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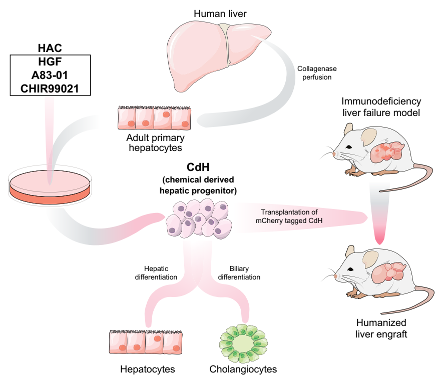
Small molecule-mediated reprogramming of human hepatocytes into bipotent progenitor cells

Graphical abstract



Highlights

- Human hepatic progenitors (hCdHs) are generated from adult hepatocytes.
- HGF is required for chemical reprogramming induced by A83-01 and CHIR99021.
- hCdHs proliferate for at least 10 passages without losing differentiation potential *in vitro*.
- Bipotent hCdHs can repopulate injured liver and acquire functional properties.

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Lay summary

Human primary hepatocytes were reprogrammed towards hepatic progenitor cells by a combined treatment with 2 small molecules, A83-01 and CHIR99021, and HGF. Chemically derived hepatic progenitors exhibited a high proliferation potential and the ability to differentiate into hepatocytes and biliary epithelial cells both *in vitro* and *in vivo*. This approach enables the generation of patient-specific hepatic progenitors and provides a platform for personal and stem cell-based regenerative medicine.

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Small molecule-mediated reprogramming of human hepatocytes into bipotent progenitor cells

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Background & Aims: Currently, much effort is directed towards the development of new cell sources for clinical therapy using cell fate conversion by small molecules. Direct lineage reprogramming to a progenitor state has been reported in terminally differentiated rodent hepatocytes, yet remains a challenge in human hepatocytes.

Methods: Human hepatocytes were isolated from healthy and diseased donor livers and reprogrammed into progenitor cells by 2 small molecules, A83-01 and CHIR99021 (AC), in the presence of EGF and HGF. The stemness properties of human chemically derived hepatic progenitors (hCdHs) were tested by standard *in vitro* and *in vivo* assays and transcriptome profiling.

Results: We developed a robust culture system for generating hCdHs with therapeutic potential. The use of HGF proved to be an essential determinant of the fate conversion process. Based on functional evidence, activation of the HGF/MET signal transduction system collaborated with A83-01 and CHIR99021 to allow a rapid expansion of progenitor cells through the activation of the ERK pathway. hCdHs expressed hepatic progenitor markers and could self-renew for at least 10 passages while retaining a normal karyotype and potential to differentiate into functional hepatocytes and biliary epithelial cells *in vitro*. Gene expression profiling using RNAseq confirmed the transcriptional reprogramming of hCdHs towards a progenitor state and the suppression of mature hepatocyte transcripts. Upon intrasplenic transplantation in several models of therapeutic liver repopulation, hCdHs effectively repopulated the damaged parenchyma.

Conclusion: Our study is the first report of successful reprogramming of human hepatocytes to a population of proliferating bipotent cells with regenerative potential. hCdHs may provide a novel tool that permits expansion and genetic manipulation of patient-specific progenitors to study regeneration and the repair of diseased livers.

Lay summary: Human primary hepatocytes were reprogrammed towards hepatic progenitor cells by a combined treatment with 2 small molecules, A83-01 and CHIR99021, and HGF. Chemically derived hepatic progenitors exhibited a high proliferation potential and the ability to differentiate into hepatocytes and biliary epithelial cells both *in vitro* and *in vivo*. This approach enables the generation of patient-specific hepatic progenitors and provides a platform for personal and stem cell-based regenerative medicine.

Introduction

Currently, liver transplantation represents the only approved standard of care for patients with end-stage liver diseases.[1] Experimental studies in rodents and clinical trials of hepatocyte transplantation have shown that direct infusion of mature hepatocytes may serve as an alternative to whole organ replacement in some cases. However, hepatocyte transplantation only results in a partial and relatively short-term correction of liver dysfunction, and has been hampered by numerous issues related to the shortage of donor tissue, limited numbers of cells suitable for transplantation, and a low efficiency of engraftment in the abnormal microenvironment of diseased livers.[2–4] In addition, human hepatocytes are difficult to maintain and expand *in vitro* because of the lack of adequate environmental signals. Typically, mature hepatocytes have low proliferative potential and easily become apoptotic in culture which reduces their therapeutic value.

To facilitate the development of cell-based therapies for treating liver disease, over the last decade much effort has been directed towards the potential use of pluripotent stem cells capable of indefinite self-renewal, including embryonic stem cells (ESCs),[5–8] induced pluripotent stem cells (iPSCs),[9–11] mesenchymal stem cells (MSCs),[12–14] and hepatic progenitor cells.[15–18] Despite the important advances in generating stem

Keywords: Human hepatocytes; Human chemically derived hepatic progenitors; Reprogramming; Small molecules.

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cell-derived hepatocyte-like cells from pluripotent cells, their clinical applications are impeded by their low efficiency of hepatic differentiation,[19] the likelihood of immune rejection,[20] high risk of cancer development,[21,22] as well as low rate of proliferation and rapid loss of differentiation potency in culture.[23] In addition, the therapeutic use of iPSCs and ESCs could be compromised by the possibility of genetic transformation and/or ethical issues.[24,25] MSCs are generally reported not to form teratomas, but they have a small total cell yield and inefficient hepatic differentiation,[26] which limits their potential use in clinic. More recently, a 3D organoid culture system for human liver has been established[27] which allows for the generation of highly stable bipotent progenitor cells capable of bi-lineage differentiation both *in vitro* and *in vivo*, but is technically challenging due to a multi-step process of cell isolation, selection and long-term expansion.

New technological advances in the direct reprogramming of somatic cells by a defined set of small molecules simplified and shortened the process of generating integration-free progenitor-type cells.[28–30] In particular, Katsuda *et al.* identified a combination of only three small molecules, Y-27632, A83-01, and CHIR99021, which was very effective in converting terminally differentiated rat and mouse but not human hepatocytes to bipotent progenitor cells.[31]

We report that mature human hepatocytes isolated from healthy and diseased donor livers could be rapidly converted into a bipotent state when treated with 2 small molecules A83-01 and CHIR99021 (AC) in combination with hepatocyte growth factor (HGF). We further show that these chemically derived human hepatocyte progenitors (hCdHs) could sustain themselves as a population of progenitor cells over a long period while maintaining chromosomal stability and the capacity to differentiate into functional hepatocytes and biliary epithelial cells (BECs) *in vitro* and *in vivo*, highlighting their potential for biomedical applications.

Materials and methods

Generation of chemically derived human hepatocyte progenitors

The study was performed according to protocols approved by the Institutional Review Board of Hanyang University, Seoul, Korea (HYI-16-229-3). Human liver tissues were obtained from six donors operated on in Hanyang University Medical Center (Table 1) with patients' informed consent. Hepatocytes were isolated using a modified 2-step collagenase perfusion technique[32] as described in detail in the supplementary material and methods section. Hepatocytes were seeded on collagen-coated dishes (STEMCELL Technologies, BC, Canada) at 5,000 cells/cm^2 in basic high glucose DMEM/F-12 media (Gibco, CA, USA) containing 1% FBS (Gibco), 10 mM nicotinamide (Sigma-Aldrich, MO, USA), 0.1 μM dexamethasone (Sigma-Aldrich), 1% insulin-transferrin-selenium (Gibco), 1% penicillin/streptomycin (Gibco), and 20 ng/ml epidermal growth factor (EGF) (Peprotech, NJ, USA). After overnight incubation, the basic medium was supplemented with 4 μM A83-01 (Gibco) and 3 μM CHIR99021 (STEMCELL Technologies), and 20 ng/ml of HGF (Peprotech), designated reprogramming medium. The reprogramming medium was changed every day. Cells were passaged using 1X TrypLE Express Enzyme (Gibco) when they reached 80% confluence and split at a ratio of 1:3–1:5 every fifth day. In total, at least 2–3 independent clonal lines were established for each of six donor liver samples.

Differentiation studies

To induce hepatic differentiation, hCdHs were plated on collagen-coated dishes (STEMCELL Technologies) at 1,000 cells/cm^2 in hepatocyte induction media consisting of the basal medium supplemented with 20 ng/ml oncostatin M (R&D Systems, MN, USA), 10^{-7} M dexamethasone (Sigma) and 20 ng/ml HGF. The medium was changed every 2 days. After 6 days, the cultures were overlaid with the hepatocyte

Table 1. Clinical characteristics of liver donors.

Donor	1	2	3	4	5	6
Ethnicity	Asian	Asian	Asian	Asian	Asian	Asian
Sex	Female	Male	Female	Male	Female	Female
Age (years)	31	56	86	75	54	80
Body mass index	24.83	30.53	27.04	22.61	24.94	27.00
Diagnosis	Gallbladder polyp	Hepatocellular carcinoma	Liver metastasis (colon cancer)	Hepatocellular carcinoma	Intrahepatic duct stone	Hepatocellular carcinoma
Operation	Laparoscopic cholecystectomy and liver biopsy	Right posterior sectionectomy	Wedge resection	Laparoscopic wedge resection	Left lateral sectionectomy	Right posterior sectionectomy
Child-Pugh score	5 (Class A)	5 (Class A)	5 (Class A)	5 (Class A)	5 (Class A)	5 (Class A)
Albumin (g/dl)	4.6	4.4	4.3	4.6	4.0	3.5
PT(INR)	0.97	1.02	1.08	1.00	1.05	1.11
Total bilirubin (mg/dl)	0.62	0.91	0.79	0.31	0.46	1.13
ALP (U/L)	50	63	126	114	67	105
AST (U/L)	19	21	47	22	24	87
ALT (U/L)	10	31	43	15	17	55
Hepatitis	None	Hepatitis C	None	Hepatitis B	None	Hepatitis B
Alcohol use	Yes	Yes	No	Yes	No	No
Smoker	No	Yes	No	No	No	No
Specimen weight (g)	0.71	5.95	0.74	1.01	0.54	4.02

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; INR, international normalized ratio; PT, prothrombin time.

induction medium containing Matrigel at a 1:7 ratio (BD Biosciences, CA, USA). On day 8, the cultures were washed with Hank's balanced salt solution (Welgene) and fixed in 4% paraformaldehyde.

For cholangiocyte differentiation, we employed a 3D culture system using collagen type 1 (BD Biosciences) according to the manufacturer's instruction. In brief, 1×10^5 hCdHs were re-suspended in DMEM/F12 medium containing 10% FBS and 20 ng/ml HGF (Peprotech, USA), designated cholangiocyte differentiation medium (CDM), and mixed on ice with an equal volume of CDM supplemented with collagen type 1 after adjusting pH to 7.0. The cell suspension was then transferred to a 6-well plate and incubated for 30 min at 37 °C to let the gel form. Thereafter, the cultures were overlaid with CDM and cultured for 7 days. At least 3 independent experiments were performed for each differentiation assay.

Further methodology may be found in the supplementary materials and methods section.

Transplantation experiments

The NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) (C57/BL background, Jackson Laboratory, USA), *Fah*$^{-/-}$/*Rag2*$^{-/-}$/*Il2rg*$^{-/-}$ on the NOD-strain background (FRGN) (C57/BL background, kind gift by Markus Grompe, Oregon Health Sciences University, Portland, OR, USA), and Alb-TRECK/SCID mice (C57/BL background, kind gift by Dr. Taniguchi, Yokohama City University, Japan) were housed and cared for under specific pathogen-free conditions with 12 h light/12 h dark cycle in accordance with the Principles of Laboratory Animal Care and the Guide for the Use of Laboratory Animals of Samsung Biomedical Research Institute (20170116003, 20160203002) and HYU Industry-University Cooperation Foundation regulations (2016-0212A). Liver damage was induced in the eight to ten week old female NSG mice by a single intraperitoneal (i.p.) injection of Jo2 antibody (BD Pharmingen, USA) at 0.2 mg/kg (NSG mice),[33] in the 8–10-week old female Alb-TRECK/SCID mice,[34] by a single i.p. injection of diphtheria toxin (Sigma) at 2 µg/kg, and in the 8–10-week old female FRGN mice by gradual withdrawal method (for 48 h) of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC).[35] FACS-sorted mCherry-positive hCdHs (P4-6, 10^6 per mouse) were transplanted into the inferior pole of the spleen 24 h after injection of Jo2 or diphtheria toxin, or 48 h after NTBC withdrawal. Liver repopulation was evaluated at 2 and 8 weeks (NSG mice), 3 weeks (Alb-TRECK/SCID mice) and 1 week (FRGN mice) after the transplantation of mCherry-tagged hCdHs (n = 3 mice, each time point/model) using Virtual Microscope AxioScan.Z1 (Zelss, German).

Statistical analysis

Quantitative data are presented as means ± standard deviations (SDs) with inferential statistics (*p* values). Statistical significance was evaluated by 2-tailed *t* tests with significances set at $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001.

Results

Generation of human chemically derived hepatic progenitors

In our initial work, we have adopted the methodology recently described by Katsuda *et al.*[31] In support of their data, we found that a cocktail of three small chemicals, Y27632, A83-01, and CHIR99021 (YAC), which was very effective in reprogramming of mouse and rat hepatocytes, did not support the conversion process in human hepatocytes (Fig. S1A, B). The YAC-treated human hepatocytes rapidly died off without proliferation.

To overcome this problem, we looked for additional hepatic factors which could increase the efficiency of the conversion process. Given the key contribution of HGF in liver organogenesis, regeneration, and the maintenance of hepatic progenitor cells,[36–38] we optimized the reprogramming medium to include HGF. After testing different sets of small molecules in the presence of HGF, the most effective was a combination of HGF and 2 small molecules, A83-01 and CHIR99021 (AC) referred to as HAC hereafter.

Exposure of human primary hepatocytes (hPHs) to HAC triggered a robust expansion of small epithelial cells with high nuclear-to-cytoplasm ratio typical of progenitor-type cells. Within the first 10–15 days of treatment with HAC, these cells, termed hCdHs, showed a steady increase in the expression of classical hepatic progenitor marker genes and proteins, such as AFP, CK19 (KRT19), EPCAM, SOX9, *etc.* (Fig. 1A, B). Consistent with effective reprogramming, the derived hCdHs expressed pluripotency stem cells markers *OCT4 (POU5F1)*, *NANOG* and *SOX2* (Fig. 1C), and endoderm markers SOX17, CXCR4 and GATA4 (Fig. S2A). Significantly, hCdHs displaying hepatic progenitor cell marker expression could be effectively generated from the frozen human hepatocytes (Fig. 1D; Fig. S2B).

To extend the analysis of lineage stage-specific transcriptional changes during the conversion process, we next compared the gene expression profiles of hCdHs with human primary hepatocytes, fetal liver, as well as human fibroblasts and hepatic stellate cells. As expected, unsupervised hierarchical clustering of gene expression data revealed that reprogrammed hCdHs clustered together with fetal liver and hPHs but not with fibroblasts or hepatic stellate cells (Fig. 1E). Notably, the following gene set enrichment analysis (GSEA) demonstrated that among the significant molecular changes found in hCdHs was a clear enrichment of several published stem cell-related gene sets (Fig. 1F).[39,40] Thus, the induction of stem cell-related genes is essential for small-molecule-mediated reprogramming of terminally differentiated human hepatocytes to a stem cell state.

We next traced the fate of single human hepatocytes plated at low density (10 cells per 6 well) and grown in the HAC reprogramming medium. Images taken every 24 h showed that a single hepatocyte went through a first division by 72 h, and produced an offspring colony containing on average 20 cells by 192 h (range 15–40, n = 10 examined cells) (Fig. S2C). Time-lapse microscopy confirmed these observations (Video S1).

In total, the cultures of hCdHs were established as independent clonal lines from hepatocytes isolated from both healthy and diseased livers (Table 1). Importantly, all clonal cell lines displayed similar hepatic progenitor phenotype, while maintaining chromosomal integrity and normal chromosome numbers (Fig. 1G; Fig. S2D). The following results refer to the hCdHs clonal lines established from a healthy donor liver (donor 1) unless otherwise indicated.

HGF facilitates generation of hCdHs through activation of ERK 1/2 signaling

To further validate the role of HGF in the generation of hCdHs, we isolated human hepatocytes and cultured them in the

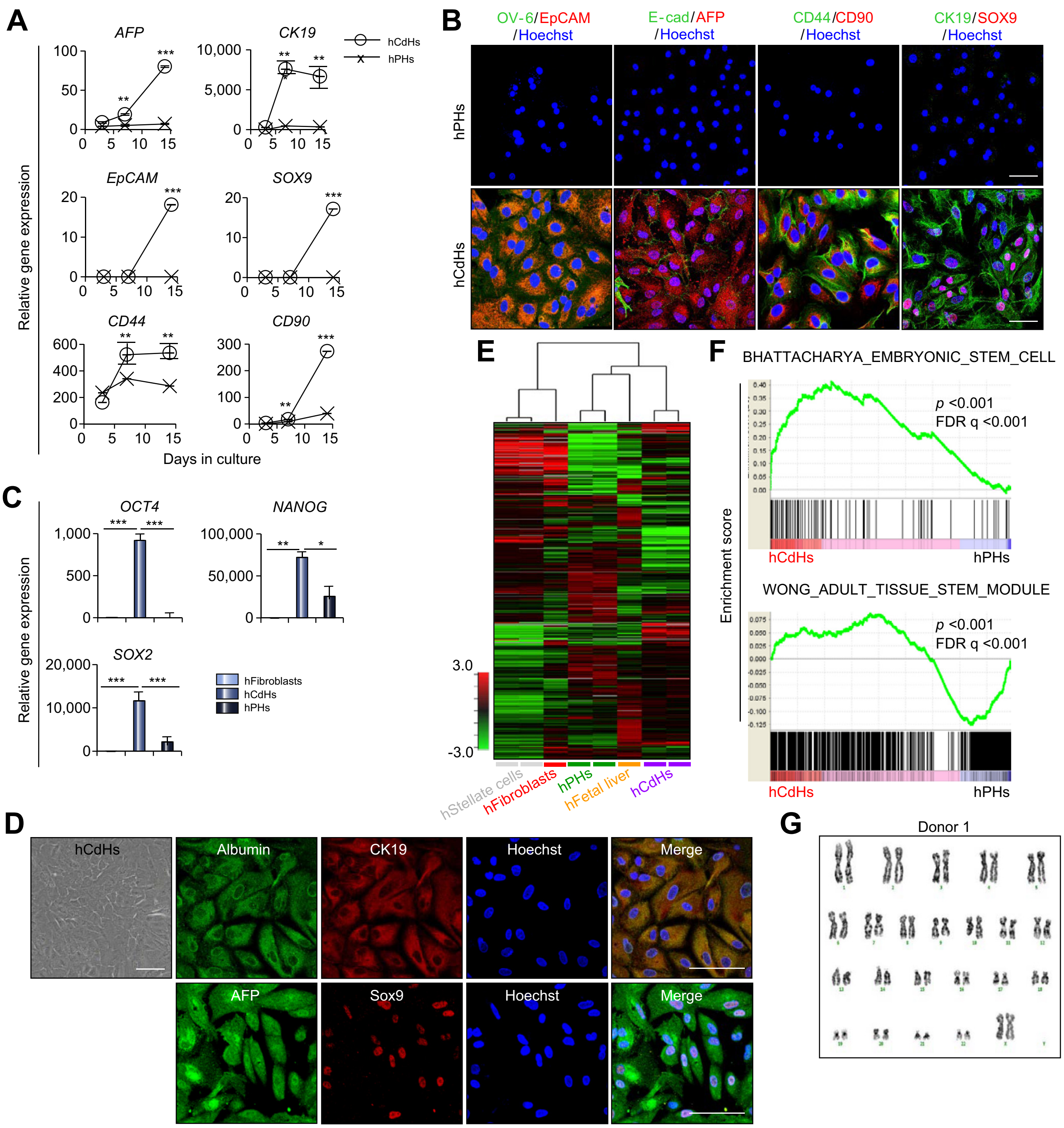

Fig. 1. hCdHs acquire the molecular features of hepatic progenitor cells. (A) Time-course induction of hepatic progenitor cell marker and pluripotency marker gene expression determined by RT-qPCR. GAPDH was used as an internal control for RT-qPCR. Data are mean ± SD (n = 3). Data analyzed by 2-tailed *t* tests, $^{**}p$ <0.01, $^{***}p$ <0.001. (B) Representative double immunofluorescence staining for hepatic progenitor markers OV-6 (green)/EPCAM (red), E-cadherin (green)/AFP (red), CD44 (green)/CD90 (red), and CK19 (green)/SOX9 (red). hCdHs were cultured in a reprogramming medium containing HGF, A83-01 and CHIR99021 (HAC) for 14 days. Nuclei were counterstained with Hoechst 33342 (blue). Scale bars, 50 µm. (C) RT-qPCR analysis of the pluripotency marker gene expression. Data are mean ± SD (n = 3). Data analyzed by 2-tailed *t* tests, $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001. (D) hCdHs generated from frozen human hepatocytes (passage 1) expressed hepatic precursor markers, albumin (green), CK19 (red), AFP (green), and SOX9 (red). Nuclei were counterstained with Hoechst 33342 (blue). Scale bars, 100 µm. (E) Unsupervised hierarchical cluster analysis of global gene expression profiles in human (h) stellate cells, fibroblasts, hPHs, fetal liver, and hCdHs cultured for 14 days. Data for the hepatic stellate cells were downloaded from GEO (GSE78853). The color bar at the left indicates gene expression in $\log_2$ scale. Red and green represent higher and lower gene expression levels, respectively. (F) Activated gene sets in hCdHs as determined by GSEA using embryonic stem cell signature (top, n = 89 genes)[39] and adult tissue stem module (bottom, n = 721 genes).[40] NES reflects the degree of over-representation for each group at the peak of the entire set. Statistical significance was calculated by nominal *p* value of the NES by using an empirical phenotype-based permutation test. False positives are calculated by the FDR. (G) Representative karyotyping image of hCdHs cultured for 14 days. No obvious chromosomal abnormalities were seen in any of the 20 analyzed images. FDR, false discovery rate; GSEA, gene set enrichment analysis; hCdHs, human chemically derived hepatic progenitors; hPHs, human primary hepatocytes; NES, normalized enrichment score; RT-qPCR, quantitative reverse transcription PCR.

reprogramming medium in the presence or absence of AC and HGF (Fig. 2A). HGF supplementation for 7 days significantly increased the proliferation of hCdHs as judged by a 5-fold increase in their number (Fig. 2B). Treatment with either HGF or AC alone failed to produce a population of progenitor-like cells (Fig. 2A, B), underlining the importance of the concurrent activation of HGF and the inhibition of TGFβ- and GSK3-mediated signaling caused by A83-01 and CHIR99021, respectively, for the reprogramming of adult human hepatocytes.

To assess the function of HGF, we then analyzed the activity/phosphorylation status of its cognate receptor MET and primary downstream effectors implicated in the control of proliferation and differentiation.[37,41,42] As anticipated, upon stimulation of hPHs with HGF, the MET receptor was tyrosine-phosphorylated in a time-dependent manner with a peak activation at 1 h (Fig. 2C). Among the known downstream targets of MET, we observed increased phosphorylation of ERK1/2 (MAPK3/1), but not AKT or STAT3 (Fig. 2C). We confirmed that HGF-mediated activation of MET and ERK was not affected by AC (Fig. 2D).

To verify the HGF/MET specific effects, we blocked the activity of MET and ERK1/2 by the selective inhibitors SU11274 and U0126, respectively. SU11274 and U0126 prevented phosphorylation of both MET and ERK1/2 (Fig. 2E) and blocked the HGF-mediated hCdHs expansion as judged by total cell count (Fig. 2F) and morphological features (Fig. S3A). The growth in the presence of increasing concentrations of SU11274 caused a dose-dependent reduction in the number of hCdHs (Fig. S3B, C). Thus, persistent HGF/MET signaling was required to establish and maintain hCdHs proliferation through a mechanism involving ERK1/2 activation.

Long-term maintenance and differentiation potential of hCdHs *in vitro*

Phenotypically, hCdHs sustained themselves as a population of undifferentiated progenitor cells for at least 10 passages. They

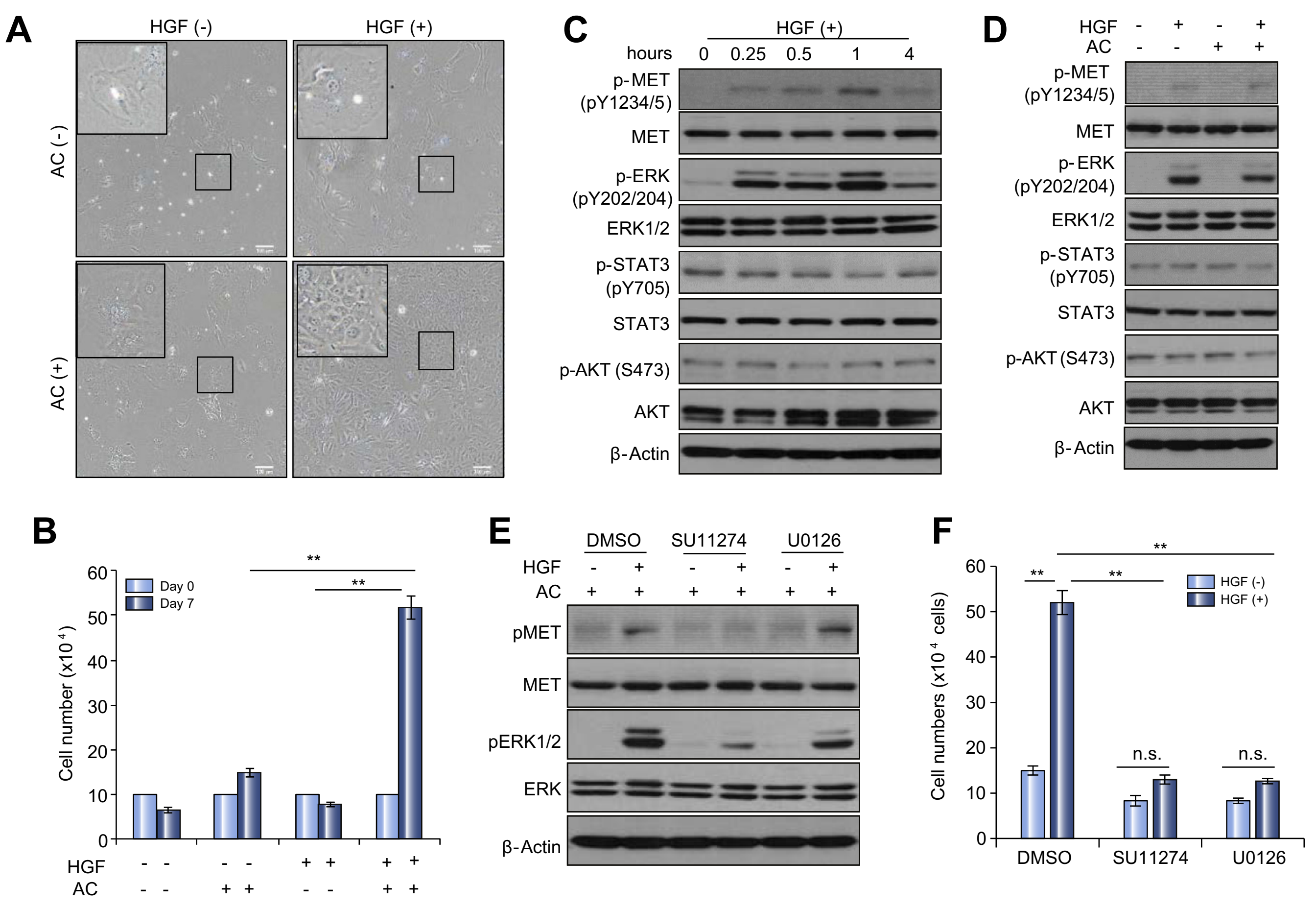

Fig. 2. HGF supplementation facilitates generation of hCdHs. (A, B) Freshly isolated human hepatocytes were cultured in a reprogramming medium containing A83-01 and CHIR99021 (AC) for 7 days in the presence or absence of HGF. (A) Representative phase contrast images. Scale bars, 100 µm. Insets, higher magnification of the boxed areas. (B) Cell number. The data are mean ± SD (n = 4). Data analyzed by 2-tailed *t* tests, ***p* <0.01. (C) Western blotting for pMET, MET, pERK1/2, ERK1/2, pSTAT3, STAT3, pAKT, and AKT. Beta-actin was used as a loading control. Freshly isolated human hepatocytes were treated with 20 ng/ml HGF for the indicated time after overnight incubation in the basic medium. (D) Western blotting for pMET, MET, pERK1/2, ERK1/2, pSTAT3, STAT3, pAKT, and AKT. Freshly isolated human hepatocytes were treated with 20 ng/ml HGF for 1 h after overnight incubation in AC medium. (E, F) Pharmacological inhibition of MET (SU11274, 10 µM) and mitogen-activated protein kinases, MEK1 (MAP2K1) and MEK2 (MAP2K2) (U0126, 10 µM) blocks the activation of MET downstream signaling (E) and suppresses hCdHs generation (F) upon HGF stimulation. Human primary hepatocytes were pre-treated with the indicated inhibitors for 30 min after overnight incubation in basic medium and then cultured for 7 days in AC reprogramming medium with and without HGF supplementation. In F, data are mean ± SD (n = 3). Data analyzed by 2-tailed *t* tests, ***p* <0.01. hCdHs, human chemically derived hepatic progenitors.

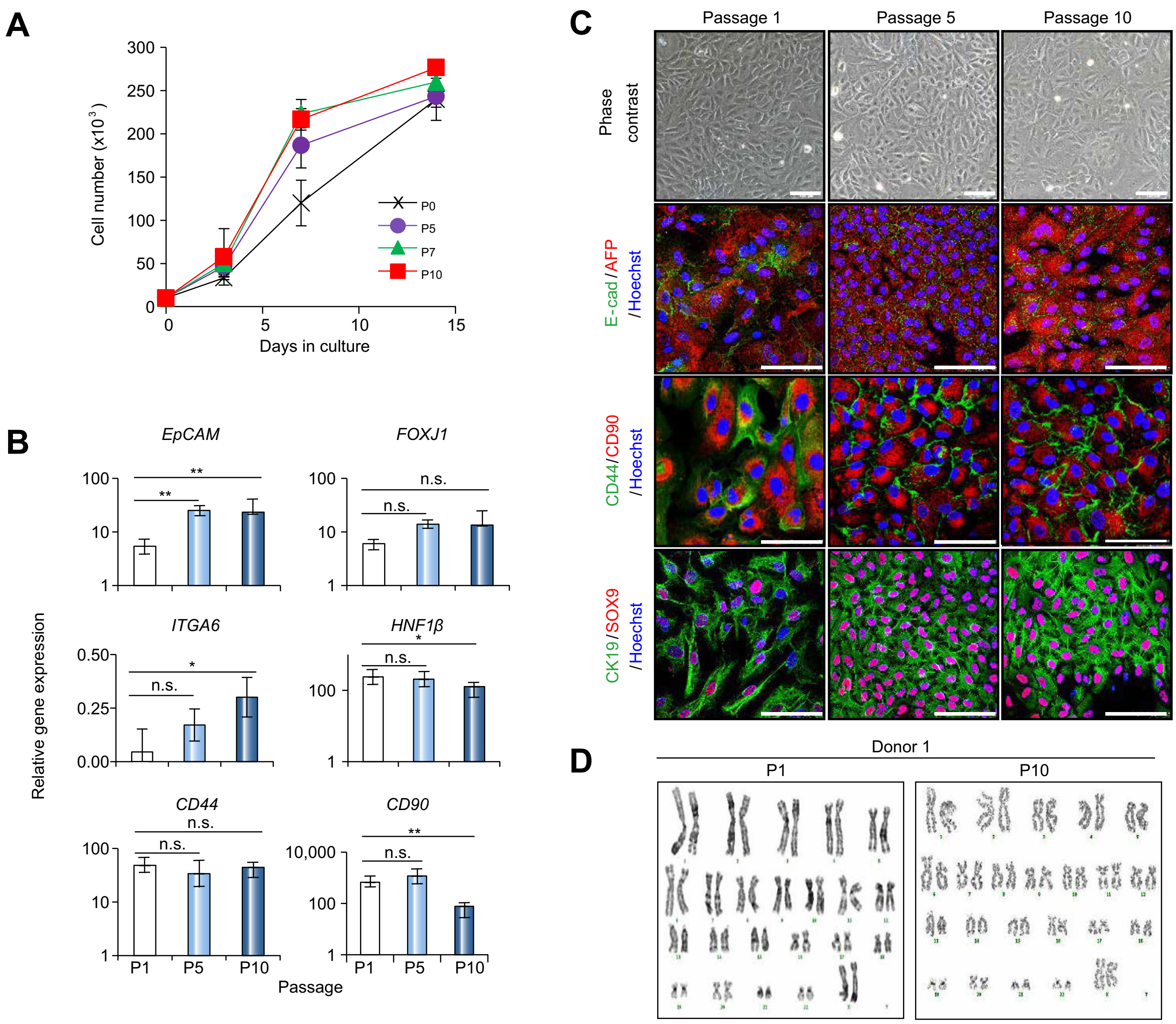

Fig. 3. hCdHs maintain properties of hepatic progenitors in long-term cultures. (A) Growth curves. The data are mean ± SD (n = 4). (B) RT-qPCR analysis of hepatic progenitor marker genes. Data are mean ± SD (n = 3). Data analyzed by 2-tailed *t* tests, $^{*}p < 0.05$, $^{**}p < 0.01$. (C) Representative phase contrast (top) and double immunofluorescence (bottom) images of staining for hepatic progenitor markers E-cadherin (green)/AFP (red), CD44 (green)/CD90 (red), and CK19 (green)/SOX9 (red). Nuclei were counterstained with Hoechst 33342 (blue). Scale bars, 100 µm. (D) Representative karyotype images of hCdHs at passage (P) P1 and P10. hCdHs, human chemically derived hepatic progenitors; RT-qPCR, quantitative reverse transcription PCR.

continued to proliferate with a similar growth rate (Fig. 3A, Fig. S4) and expressed comparable levels of hepatic progenitor markers as shown by reverse transcription quantitative PCR (RT-qPCR) analysis (Fig. 3B) and fluorescence staining (Fig. 3C). Karyotyping of metaphase cells at passages 1 and 10 also did not reveal any chromosomal alterations (Fig. 3D).

Since the defining attribute of progenitor cells is their ability to multi-lineage differentiate to their tissue of origin,[43,44] we then examined the differentiation potency of hCdHs using standard lineage-specific differentiation assays *in vitro*. Notably, during the transition of hCdHs to hepatocyte-like cells (hCdH-Heps), the expression of endoderm-associated markers SOX17, CXCR4 and GATA was decreased (Fig. S2A) while expression of mature hepatocyte-related genes and proteins was strongly induced as assessed by RT-qPCR analysis (Fig. 4A, Fig. S5A, B), double fluorescence confocal microscopy, glycogen and MitoTracker staining (Fig. 4B). In support of these findings, the hCdH-Heps also displayed significant increases in albumin secretion (Fig. 4C), CYP1A2 activity (Fig. 4D), and urea synthesis (Fig. 4E). Likewise, the differentiation towards hCdH-Heps was associated with improved canalicular function as demonstrated by the uptake and release of indocyanine green (Fig. S5C), fluorescein diacetate (Fig. S5D), and a parallel upregulation of the superfamily of ATP-binding cassette transporters such as *MRP2 (SYCE1L)* (Fig. 4A) and *BSEP* (*ABCB11*) (Fig. S5A). Consistent with the acquisition of a more mature hepatic state, hCdH-Heps showed an increase in mitochondria mass (Fig. 4B, *k-l*) as well as a strong induction of key genes involved in the control of mitochondrial function, such as *ATP5G1 (ATP5MC1)*, *POLG*, *POLG2*, *TFAM*, and *UCP2*, which were undetectable in hCdHs (Fig. S5B).

Importantly, hCdHs retained the differentiation potency upon long-term culture. hCdHs subjected to the hepatocyte differentiation protocol at different passages showed a comparable induction of the mature hepatocyte markers (Fig. 4F, G). hCdHs

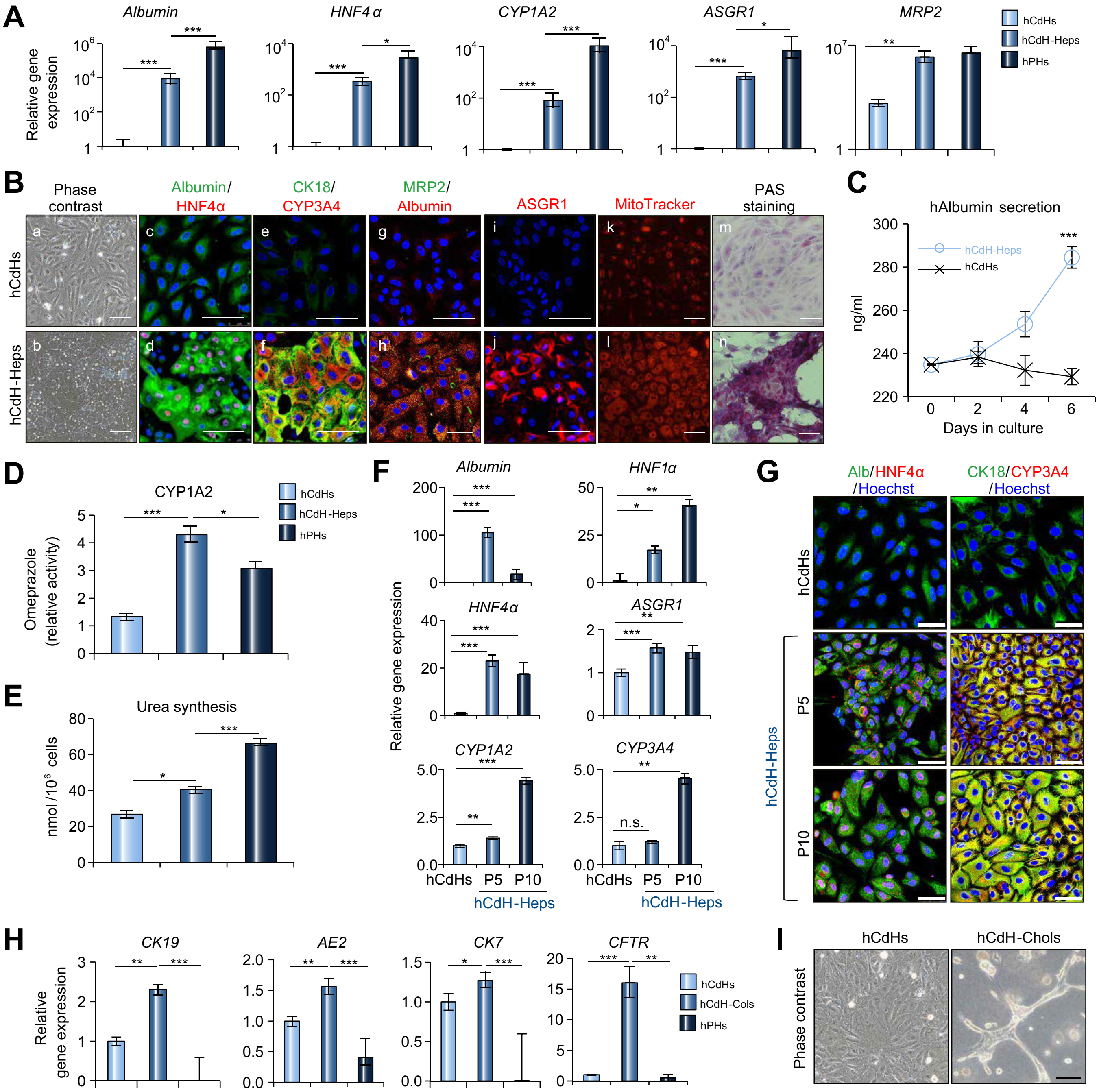

Fig. 4. Bipotent differentiation potential of hCdHs *in vitro*. Differentiation of hCdHs into hepatic (hCdH-Heps) lineage *in vitro*. (A) Increased expression of hepatocyte-specific marker genes determined by RT-qPCR analysis of indicated genes. GAPDH was used as an internal control for RT-qPCR. Data are mean ± SD (n = 3). Data analyzed by 2-tailed *t* tests, **p* <0.05, ***p* <0.01, ****p* <0.001. (B) hCdH-Heps acquire characteristics of mature hepatocytes. Phase contrast images (*a*, *b*); double immunofluorescence staining for mature hepatocyte proteins albumin (green)/HNF4 (red) (*c*, *d*), CK18 (green)/CYP3A4 (red) (*e*, *f*) and MRP2 (green)/albumin (red) (*g*, *h*); immunofluorescence staining for ASGR1 (red) (*i*, *j*); MitoTracker (*k*, *l*); and Periodic Acid-Schiff staining (purple) (*m*, *n*); Nuclei were counterstained with Hoechst 33342 (blue). Scale bars, 100 µm. (C) Human albumin secretion in culture media. Data are mean ± SD (n = 3). ****p* <0.001. (D) CYP1A2 activity. (E) Urea synthesis. Data are mean ± SD (n = 3). Data analyzed by 2-tailed *t* tests, **p* <0.05, ****p* <0.001. (F, G) hCdHs retain their differentiation potency after long-term passaging. (F) Relative mRNA expression levels of indicated genes determined by RT-qPCR. Data are mean ± SD (n = 3). Data analyzed by 2-tailed *t* tests, **p* <0.05, ***p* <0.01, ****p* <0.001. (G) Double immunofluorescence staining of mature hepatocyte proteins albumin (green)/HNF4α (red), and CK18 (green)/CYP3A4 (red). Nuclei were counterstained with Hoechst 33342 (blue). Scale bars, 50 µm. (H) Increased expression of cholangiocyte-specific marker genes determined by RT-qPCR analysis. Data are mean ± SD (n = 3). hPHs were used as a negative control. (I) hCdHs subjected to the cholangiocyte differentiation protocol acquire typical 3D budding structures. Scale bars, 100 µm. hCdHs, human chemically derived hepatic progenitors; hPHs, human primary hepatocytes; RT-qPCR, quantitative reverse transcription PCR.

were also capable to differentiate towards the biliary epithelial cell lineage when grown in a tree-dimensional culture system.[45] They acquired biliary marker expression (Fig. 4H; Fig. S5E) and formed a tube-like branching morphology characteristic of cholangiocyte differentiation as shown by morphology and staining with fluorescein diacetate (Fig. 4I; Fig. S5F).

Transcriptional remodeling during differentiation of hCdHs towards hepatocytes

To gain a more extensive understanding of the global gene expression changes during hCdHs differentiation towards hepatocytes, we compared transcriptomes of hCdHs and hCdH-Heps with human hepatocytes and fetal liver. Unsupervised hierarchical clustering revealed a close relationship between hCdH-Heps and adult hepatocytes (Fig. 5A). Furthermore, GSEA generated using a liver specific gene list of 244 genes[46] confirmed a strong induction of the hepatic gene expression program in hCdH-Heps, whereas hCdHs showed a negative correlation, consistent with a less differentiated phenotype (Fig. 5B). Many of the differentially expressed genes in hCdH-Heps were involved in the diverse biological processes associated with hepatocyte functions, including glucose, lipid, cholesterol and xenobiotic metabolism (Fig. S6).

To further characterize our hCdH-Heps, we compared their global similarities with different cohorts of hepatocyte-like cells and primary hepatocytes. Hepatocyte-like cells were generated by different strategies and from different cell sources such as ESCs (hESC-Heps),[47] iPSCs (hiPSC-Heps), direct reprogramming of human fibroblasts (hiHeps)[48] and liver progenitor-like cells (hep-LPCs-Heps). A clustering analysis of gene expression patterns of our samples and publicly available sequencing data from GEO (https://www.ncbi.nlm.nih.gov/geo/) revealed that among the hepatocyte-like cells of various cell origin, the hCdH-Heps showed the closest clustering and highest correlation with primary hepatocytes in the correlation map (Fig. 5C, D). Of note, hep-LPCs-Heps clustered closer to our hCdH-Heps and human hepatocytes than to hepatocyte-like cells obtained from ESCs, iPSC, or by direct reprogramming of fibroblasts, most likely because of the retention of residual transcriptional memory of donor cells.[49] The expression patterns of hiPSC-derived hepatocytes were the most distant from primary hepatocyte gene sets.

As an indicator of unbiased clustering, the transcriptomic profiles of primary human hepatocytes sequenced in this study and published by Gao *et al.*[48] were remarkably similar despite the differences in the sequencing platforms (HiSeq2000 *vs.* HiSeq2500), type of sequencing (paired-end *vs.* single end), and amount of raw throughput (~7GB *vs.* ~1.3 GB). These results establish that our hCdH-Heps most faithfully recapitulate the transcriptomic profile of human hepatocytes compared to hiPSC- and hESC-derived hepatocytes, and thus could represent a better model system for regenerative medicine.

Differentiation potential of hCdHs *in vivo*

Finally, we tested whether hCdHs can differentiate into functional hepatocytes and cholangiocytes when implanted *in vivo*. For this purpose, we used a novel model of Alb-TRECK/SCID

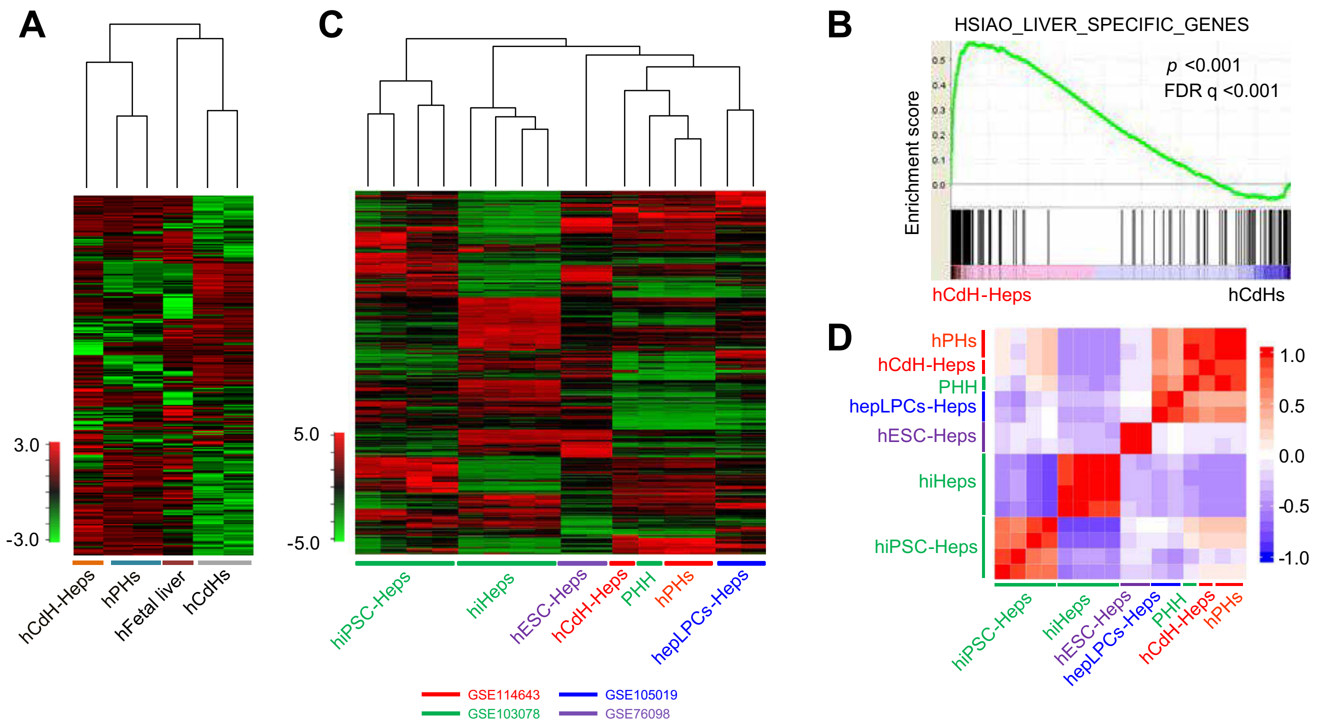

Fig. 5. Transcriptional analysis of hCdHs. (A) Unsupervised hierarchical cluster analysis of global gene expression profiles in hCdHs, hCdH-Heps, fetal liver, and hPHs. The color bar at the left indicates gene expression in log2 scale. Red and green represent higher and lower gene expression levels, respectively. (B) Activation of liver specific gene-set (n = 244 genes)[46] in hCdH-Heps were identified by GSEA. NES reflects the degree of over-representation for each group at the peak of the entire set. Statistical significance was calculated by nominal *p* value of the NES by using an empirical phenotype-based permutation test. False positives are calculated by the FDR. (C) Global similarities of hCdH-Heps with different cohorts of hepatocyte-like cells and primary hepatocytes. Shown is a clustering analysis of gene expression patterns of hCdH-Heps and publicly available sequencing data from GEO (https://www.ncbi.nlm.nih.gov/geo/). Primary hepatocytes (pPHs, GSE114643; PHH, GSE103078), hepatocytes derived from human ESCs (hESC-Heps, GSE76098), human hepatic liver progenitor cells (hepLPCs-Heps, GSE105019), human induced pluripotent stem cells (hiPSC-Heps, GSE103078), and direct reprogramming of human fibroblasts (hiHeps, GSE76098) and hCdH-Heps (GSE114643). The color bar at the lower left indicates gene expression in log2 scale. Red and green colors represent higher or lower than the median across samples. (D) Pearson correlation map. The color bar at the right indicates a value of correlation between 2 samples. Red and blue colors represent higher or lower correlation coefficient between samples. FDR, false discovery rate; GSEA, gene set enrichment analysis; hCdHs, human chemically derived hepatic progenitors; hPHs, human primary hepatocytes; NES, normalized enrichment score; RT-qPCR, quantitative reverse transcription PCR.

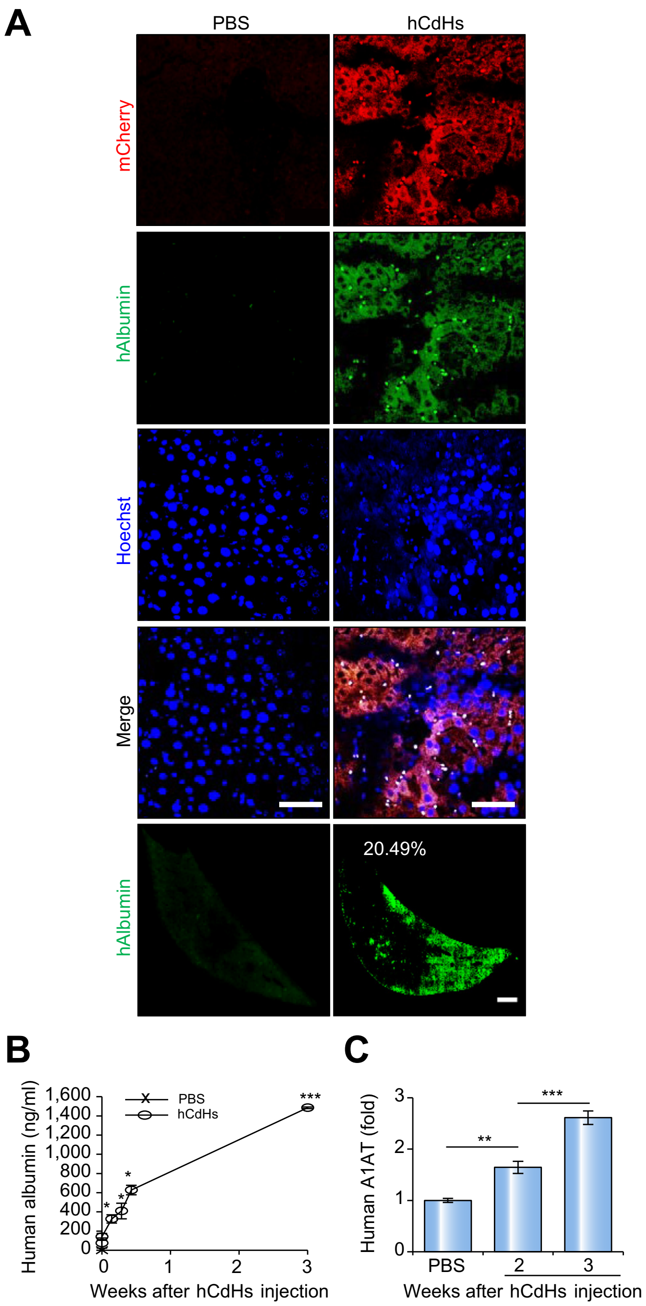

Fig. 6. Liver repopulation by hCdHs in Alb-TRECK/SCID mice. (A) Liver repopulation by mCherry-tagged hCdHs in Alb-TRECK/SCID mice 21 days after transplantation. Cells (1×10^6) were injected via the spleen 24 h after liver injury with diphtheria toxin. Paraffin liver sections were stained with anti-human albumin. Repopulation efficiency was evaluated based on human albumin staining (green) using the Virtual Microscope AxioScan.Z1. Nuclei were counterstained with Hoechst 33342 (blue). Upper scale bars, 100 µm. Bottom scale bars, 1 mm. (B) Detection of human albumin in mouse serum on 0, 1, 24, 48, 96 h, and 21 day after hCdHs injection. Mice injected with PBS served as a negative control. Data analyzed by 2-tailed *t* tests, $^{*}p$ <0.05, $^{***}p$ <0.001. (C) Detection of human A1AT in mouse serum 2 and 3 weeks after hCdHs injection. Data analyzed by 2-tailed *t* tests, $^{**}p$ <0.01, $^{***}p$ <0.001. hCdHs, human chemically derived hepatic progenitors.

mice which develop a fulminant hepatic failure after 1 dose of diphtheria toxin.[34] One million of hCdHs transfected with mCherry reporter gene for easy tracking were transplanted via the spleen into Alb-TRECK/SCID mice 24 h after diphtheria toxin injection. Liver repopulation was assessed by confocal microscopy and the presence of human albumin and A1AT in the recipient mouse serum. hCdHs successfully engrafted and repopulated about 20% of the diseased parenchyma by 3 weeks after transplantation into Alb-TRECK/SCID mice (Fig. 6A) which was paralleled by a steady increase in secretion of human albumin (Fig. 6B) and A1AT (Fig. 6C). Significantly, the albumin levels reached >1 µg/ml which was similar to that found in the Alb-TRECK/SCID mice at 8 weeks after transplantation of human hepatic stem cells[34] and about 10-fold higher than in FRGN mice 2 months after they received injections of 5×10^6 iPSC-Heps (114 ± 50 ng/ml) or hiHeps (153 ± 42 ng/ml).[48] In comparison, blood concentrations of human albumin in FRGN mice repopulated with 1×10^6 human hepatocytes varied between 5–10 µg/ml at later time after transplantation (4–5 weeks).[35] Given that the transplanted hCdHs were undifferentiated when engrafted in mouse liver and their continuing expansion and maturation in the hepatic microenvironment, the secretion of human albumin is expected to rise in parallel with increasing repopulation.

As a proof of principle, we also used 2 additional mouse models of hepatic xenorepopulation, including NSG (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) and FRGN mice. In these models, liver injury was induced either with a single injection of Jo2 antibody in NSG mice or caused by a withdrawal of NTBC in FRGN mice. Functional characterization of NSG mice injected with 10^6 mCherry-positive hepatocytes showed that the majority of mCherry-positive cells acquired properties characteristic of mature hepatocytes. They stained positive for HNF4α, a key driver of hepatocyte maturation, and expressed comparable levels of human albumin as the neighboring mCherry-negative hepatocytes at 2 and 8 weeks after injection (Fig. S7A). Some engrafted hCdHs differentiated towards cholangiocytes as shown by co-expression of mCherry with CK7, a lineage-specific marker of biliary epithelial differentiation (Fig. S7A). No tumors were found at 8 weeks after transplantation (end-point of observation). Likewise, albumin-expressing mCherry-positive hepatocytes were found in FRGN mice 1 week after transplantation (Fig. S7B) while some hCdHs differentiated towards cholangiocytes as shown by CK7 staining (Fig. S7C). As a further demonstration of the successful engraftment and repopulation, we detected a steady increase in the expression levels of human sex-determining region on Y chromosome (hSRY) in the recipient FRGN mouse livers (Fig. S7D). These results indicate that bipotent hCdHs can repopulate injured liver and acquire functional properties of hepatocytes and cholangiocytes upon exposure to an adequate hepatic microenvironment.

Discussion

In this study, we used a recently developed strategy for cell fate modification by small molecules to directly convert human hepatocytes to bipotent hepatic progenitor cells with potential for transplantation therapy.[31] Specifically, 2 small molecules, including A83-01 and CHIR99021, which inhibit TGFβ and GSK3 signaling, respectively, were effective in the reprogramming of mouse and rat hepatocytes[31] and mouse fibroblasts[50] into bipotent progenitor-type cells. However, the attempt to reprogram human hepatocytes to a hepatic progenitor state using the defined set of small molecules was ineffective, suggesting a requirement for additional conversion factors.[31]

Consequently, we tested different combinations of growth factors frequently used in the diverse culture systems to facilitate proliferation and lineage commitment of hepatic precursors of rodent and human origin, including HGF, BMP4, FGF4, retinoic acid, *etc.*[5,12] The addition of HGF, a key driver of liver stem cells, to a cocktail of only 2 small molecules A83-01 and CHIR99021 (AC), proved to be essential for the conversion of adult human hepatocytes to bipotent progenitors hCdHs. Treatment with any single factor, either HGF or AC, did not support the conversion process. Furthermore, blocking the HGF-mediated activation of the MET signaling pathway by the specific tyrosine kinase inhibitor SU11274 dramatically attenuated the generation of the reprogrammed hCdHs. In particular, inhibition of the MET-initiated activation of ERK1/2 by U0126 was found to significantly decrease the number of hCdHs, consistent with the role of ERK1/2 signaling in supporting regenerative proliferation and self-renewal of hepatic progenitor cells.[51,52] The highly efficient generation of hCdHs by a combination of 2 small molecules and HGF may be related to a partial reprogramming, unlike the full reprogramming of somatic cells of different origin which requires more reprogramming factors.[45] Further work is needed to address the molecular mechanisms underlying direct conversion of hepatocytes to a pluripotent state.

The reprogrammed hCdHs acquired characteristics ascribed to hepatic progenitor-like cells. They formed spheroids in ultra-low attachment culture dish (data not shown) and expressed both hepatic (AFP) and cholangiocytic (CK19) marker genes and proteins. Importantly, hCdHs displayed high proliferative potential, and could maintain themselves as a population of undifferentiated precursors without obvious chromosomal abnormalities for at least 10 passages. When subjected to differentiation assays *in vitro*, these cells were capable of differentiating into both hepatocytes and BECs, as assessed by the expression of lineage-specific markers and acquisition of mature functions. Importantly, the differentiation potency of hCdHs did not change upon long-term culture.

Transcriptome profiling corroborated these findings showing extensive transcriptional remodeling in the reprogrammed hCdHs. Thus, GSEA performed to assess lineage stage-specific transcriptional memory revealed the activation of progenitor genes as an essential element of the small molecule-mediated hepatocyte fate conversion process. In comparison, a strong induction of the hepatic gene expression program upon hepatic maturation *in vitro* indicated the efficient differentiation of the commitment of hCdHs towards hepatic lineage. Functional annotation of key expression changes in hCdH-Heps established a significant enrichment of genes involved in glucose, lipid, cholesterol and xenobiotic metabolism.

Finally, we provide evidence that hCdHs could differentiate into both functional hepatocytes and cholangiocytes when implanted *in vivo*. Upon transplantation into an acute hepatic failure Alb-TRECK/SCID mouse model,[34] hCdHs acquired mature hepatocyte properties and secreted more serum albumin in mouse serum compared to either hepatic progenitor cells or hESC- or hPSC-derived hepatocytes,[52] implying that hCdHs possess a greater hepatocyte-forming potential *in vivo*.

Remarkably, the gene expression profiles of hCdH-Heps clustered closer to adult human hepatocytes than any of the hESC- or hiPSC-derived hepatocyte-like cells suggesting that retention of lineage-specific donor memory may facilitate hepatic differentiation for therapeutic application.[49] These data were corroborated by similar findings in 2 additional mouse models of therapeutic liver repopulation, including a model of acute liver injury induced by Jo2 antibody[53] in NSG mice and $Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}$ mice.[33–35] Of note, in the Jo2 model, the transplanted cells did not have a competitive growth advantage because the remaining healthy hepatocytes were capable of proliferating and regenerating the injured parenchyma. Nevertheless, hCdHs successfully integrated into the diseased parenchyma and acquired properties characteristic of mature hepatocytes and BECs. The latter is consistent with the findings by Katsuda *et al.*[31] using the mouse hepatic progenitor cells (CLiPs) reprogrammed by a similar strategy as our hCdHs for liver repopulation in the uPA/SCID mouse model.

In conclusion, we describe the first successful culture system for reprogramming human hepatocytes into bipotential progenitor cells with regenerative potential. Our approach of combining HGF with 2 small molecule inhibitors allows for the generation of a significant number of patient-specific hepatic progenitors and opens new avenues for development of personalized care strategies in cell-based regenerative medicine.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

YK and KK performed the experiments, analyzed the data, and wrote the manuscript. SBL and SY performed the experiments and analyzed the data. DS performed next-generation sequencing experiments, analyzed the data, and wrote the manuscript. SJK performed the animal experiments and analyzed the data. KJ, YKJ and KGL provided the human liver tissue and analyzed the data. VMF provided helpful discussions, and wrote the manuscript. JJ and DC designed the experiments, analyzed the data, and wrote the manuscript.

Supplementary data

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