comparisons using Tukey test with significances set at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Unless otherwise specified in the figure legends.

3. Results

3.1. hCdHs as a cell source for liver organoids

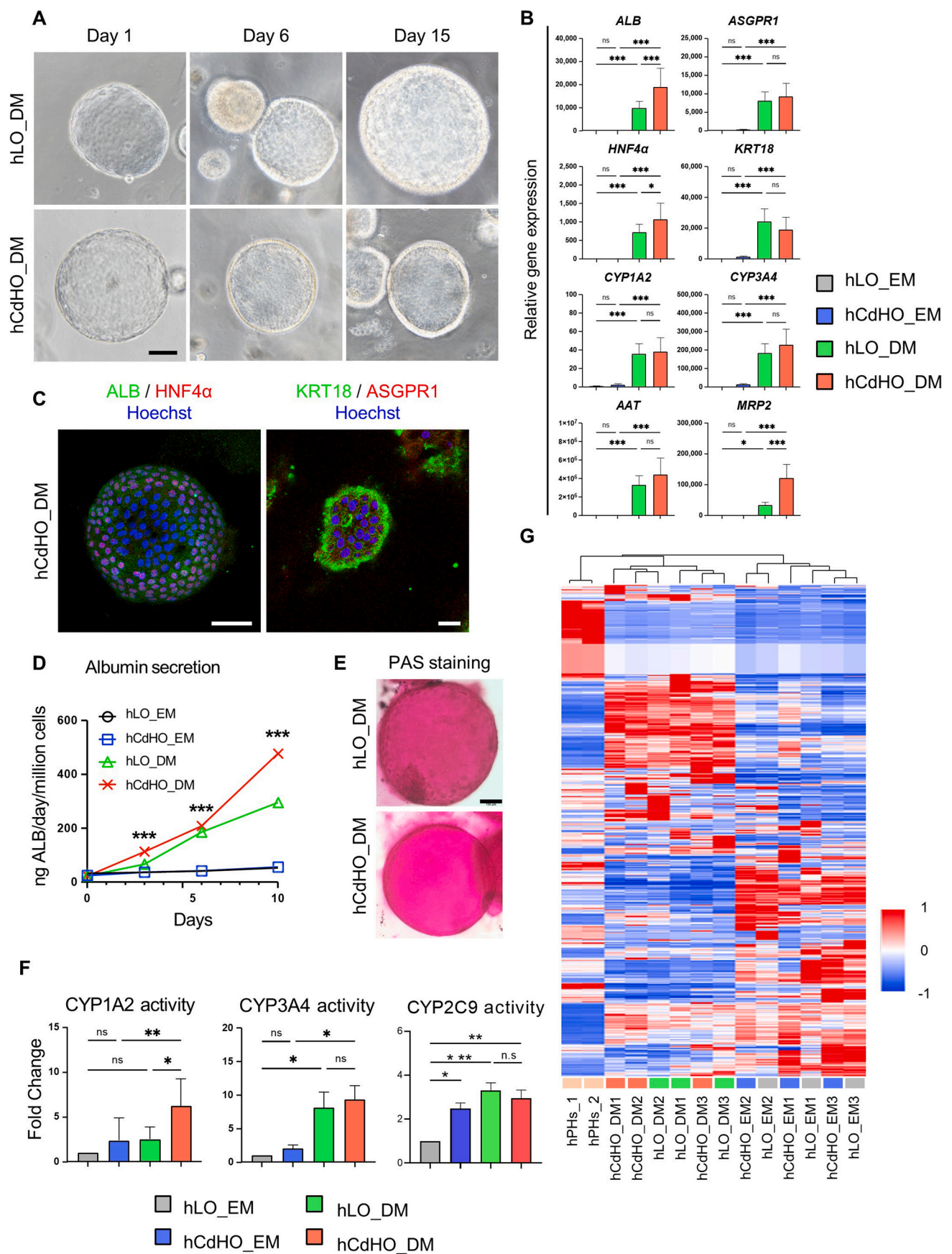
To generate hCdHs, we utilized our recently described methodology using direct small molecule-mediated reprogramming of human primary hepatocytes (hPHs) [16]. In total, hPHs were isolated from twenty-one donor liver samples (Table 1) and cultured in the reprogramming medium containing two small molecules, A83-01 and CHIR99021, in combination with EGF and HGF (for details, see Material and Methods). The treatment triggered a robust expansion of small epithelial cells which formed a dense monolayer by the end of the first week (Fig. 1A) and showed a steady upregulation of key hepatic progenitor marker genes and proteins including KRT19, EpCAM and SOX9 while expression of albumin was significantly reduced (Fig. 1B and C). When reprogramming medium was switched to the lineage-specific differentiation medium (DM), hCdHs acquired phenotypic features of hepatocytes and cholangiocytes (Fig. S1A, B). Flow cytometric analysis revealed that 85.2 % of hCdHs population expressed EpCAM versus 0.2 % of human primary liver cells demonstrating a high efficiency of reprogramming (Fig. 1D).

Hierarchical-clustering analysis comparing the global gene expression profiles of hCdHs and hPHs confirmed a strong induction of hepatic progenitor marker genes in hCdHs (EpCAM, LGR5, SOX9, KRT7 and KRT19) and absent expression of mature hepatocyte markers (Fig. 1E).

Consistent with the high potency of EpCAM⁺ cells for liver organoid formation [12], hCdHs embedded in Matrigel and cultured in medium designed for liver organoids [21] rapidly formed typical cyst-like structures by day 3 which could be maintained in culture for more than six months by passaging at 1:4–1:8 ratio every 7–9 days (Fig. 1F). To determine the hCdHOs origin cells, the liver cells were subjected to EpCAM⁺ selection, and then EpCAM⁺ cells were embedded into Matrigel. As expected, EpCAM⁺ cells gave rise to cystic organoids, providing evidence that hCdHOs and hLOs organoids share a common cell origin from EpCAM-positive ductal liver cells (Figs. S1C and D). Altogether, we successfully established long-term (over 6-months) hCdHOs cultures from nineteen hCdH-derived progenitor cell lines (Table 1, Fig. S1E).

3.2. Characterization of hCdH-derived liver organoids

To define the advantages of growing organoids from the chemically derived hepatic progenitor cells, we subsequently generated and directly compared hCdHOs and hLOs cultures from the same human donor liver tissues ($n = 19$) (Fig. 2A). For each organoid culture we used 10,000 to



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Fig. 3. Hepatic differentiation potential of hCdHOs *in vitro*. (A) Brightfield microscopy images of hLOs and hCdHOs cultured in differentiation medium (DM) for 15 days. Scale bars, 100 μ m (B) Induction of hepatocyte-specific marker genes was evaluated by RT-qPCR. Data are mean $\pm$ SD. At least 3 independent experiments with triplicate measurements were performed and analyzed by one-way ANOVA followed by Tukey's multiple comparisons test, *, $p < 0.05$ and ***, $p < 0.001$. (C) Double immunofluorescence staining for mature hepatocyte-specific proteins albumin (ALB) and HNF4 α , and KRT18 and ASGPR1. Nuclei were counterstained with Hoechst 33342. Scale bars, 75 μ m and 100 μ m. (D) ALB secretion was measured in supernatant every three days for ten days. Data are mean $\pm$ SD of three independent hLOs and hCdHOs cultures. *** $p < 0.001$ compared with the equivalent data point of other cultures analyzed by *t*-test. (E) Glycogen accumulation was detected by Periodic acid-Schiff staining (PAS). Scale bars, 100 μ m. (F) CYP1A2, CYP3A4 and, CYP2C9 activities after 72 h of omeprazole and rifampin treatment. Data are mean $\pm$ SD from triplicate experiments in three independent donor-derived cultures analyzed by one-way ANOVA followed by Tukey test, *, $p < 0.05$ and **, $p < 0.01$. (G) Heatmap of differentially expressed genes determined by mRNA sequencing in human primary hepatocytes (hPHs), hCdHOs and hLOs in EM and DM medium used in triplicates. Red and blue represent higher and lower gene expression levels, respectively. Organoid cultures from two independent donors were analyzed. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

20,000 liver-resident cells or hCdHs. When organoid size reached $\geq 100 \mu$ m, they were collected and analyzed by various cell biology methods including immunohistochemistry, RT-qPCR, microarray, and functional analyses.

When grown in Matrigel, both hCdHs and liver resident cells formed cystic structures that were morphologically undistinguishable (Fig. 2B). However, hCdHs displayed 15-folds greater efficiency of organoid generation as compared to liver resident cells ($12.9 \pm 4.21\%$ vs $0.85 \pm 0.19\%$, respectively, $p < 0.001$) (Fig. 2C), the difference that could be attributed to a high frequency of EpCAM expression in the population of hCdHs (Fig. 1D). The superior efficacy of hCdHOs generation was paralleled by a higher proliferative potential as judged by the analysis of doubling time in hCdHOs and hLOs (30.8 ± 1.8 vs 39.3 ± 0.8 , respectively, $p < 0.01$) (Fig. S2). Furthermore, hCdHOs expressed higher levels of classical hepatic progenitor markers than hLOs, such as *EpCAM*, *KRT19* and *SOX9*, and the cholangiocyte marker *CFTR*, and displayed similar levels of cholangiocyte markers, *AE2* and *AQPR1* (Fig. 2D and E).

The following comparison of global gene expression using RNAseq analysis (Fig. 2F) showed that hCdHOs and hLOs shared a core transcriptional profile while hCdHs remained distinct from both hCdHOs and hLOs and clustered more closely with fetal liver (Fig. 2F). Despite global transcriptomic similarities between hCdHOs and hLOs, the gene set enrichment analysis (GSEA) [26,27] revealed a significant positive enrichment of the gene set associated with EpCAM expression in hCdHOs (Fig. 2G).

To extend the analysis of hCdHOs, we also generated organoids from human iPSCs (hiPSOs) using a technique described by Wang and collaborators [2]. The hiPSOs showed comparable doubling time (Fig. S2C) as well as efficiency of organoid generation (Fig. S3B) and the same morphological characteristics (Fig. S3C) to hCdHOs. At the gene expression level, hiPSOs expressed higher (*AFP*, *EpCAM*), similar (*KRT19*, *CFTR*) or reduced (*SOX9*, *AE2*, and *AQPR1*) levels of the selected hepatic progenitor and cholangiocyte markers than hCdHOs (Figs. S3A and C), but different morphological and time generation features being hCdHOs a better tool for *in vitro* analysis. On the other hand, hCdHO have advantage over hLOs in terms of efficiency of organoid generation and EpCAM expression. Together, these data demonstrate that hCdHOs and hiPSOs display comparable generation efficiency (95 %) and biochemical characteristics.

3.3. Hepatic differentiation potential of hCdHOs *in vitro*

To further characterize our hCdHOs, we compared their hepatic differentiation potency with that of hLOs using the protocol developed by Huch and colleagues [12]. All organoids were collected at passages 3–10 and cultured in the expansion media (EM) containing BMP7. After 5 days, the media was changed to the hepatic differentiation media (DM) consisting of advanced DMEM/F-12 supplemented with B27, N2, EGF,

HGF, A83-01, DAPT, dexamethasone, BMP7 and FGF19 for 15 days. At the end of treatment, hCdHOs grown in DM (hCdHO_DM) acquired a more compact morphology and in general became smaller than hLO_DM (325.7 ± 31.07 vs $474.6 \pm 12.07 \mu$ m, respectively, $p < 0.01$) (Fig. 3A). Double immunofluorescence and RT-qPCR analyses confirmed a strong induction of key hepatocytic markers in both organoid cultures fated to hepatocyte differentiation, such as *ALB*, *HNF4 α* , and *ASGPR1*. Another upregulated marker was *KRT18* gene encoding the main cytoskeletal protein expressed in liver, as well as genes related to drug metabolism (*CYP1A2*, *CYP3A4*), acute phase response (*AAT*), and bile acid transport (*MRP2*) (Fig. 3B and C). In comparison, hiPSOs grown in the differentiation medium (hiPSO_DM) displayed a similar induction of several hepatocyte-specific genes (*ALB*, *ASGPR1*, *HNF4 α* , and *CYP3A4*). However, unlike the hCdHOs, hiPSOs expressed reduced levels of *KRT18* encoding one of the key structural proteins differentiating cells of epithelial origin, as well as *MRP2* encoding a drug-resistance protein involved in biliary transport, while the levels of *AAT*, a member of the serine proteinase inhibitor family associated with stress response and biliary transport, were considerably increased (Figs. S4A and B).

Hepatic differentiation of hCdHOs was accompanied by a progressive increase in hepatocyte-specific functions such as albumin secretion (Fig. 3D), glycogen storage (Fig. 3E) and cytochrome p450 metabolic activities (Fig. 3F, Fig. S4C). Notably, the levels of secreted albumin in hCdHO_DM cultures were about 1.6-fold higher than that produced by hLO_DM. Similarly, hCdHO_DM displayed a higher activity of P450 enzymes CYP1A2, CYP3A4 and, CYP2C9 playing a major role in the drug and lipid metabolism *in vivo*. Both hCdHOs and hLOs cultures showed a strong periodic acid-Schiff (PAS) staining indicative of glycogen accumulation. To better assess the differentiation status, we performed mRNA sequencing of hCdHOs and hLOs grown in EM and DM medium in comparison with hPHs used as a reference data set (Fig. 3G). Unsupervised hierarchical cluster analysis classified all samples into 3 major classes (Fig. 3G). hCdHOs and hLOs cultured under the same conditions showed overlapping transcriptional profiles and clustered together. As expected, the transcriptome of organoids grown in DM showed a closer proximity to a reference data set than did hCdHO_EM. The diverse biological processes related to hepatocyte differentiation, cytochrome p450, glucose, cholesterol, and xenobiotic metabolism were upregulated after differentiation (Fig. S5).

3.4. Therapeutic potential of hCdHOs *in vivo*

To test the capacity of hCdH-derived organoids to engraft as functional hepatocytes *in vivo*, we used Fah^{-/-}–Rag2^{-/-}–Il2rg^{-/-}–(FRG) mutant mice as a model of therapeutical repopulation. hLOs and hCdHOs were established from 3 donor liver tissues, and transplanted intrasplenically three days after induction of acute liver damage by the NTBC withdrawal (Fig. 4A). The comparison of the survival rate showed

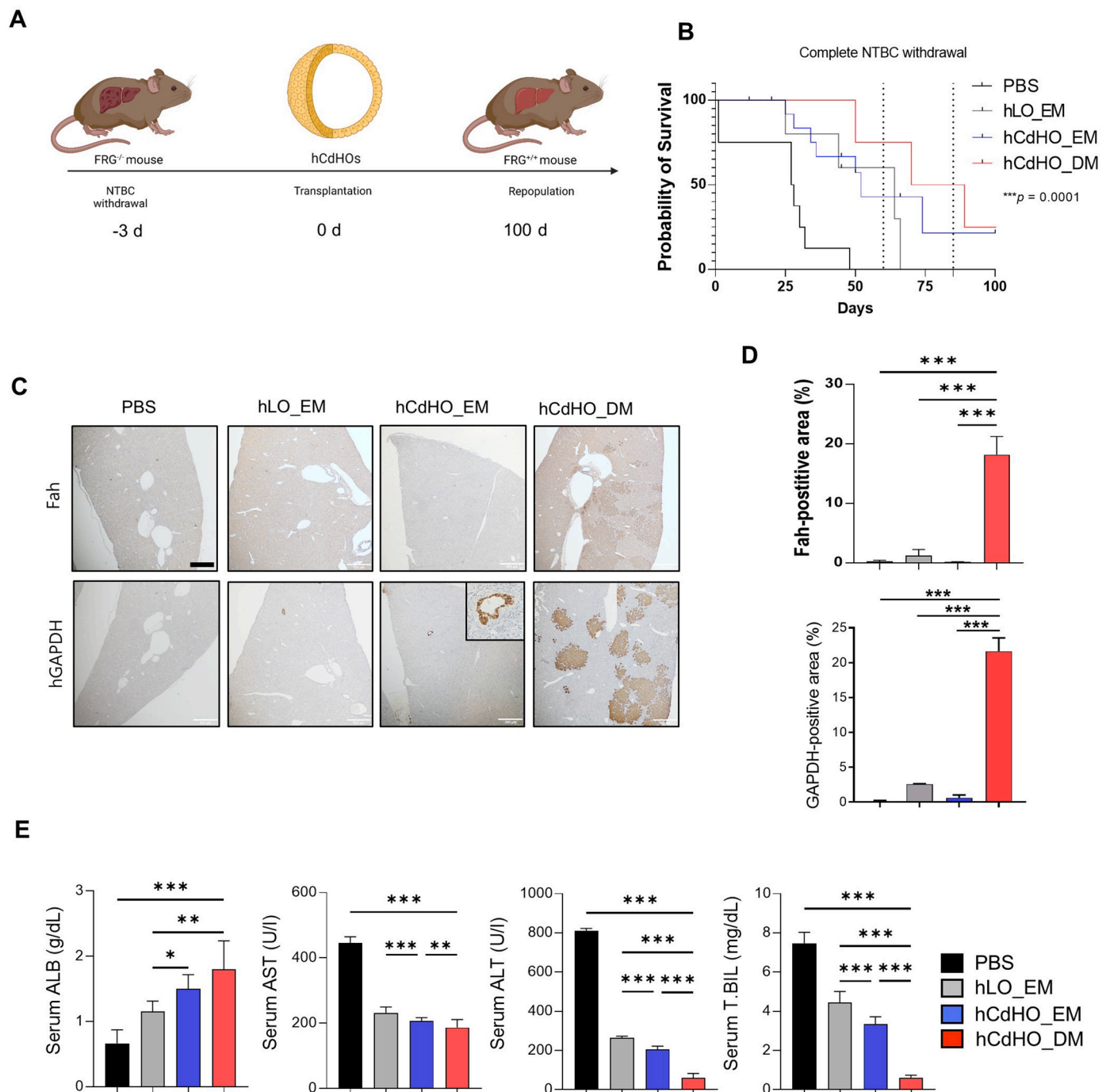
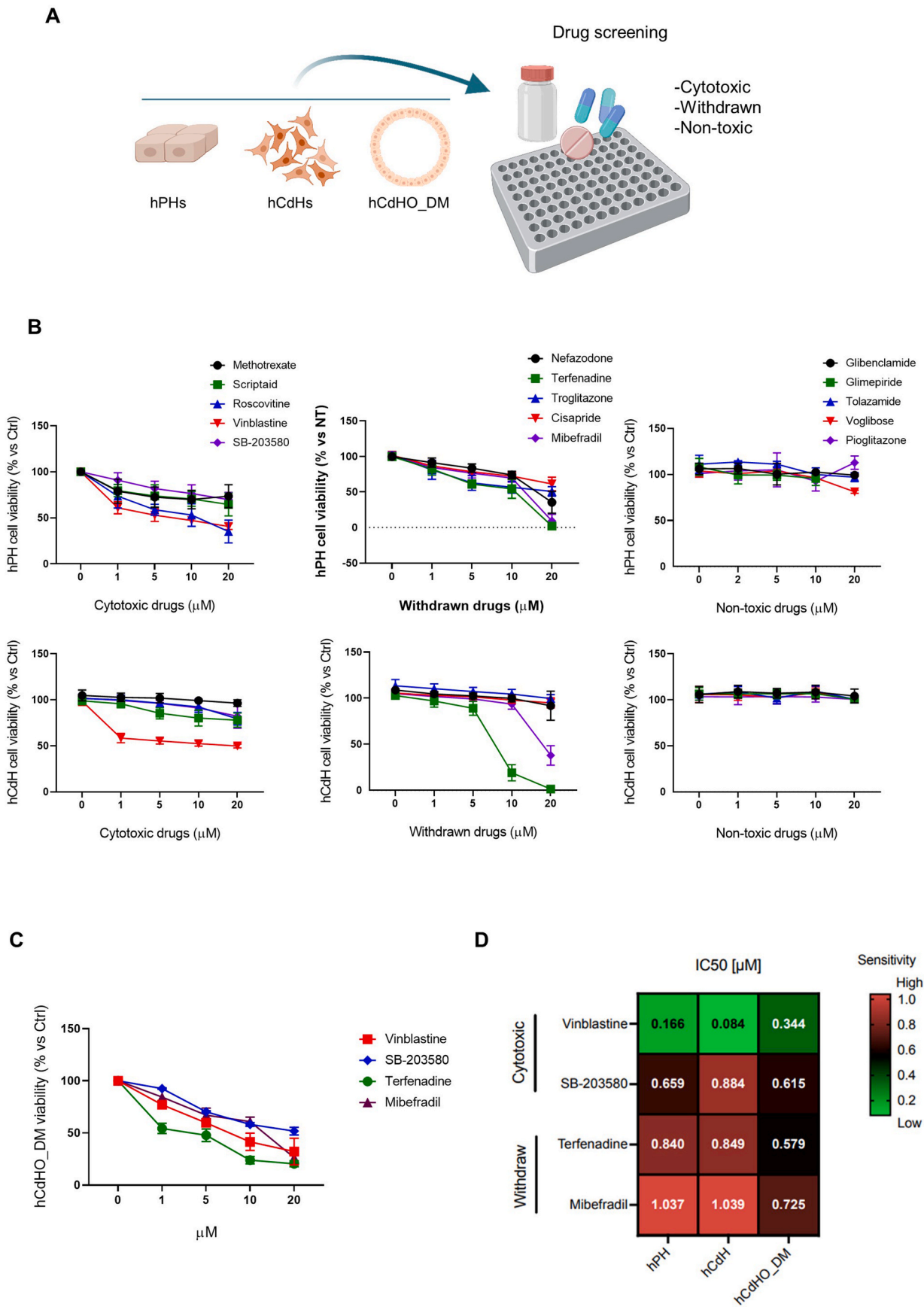


Fig. 4. Liver repopulation by hCdHOs in FRG mice. (A) Experimental design. Fah^{-/-}Rag2^{-/-}Il2rg^{-/-} (FRG) mice were used for transplantation of liver organoids. hLOs and hCdHOs from three independent donor-derived clones were maintained in expansion medium for 7 days before cell isolation and intrasplenic transplantation. To induce hepatic differentiation, hCdHOs were cultured in differentiation medium for 15 days (hCdHO_DM). (B) Kaplan-Meier survival curves followed by Log-rank (Mantel-Cox) test. Mice were maintained with NTBC (2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione) on and off after transplantation. Results are shown for FRG mice (n = 8, PBS, negative control group), hLOs (n = 5), hCdHO_EM (n = 13), and hCdHO_DM (n = 5). (C) Representative DAB staining of liver sections with anti-Fah and anti-hGAPDH on days 48 for PBS, 66 for hLOs, 100 for hCdHO_EM, and 102 for hCdHO_DM. Scale bars, 100 μ m. (D) Quantification of Fah- and GAPDH-positive areas by Image J. Data are mean $\pm$ SD (n = 3) analyzed by one-way ANOVA, followed by Tukey test, $***p < 0.001$. (E) Serum levels of albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (T.BIL) at seven weeks after organoid transplantation. Data are mean $\pm$ SD analyzed by one-way ANOVA, followed by Tukey test, $***p < 0.001$.

that control FRG mice injected with phosphate-buffered saline (PBS) died by week 7 (Fig. 4B). Mice receiving cell suspensions from hLOs lived longer but did not survive beyond 9 weeks. In contrast, mice transplanted with hCdHO-derived cells survived longer, up to about 14 weeks. The FRG mice receiving the hCdHO_DM cell transplant showed the best survival rate even under a complete NTBC removal between

days 60 and 85.

To analyze the repopulation capacity of the transplanted cells, we stained liver sections with anti-Fah and anti-human specific -hGAPDH antibodies. In mice injected with hCdHO_DM cells, Fah- and hGAPDH-positive areas were significantly more abundant at 100 days after transplantation and occupied up to 20 % of liver parenchyma as



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Fig. 5. hCdHO_DM as a drug-screening tool. (A) Experimental design. Drug-response was analyzed in hPHs, hCdHs and hCdHOs cultured in differentiation medium (DM) for 15 days (hCdHO_DM). hPHs were cultured in Williams medium, hCdHOs in HAC medium, and hCdHO_DM in differentiation medium. (B) Dose-response curves after treatment with indicated compounds for 24 h. Cell viability was detected by Cell Counting Kit-8 Assay. The values were expressed as percentages versus control (treated with DMSO). Data are mean $\pm$ SD of 3–4 independent experiments performed in triplicate and analyzed by one-way ANOVA followed by Tukey test. (C) Cell viability of hCdHO_DM after drugs treatment following the specifications of the previous paragraph. (D) Heat map representation of IC₅₀ values at 24 h. The color scale displays low and high IC₅₀ values in green (high sensitivity) and red (low sensitivity), respectively. Dose-response curves were fitted using nonlinear least-squares regression fitting and plotted relatively to the non-treated controls. Data are mean of 3–4 independent experiments performed in triplicate and analyzed by one-way ANOVA followed by Holm-Sidak test. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

compared to less than 3 % in mice transplanted with hLO cells (Fig. 4C and D). Notably, hCdHO_DM cells engraftment has significantly improved liver function by 7 weeks as judged by increased production of serum ALB and reduced secretion of liver damage enzymes, such as aspartate transaminase (AST), alanine transaminase (ALT), and total bilirubin (T.BIL) (Fig. 4E). These results indicate that hCdHO_DM outperform hLOs in the *in vivo* transplantation assay and thus may provide a better candidate for regenerative medicine.

3.5. hCdHO_DM as a hepatotoxicity screening tool

We then assessed whether hCdH-derived organoids grown in DM (hCdHO_DM) could serve as an *in vitro* screening platform for drug sensitivity testing (Fig. 5A). The initial screens were performed in hPHs and hCdHs using several well-known cytotoxic compounds, including drugs that were recently withdrawn from the market due to the severe hepatic side effects, as well as selected non-toxic drugs (Fig. 5B). None of the tested non-toxic compounds had an impact on cell survival at 24 h (Fig. 5B). In comparison, all cytotoxic drugs used at concentrations within 1–20 μ M range reduced survival of hPHs and hCdHs as measured by cell viability assay at 24, 48 and 72 h after treatment albeit to a different degree (Fig. 5B and data not shown). Four out of 10 cytotoxic drugs, including Vinblastine, SB-203580, Terfenadine and Mibefradil, which were the most effective in reducing cell viability (>40 %), were selected for a more detailed analysis of the cell type-specific drug responses (Fig. 5C, Fig. S6A). The comparative analysis of IC₅₀ values for all four tested drugs in all three examined cell types showed that hCdHO_DM were more resistant to Vinblastine, comparable to SB-203580, and more sensitive to Terfenadine and Mibefradil than both hPHs and hCdHs (Fig. 5C–D, Fig. S6B). The relatively higher sensitivity of hCdHO_DM to drug treatments could provide an advantage for selecting safe drug concentrations and avoiding toxicity as reported in studies using hPHs as a test model [28,29].

The comparison of the differences between IC₅₀ values in all three examined cell types showed that the IC₅₀ in hCdHO_DM treated with Terfenadine and Mibefradil are lower compared to that in hPHs and hCdHs suggesting a higher drug sensitivity (Fig. 5D). The latter could help to avoid errors in selecting optimal doses as reported in studies using primary hepatocytes which presented a greater resistance to drugs, such as Terfenadine and Mibefradil [28] or troglitazone [29]. Thus, hCdHO_DM may provide a novel model for toxicological studies since they display a comparable and sometimes greater sensitivity than hPHs.

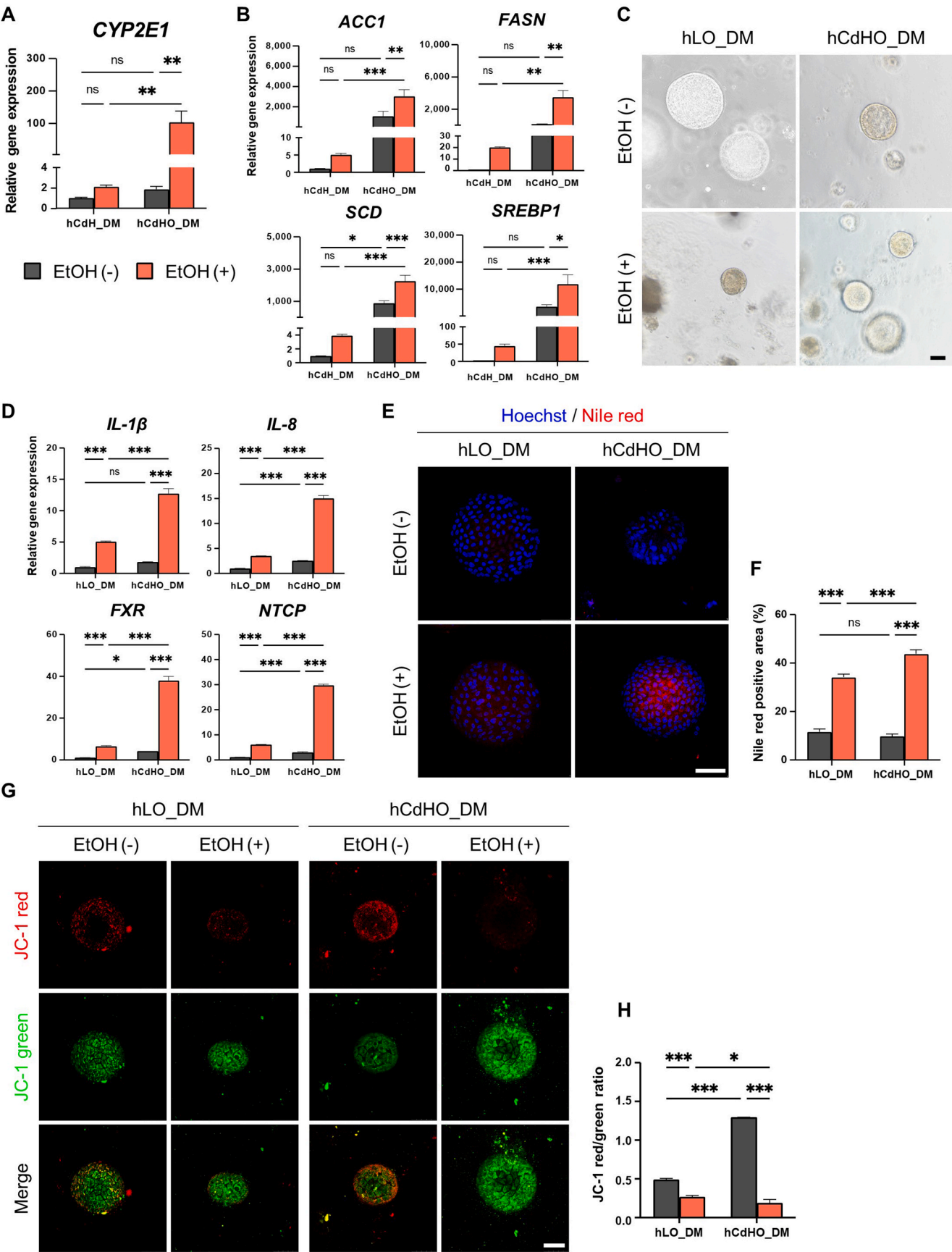
3.6. hCdHOs as a model to study alcoholic-related liver disease

Finally, we examined the possibility of using hCdHOs as an alternative *in vitro* system to model alcohol-related liver disease (ARLD), a global disease problem and leading cause of cirrhosis and liver-related death [2]. To demonstrate that hCdHOs allow more effective

pathophysiological modeling of ARLD than cells grown in 2D cultures, we first compared the induction of *CYP2E1*, a key feature of alcohol-induced liver injury responsible for conversion of ethanol to its toxic metabolites acetaldehyde and acetate [30,31]. Indeed, hCdHO_DM grown in hepatocyte differentiation medium for 15 days in the presence of 100 mM EtOH demonstrated about 49-fold higher upregulation of *CYP2E1* expression than EtOH-treated 2D hCdH_DM cultures (Fig. 6A). This was paralleled by a concomitant strong induction of lipogenic-associated enzymes (*ACC1*, *FASN* and *SCD*) as well as hyper-activation of critical transcription factor *SREBP1* essential for alcohol-induced lipid metabolic abnormalities and development of steatosis indicating that hCdHO_DM have high potential for liver disease modeling (Fig. 6B).

Next, we compared the response of hLOs and hCdHOs organoids derived from the same donor tissues to ethanol treatment. Exposure to ethanol reduced organoid size without affecting shape or morphology in both 3D systems as compared to the corresponding untreated controls (Fig. 6C). However, induction of inflammatory markers was significantly higher in EtOH-treated hCdHO_DM than in hLO_DM (Fig. 6D). Furthermore, EtOH treatment caused a stronger accumulation of lipids in hCdHO_DM than in hLO_DM as measured by Nile Red fluorescence detecting neutral lipids and phospholipids (Fig. 6E and F). To further confirm that hCdHO_DM develop a strong ARLD phenotype, we compared the mitochondria membrane potential (MMT), a well-known target of acetaldehyde, the primary oxidative product of ethanol breakdown by *CYP2E1* [30,31]. To assess MMT, we used the ratio between the red and green fluorescence of cells loaded with JC-1 [2]. The results of immunofluorescence staining revealed a stronger decrease of MMT in hCdHO_DM than in hLO_DM as compared to the corresponding untreated controls (6.8- vs 1.8-fold, respectively, $p < 0.001$) (Fig. 6G and H), providing evidence for mitochondrial membrane depolarization [2, 32]. Together, these results show that hCdHO_DM recapitulate the pathological features of ARLD better than hLO_DM.

To examine the spectrum of ethanol-induced pathological changes in greater depth, we performed RNA sequencing. The principal component analysis (PCA) plot showed a clear separation in the mRNA expression patterns between the hCdHO_DM exposed to ethanol and untreated hCdHO_DM controls (Fig. 7A). Pearson correlation map confirmed a strong correlation between the samples from the same group (Fig. 7B). Likewise, hierarchical clustering analysis revealed the substantial differences in the genome-wide gene expression profiles between these two groups (Fig. 7C). More specifically, exposure to ethanol caused upregulation of the alcohol metabolism-related genes, including *ADH4*, *ADH1A1*, *CYP2D6*, *CYP3A4*, *SULT1A1*, *SULT1A3*, *SULT1A4*, and *SULT1E1* (Fig. 7D). Accordingly, among the most significant Gene Ontology (GO) terms were oxidation-reduction processes, xenobiotic metabolism, fatty acid beta-oxidation and lipid homeostasis (Fig. 7E). The following GSEA confirmed that ethanol-induced molecular changes found in hCdHO_DM were characterized by a statistically significant enrichment of several published gene sets associated with ARLD



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Fig. 6. hCdHOs as a model to study alcoholic liver disease (ARLD). hCdHs, hCdHO_DM and hLO_DM were cultured in a differentiated medium (DM) in the presence or absence of 100 mM ethanol (EtOH) for 15 days. (A, B) RT-qPCR analysis of (A) *CYP2E1* and (B) indicated genes encoding proteins involved in EtOH metabolism and associated with ARLD. Data are mean $\pm$ SD of $n = 3$ independent organoid cultures with triplicate measurements and analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$. (C) Brightfield microscopy images of hLO_DM and hCdHO_DM organoids. Scale bars, 100 μ m. (D) RT-qPCR analysis of indicated genes associated with ARLD. Data are mean $\pm$ SD of 3 independent organoid cultures with triplicate measurements and analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$. (E) Representative confocal fluorescence images of organoids stained with Nile red. Scale bars, 75 μ m. (F) Relative Nile red intensity. Data are mean $\pm$ SD of three independent experiments analyzed by Image J. Statistics are analyzed by multiple unpaired t -test. ***, $p < 0.001$. (G) Representative JC-1 fluorescence mitochondrial imaging was used to study mitochondrial membrane potential (MMP) change. Scale bars, 75 μ m. (H) Ethanol-induced changes in MMP were evaluated by comparing the intensity of JC1-red to JC1-green fluorescence. Data are mean $\pm$ SD ($n = 3$) and analyzed by multiple unpaired t -test, *** $p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

phenotype (Fig. 7F). From these results we conclude that hCdH-derived organoids exposed to ethanol reproduce the pathological characteristics of alcoholic liver injury and thus can be suited for *in vitro* modeling of ARLD.

Finally, we treated hCdHO_DM with troglitazone, a cholestatic drug known to induce liver damage via oxidative stress, mitochondrial damage and inhibition of the bile salt excretory function [33]. The exposure to troglitazone caused a rapid loss of the ability to uptake the bile salt analogue (CDF) (Fig. S7) suggesting the utility of hCdHOs for modeling different liver diseases.

4. Discussion

Organoids are 3D self-organized structures commonly derived from a clonal expansion of a single pluripotent (i.e., hES and hiPSC) or adult tissue-specific stem cell under defined culture conditions [34,35]. They recapitulate morphology and functions of human organs better than 2D cultures providing powerful *in vitro* models to study human organ development and disease [2,10,32,36].

In 2019, we introduced a method that allows rapidly and consistently obtain large quantity of adult human bipotent progenitor cells (hCdHs) by a short-term culture of primary hepatocytes in medium containing a combination of growth factors with two small molecules, including Wnt agonist CHIR99021 and A83-01, a potent inhibitor of TGF- β /Activin/Notch signaling pathway. The overall objective of this study was to extend these results to generate hCdH-derived organoids (hCdHOs) as a platform for diverse biomedical applications.

Remarkably, 85.2 % of hCdHs established from the healthy adult donor liver tissues expressed EpCAM (Fig. 1D). When embedded in Matrigel, the cells efficiently produced hCdHOs that could be expanded long-term in culture while retaining hepatic progenitor properties and lineage-specific differentiation potential (Fig. 1F).

In parallel, we developed organoids from the cells isolated from the same donor livers (hLOs) and cultured under the same conditions for a direct comparison. Notably, the frequency of the EpCAM⁺ cells and efficiency of hLOs formation were considerably lower (Fig. 2C). Previously, a high degree of concordance between EpCAM⁺ expression and ability to form organoids was reported for human biliary duct-derived progenitor cells [12], although in this system, the initial low frequency of EpCAM⁺ freshly isolated cells required enrichment by FACS sorting. Consistent with the high efficiency of organoid formation, hCdHOs also displayed a shorter doubling time than hLOs organoids (Fig. S2) derived from the liver ductal cells [5].

Although the growth rate and organoid generation efficiency were similar in hCdHOs and hiPSOs, it takes less time to make hCdHOs from the seeded primary patient cells and the yield is greater as compared to hiPSOs (10 versus 20 days, and 85 % versus 20 %, respectively). Though we have not performed more detailed comparisons with hiPSC-derived organoids, our results suggest that hCdH-derived organoids can provide certain advantages in terms of faster generation and simplicity of the cost-effective culture technique. More importantly, hCdHOs origin from the hepatic progenitor cells may facilitate hepatic differentiation for therapeutic applications and a better understanding of liver disease.

In functional analyses studies, hCdHOs outperformed hLOs. When

grown in hepatocyte differentiation medium, hCdHOs manifested more mature hepatocyte phenotypes as evidenced by a stronger induction of lineage-specific functions, including albumin secretion, glycogen storage, and activity of drug-metabolizing enzymes (Fig. 3). Similarly, hCdHOs were more suitable than hLOs for pathological modeling of ARLD as documented by transcriptomic, biochemical, and functional assessment (Fig. 6). Exposure of hCdHOs to ethanol triggered stronger *CYP2E1* expression responsible for alcohol metabolism as well as upregulation of inflammatory markers, deregulation of lipid metabolism, and mitochondrial dysfunction, all hallmark features of ARLD [29, 37–41].

To explore the clinical relevance of our organoid system, we used therapeutic transplantation of hLOs and hCdHOs into the diseased livers of the immunodeficient FRG (*Fah*^{−/−}) *Rag2*^{−/−} *Il2rg*^{−/−} mice. The best-fit model to repopulate the *Fah*-deficient livers and restore hepatic functions were hCdHOs which grew in the hepatocyte differentiation medium before intrasplenic transplantation (Fig. 4). The mice injected with hCdHO_EM also lived longer and displayed higher levels of albumin secretion than the hLO_EM recipients, pointing to a greater value of hCdHOs for cell therapy application. These data are consistent with our earlier reports that hCdHs can serve as an alternative cell source to primary hepatocytes for therapeutic liver repopulation based on the comparable increase in albumin production after integration into the diseased parenchyma [16,42]. Furthermore, recently, our group successfully corrected the pathogenic *Fah* gene mutation by adenosine base editing and prime editing in chemically derived hepatic progenitors generated from the Hereditary tyrosinemia type 1 (HT1) mouse livers [38] indicating that small molecule-mediated reprogramming offers a reliable *in vitro* model for the *ex vivo* gene correction studies with potential for clinical applications.

Finally, in drug-induced toxicity studies, hCdHO_DM displayed the same or greater sensitivity as compared to hPHs. To underscore the potential utility of hCdHOs as a robust tool for drug testing, we opted to utilize a concentration range of 1–20 μ M to encompass a wide spectrum of reported concentrations and assess their effects at both lower and higher levels. The latter consideration is relevant considering the reported differences in C_{max} values for Vinblastine, Terfenadine and, Mebexradil (7.95 μ g/mL, 1.54 ng/mL and 0.5–1.8 μ M respectively). Toxicological studies at high concentrations can help to answer questions of vital importance in the search for treatment for liver disease (Fig. 5). Given the potential for long-term expansion in culture, this organoid system allows drug toxicity evaluation and selecting optimal drug concentrations for personalized therapy.

5. Conclusion

In conclusion, we provide evidence that human chemically derived hepatic progenitors represent a valuable and sustainable resource for organoid formation. The ability to generate liver organoids rapidly and with high efficiency from a variety of individuals with healthy and diseased livers makes this novel organoid model uniquely suitable for basic and translational studies.

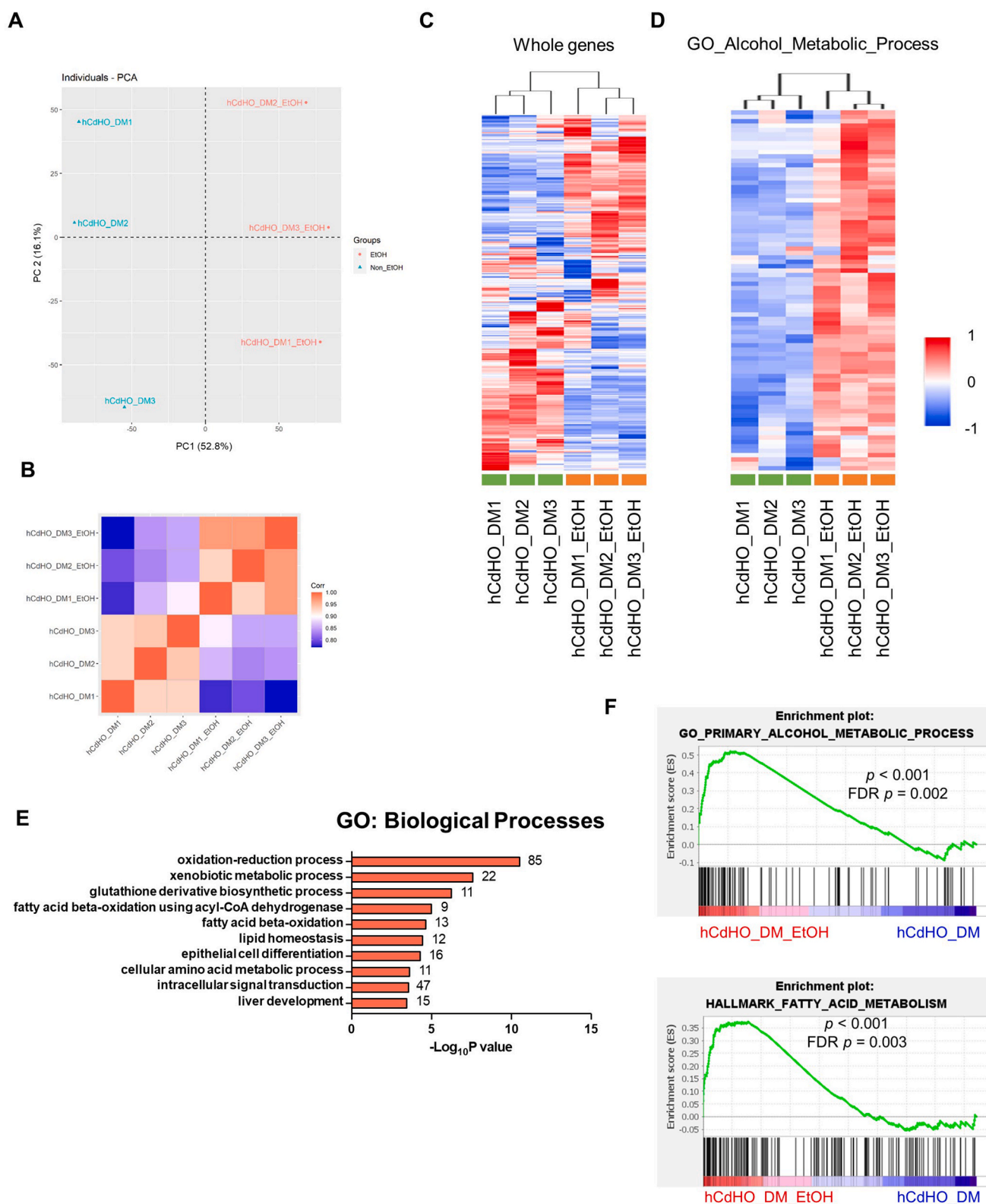


Fig. 7. Ethanol-treated hCdHOs recapitulate transcriptomic profiles of ARLD. (A) Principal component analysis (PCA) plot showing a clear distinction between hCdHOs grown in differentiation medium (DM) in the presence or absence of 100 mM ethanol (EtOH) for 15 days. Each data point represents one sample (red circle, EtOH-treated; green triangle, untreated). PC1 was strongly correlated with the sample type (52.6 %). (B) Correlation plot showing Pearson's correlation coefficient between biological replicates of hCdHO_DM and hCdHO_DM_EtOH. The color bar at the right indicates a correlation coefficient value between two samples. Red and blue colors represent higher or lower correlation coefficients between samples. (C) Heatmap of 11,431 differentially expressed genes determined by mRNA sequencing. Red and green represent higher and lower expressions, respectively. (D) Heatmap of 315 differentially expressed genes involved in Alcohol and Metabolic processes as defined by Gene Ontology (GO) tool. (E) Top ten GO terms showing induction of functional pathways associated with alcoholic liver disease. (F) Gene set enrichment plots showing strong enrichment of gene signatures of alcohol metabolic process and fatty acid metabolism in hCdHO_DM treated with EtOH. FDR, false discovery rate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

CRediT authorship contribution statement

Soraya Salas-Silva: Conceptualization, Methodology, Software, Writing – original draft. **Yohan Kim:** Conceptualization, Methodology, Software, Writing – original draft. **Tae Hun Kim:** Methodology. **Myounghoi Kim:** Methodology. **Daekwan Seo:** Methodology, formal analysis, software, validation. **Jeonghoon Choi:** Methodology, Formal analysis, Software, Validation. **Valentina M. Factor:** Data curation, formal analysis, validation, and writing-original draft. **Haeng Ran Seo:** Supervision, validation. **Yeonhwa Song:** Supervision, Validation. **Gyu Sung Choi:** Resources. **Yun Kyung Jung:** Resources. **Kungsik Kim:** Resources. **Kyeong Geun Lee:** Resources. **Jaemin Jeong:** Data curation, Formal analysis, Validation, Writing – original draft. **Ji Hyun Shin:** Conceptualization, project administration, supervision, funding acquisition. **Dongho Choi:** Conceptualization, Project administration, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

I have shared the link to our data. We would appreciate it if other researchers could endeavor to replicate our methods and achieve comparable results. This will foster discussions and collaboration among scientists worldwide.

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Abbreviations

hCdHs	human chemically derived hepatic progenitors
hCdHOs	human chemically derived hepatic progenitors-derive organoids
hPHs	human primary hepatocytes
hLOs	human liver organoids
EM	expansion media
DM	differentiation media

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biomaterials.2023.122360>.

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