

Generation of Functional Cholangiocyte-Like Cells From Human Pluripotent Stem Cells and HepaRG Cells

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Cholangiocytes are biliary epithelial cells, which, like hepatocytes, originate from hepatoblasts during embryonic development. In this study we investigated the potential of human embryonic stem cells (hESCs) to differentiate into cholangiocytes and we report a new approach, which drives differentiation of hESCs toward the cholangiocytic lineage using feeder-free and defined culture conditions. After differentiation into hepatic progenitors, hESCs were differentiated further into cholangiocytes using growth hormone, epidermal growth factor, interleukin-6, and then sodium taurocholate. These conditions also allowed us to generate cholangiocytes from HepaRG-derived hepatoblasts. hESC- and HepaRG-derived cholangiocyte-like cells expressed markers of cholangiocytes including cytokeratin 7 and osteopontin, and the transcription factors SOX9 and hepatocyte nuclear factor 6. The cells also displayed specific proteins important for cholangiocyte functions including cystic fibrosis transmembrane conductance regulator, secretin receptor, and nuclear receptors. They formed primary cilia and also responded to hormonal stimulation by increase of intracellular Ca²⁺. We demonstrated by integrative genomics that the expression of genes, which signed hESC- or HepaRG-cholangiocytes, separates hepatocytic lineage from cholangiocyte lineage. When grown in a 3D matrix, cholangiocytes developed epithelial/apicobasal polarity and formed functional cysts and biliary ducts. In addition, we showed that cholangiocyte-like cells could also be generated from human induced pluripotent stem cells, demonstrating the efficacy of our approach with stem/progenitor cells of diverse origins. **Conclusion:** We have developed a robust and efficient method for differentiating pluripotent stem cells into cholangiocyte-like cells, which display structural and functional similarities to bile duct cells in normal liver. These cells will be useful for the *in vitro* study of the molecular mechanisms of bile duct development and have important potential for therapeutic strategies, including bioengineered liver approaches. (HEPATOLOGY 2014;60:700-714)

In the early stages of liver organogenesis, the major components of hepatic parenchyma are hepatic progenitor cells (hepatoblasts, HB), which can differentiate into hepatocytes and cholangiocytes. Cholan-

giocytes are biliary epithelial cells that line the intra- and extrahepatic ducts of the biliary tree. Around the eighth week of gestation in humans, hepatoblasts in the vicinity of the portal mesenchyme form a single

Abbreviations: AChR M3, M3 muscarinic cholinergic receptor; AE2, Cl/HCO₃⁻ anion exchanger 2; AFP, α -fetoprotein; AQP1, aquaporin 1; ASBT, apical Na⁺-dependent bile acid transporter; BMP4, bone morphogenetic protein 4; CFLD, cystic fibrosis liver disease; CFTR, cystic fibrosis transmembrane conductance regulator; CK19, cytokeratin 19; CLE, cholyl-lysyl-fluorescein; EpCAM, epithelial cell adhesion molecule; ESRI, estrogen receptor 1; FOXM1B, Forkhead (fox) factor M1B; FXR, farnesoid X receptor; GEO, gene expression omnibus; GGT1, γ -glutamyltransferase 1; GH, growth hormone; HB, hepatoblast; HepaRG-cHep, hepatocyte-committed HepaRG cells; hESC, human embryonic stem cell; hESC-HB/hiPSC-HB/HepaRG-HB, hESC/hiPSC/HepaRG-derived hepatoblast; hESC-Chol/hiPSC-Chol/HepaRG-Chol, hESC/hiPSC/HepaRG-derived cholangiocyte; HIBEC, human intrahepatic biliary epithelial cell; hiPSC, human induced pluripotent stem cell; HNF3 β , hepatocyte nuclear factor 3 β ; InsP3R type III, inositol 1,4,5-triphosphate receptor type 3; IPA, ingenuity pathway analysis; JAG1, JAGGED 1; MDR1, multidrug resistance protein 1; NaBut, sodium butyrate; NCAM, neural cell adhesion molecule; P2RY1, purinergic receptor P2Y G-protein coupled 1; SCTR, Secretin receptor; SSTR2, somatostatin receptor 2; RXR, retinoid X receptor; SALL4, Sal-like protein 4; TGF β 1, transforming growth factor β 1; TGR5, G protein-coupled bile acid receptor 1; VDR, vitamin D receptor; VEGF, vascular endothelial growth factor.

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layer ring of cells called the “ductal plate,” which give rise to cholangiocytes.¹ The ductal plate is also assumed to be the compartment of hepatic stem cells in fetal and neonatal livers, although this is still controversial.^{2–4} One of the first pathways that prime biliary commitment and formation of the ductal plate is the NOTCH pathway: JAGGED1-positive mesenchymal cells in periportal areas interact with adjacent NOTCH2-positive hepatoblasts and induce cholangiocyte differentiation. NOTCH pathway activation induces SOX9 expression, which is the most specific and earliest marker of biliary cells in developing liver and controls bile duct morphogenesis.¹ Sal-like protein 4 (SALL4) is also reported as a key transcription factor controlling the lineage commitment of hepatoblasts, not only by inhibiting their differentiation into hepatocytes but also by driving their differentiation toward cholangiocytes.

The main physiological function of cholangiocytes is to actively regulate bile composition by modification of hepatocyte-derived bile components through a series of secretory and reabsorptive events. In addition to their role in the modification of ductal bile, cholangiocytes participate in the detoxification of xenobiotics.⁵ They are also the primary targets of injury in a variety of cholestatic liver diseases, ranging from inherited disorders (Alagille syndrome and cystic fibrosis) or autoimmune cholangitis to primary biliary cirrhosis, which represent the main indications for liver transplantation in pediatrics.⁶ Despite the physiological and pathological importance of cholangiocytes, their limited number (3% of the total liver mass) and their intrahepatic localization have limited the development of *in vitro* cell models in order to gain molecular insights into their function. At present, few human cholangiocyte cell lines are available. They are derived from either cholangiocarcinoma or normal cells immortalized by SV40,⁷ and most of the cells studied *in vitro* are

derived from rat cholangiocytes.⁸ However, important dissimilarities exist between rodent and human models: in particular, only human, but not rodent, small cholangiocytes express cystic fibrosis transmembrane conductance regulator (CFTR).⁹

Pluripotent stem cells, which can differentiate into various cell types and display an infinite ability to proliferate, have appeared as an alternative and reproducible source of differentiated cells with therapeutic interest.¹⁰ To date, numerous studies have focused on the development of efficient protocols to generate hepatocyte-like cells, and several groups, including ours, have already generated hepatocyte-like cells from human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) *in vitro*. Our approach recapitulated the key stages of liver development and enabled us to stepwise differentiate hESCs and hiPSCs into definitive endoderm cells, hepatic progenitors, and hepatocyte-like cells in a fully defined medium without feeder cells or serum.^{11,12} However, the question remains as to whether hepatic progenitors generated by way of different protocols, and in particular in our defined conditions, are able to generate cholangiocytes. In other respects, HepaRG is a well-characterized human hepatoma cell line which is permanently able to differentiate into mature hepatocytes from a stage of bipotent progenitors¹³ and which is widely used for pharmacotoxicology assays.¹⁴

In this study we investigated the potential of pluripotent stem cells, hESC and hiPSC, and HepaRG-derived hepatic progenitors (hepatoblasts) to differentiate further along the cholangiocytic lineage. We developed for the first time robust conditions to drive differentiation of different types of progenitors into functional cholangiocyte-like cells. We found that these cells displayed a variety of specific functionalities including those involved in bile acid transport. They formed cilia and responded to stimulation of

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the calcium signaling pathway. We also found that, under 3D maturation conditions, cholangiocytes formed cysts and tubule-like structures with apico-basal polarity.

Materials and Methods

This study was performed in agreement with the French and European regulations.

Differentiation of hESC/hiPSC-Derived Hepatoblasts Into Cholangiocytes. At days 10-12 of differentiation, hESC/hiPSC-hepatoblasts were harvested with cell dissociation buffer (0.1 mg/ml EDTA, 0.5 mg/ml BSA in PBS) and seeded onto 12-well collagen I-coated plates (BD Biosciences) with plating medium (William's E/Ham F12 1:1, 10% FBS [PAA Laboratories], 1 mg/ml fraction V fatty acid-free BSA [Sigma], 1mM L-glutamine) for 4 hours. Cells were then incubated overnight with biliary differentiation medium (BDM) (William's E/Ham F12 1:1, 10^{-5} M linoleic acid-Albumin [Sigma L9530], 5.10^{-8} M 3,3',5-Triiodo-L-thyronine [Sigma T2752], 0.2 IU Insulin, 6.10^{-4} M Vitamin C [Boyer], 6.10^{-4} M human apo-transferrin [Sigma T5391], 1mM sodium pyruvate [Gibco]). The next day, cells were incubated with BDM supplemented with 50ng/ml human growth hormone (GH, Sigma H5916) and 25 ng/ml EGF for three days. Cells were then incubated with 10ng/ml Interleukin 6 (IL-6, Miltenyi 130-093-929) for another three days. At day 17, cells were passaged on collagen I-coated 12-well plates as described for the first passage. At day 18, BDM medium was supplemented with 10 ng/ml IL-6 for three days. Finally, the cells were incubated in $10\mu\text{M}$ sodium taurocholate hydrate (Sigma 86339) for 2 days. For the transcriptome analysis, $1\mu\text{M}$ Sodium Butyrate (NaBut, Sigma 303410) was added to the medium between days 21 to 23 (2 days). Three independent differentiation experiments were performed for hESCs and hiPSCs. Phase-contrast images were taken with a Nikon Eclipse microscope.

Differentiation of HepaRG Progenitors Into Cholangiocytes. HepaRG-HB were treated for 2 days with IL-6 (10 ng/ml), then for 2 days with sodium taurocholate hydrate (10 nM) and then for 2 days with sodium taurocholate hydrate (10 nM) and sodium butyrate (1.8 μM) to prevent spontaneous differentiation along the hepatocytic lineage. Independent culture experiments were performed 4 times.

See Supporting Information for human pluripotent and HepaRG cell culture, hepatoblast differentiation, qRT-PCR and RT-PCR, 3D cell culture assay, immunocytochemistry, transport of fluorescent dye test, flow

cytometry, cell cycle analysis, western blot analysis, and all transcriptomic data analysis.

Results

Differentiation of hESCs Into Hepatoblasts. Our strategy to induce differentiation of hESCs into hepatoblasts was based on a previously published protocol^{11,12} with some modifications (see Supporting Materials for details). The differentiation protocol is illustrated in Fig. 1A. At day 0, hESCs displayed typical colony morphology of stem cells (Fig. 1B, panel i). The cells expressed pluripotency markers including OCT4, NANOG, TRA-1-60, and SSEA4 and were negative for GATA4, confirming the absence of endodermal differentiation (Fig. 1C, panel i). Flow cytometry analysis showed that more than 70% of the cells expressed TRA-1-81 (Fig. 1D, panel i). Five days of endoderm induction treatment resulted in a homogeneous monolayer of polygonal endodermal cells (Fig. 1B, panel ii). Immunostaining revealed that most of the cells were positive for the definitive endoderm markers GATA4, CXCR4, hepatocyte nuclear factor 3 β (HNF3 β), and SOX17 (Fig. 1C, panel ii). Flow cytometry analysis confirmed that more than 90% of the cells expressed CXCR4 (Fig. 1D, panel ii). Following the endodermal induction, cells were treated with the hepatic commitment factors for 3 days, which gave rise to specified hepatic endoderm/liver bud (Fig. 1B, panel iii). Immunostaining of these cells confirmed that they expressed characteristic markers HNF3 β , HNF4 α , cytokeratin 19 (CK19), CK18, SOX9, SOX17, GATA4, and neural cell adhesion molecule (NCAM). In addition, these cells were negative for α -fetoprotein (AFP) and CK7, the cytokeratin specifically expressed by cholangiocytes (Fig. 1C, panel iii; Supporting Fig. 1). Most of them (93%) were also positive for epithelial cell adhesion molecule (EpCAM) as assessed by flow cytometry analysis (Fig. 1D, panel iii). Finally, the medium was supplemented with factors inducing differentiation of hepatic endodermal cells into hepatoblasts with a polygonal morphology (Fig. 1B, panel iv). Immunostaining of cells at day 10 showed that they were positive for AFP and CK19. hESC-derived hepatoblasts (hESC-HB) also expressed the transcription factors HNF3 β , GATA4, HNF6, and HNF4 α (Fig. 1C, panel iv). Flow cytometry analysis revealed that most of the cells (87%) remained positive for EpCAM (Fig. 1D, panel iv).

hESC-Derived Hepatoblasts Exhibit the Potential for Commitment Toward Cholangiocyte Precursors. Like NOTCH2 and SALL4, forkhead factor M1B (FOXM1B) is also reported to be critical for

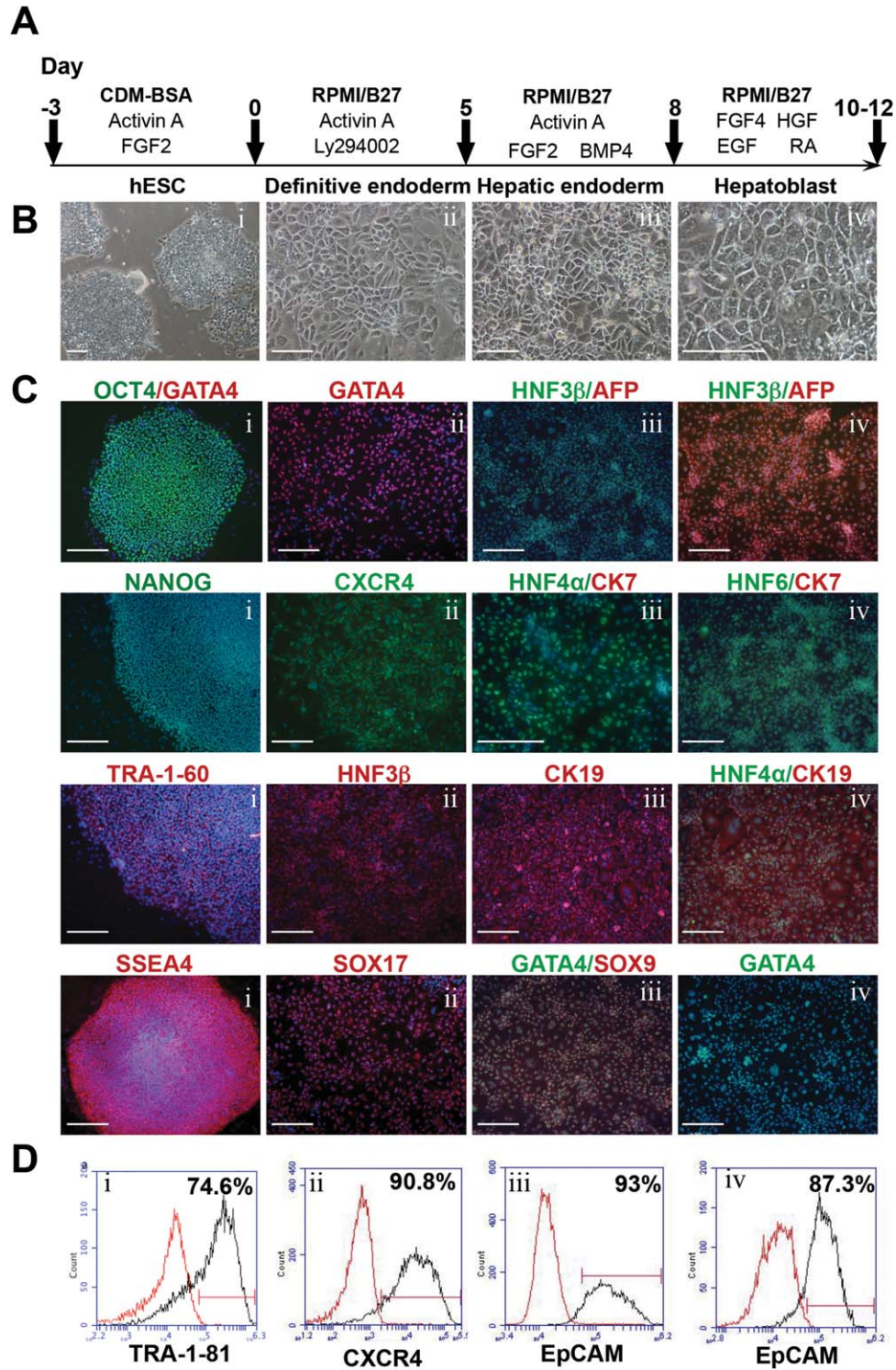


Fig. 1. Generation of hepatoblasts from human embryonic stem cells (hESCs). (A) Protocol to differentiate hESCs into progenitors. (B) Images showing the sequential morphological changes that occur to give a polygonal shape after 10 days of culture in appropriate conditions. (C) Immunocytochemistry showing the expression of pluripotency markers (OCT4, NANOG, TRA-1-60, SSEA4) at day 0 (Panel i) followed by the expression of definitive endoderm markers (GATA4, CXCR4, HNF3 β , SOX17) at day 5 (Panel ii) and the expression of hepatic endodermal cells markers (HNF3 β , HNF4 α , CK19, GATA4, SOX9) at day 8 (Panel iii). At day 10, cells are positive for HNF3 β , AFP, HNF6, HNF4 α , CK19, and GATA4 and negative for CK7 (Panel iv). (D) Flow cytometry analysis of pluripotency marker TRA-1-81 (Panel i), definitive endoderm marker CXCR4 (Panel ii) at day 5, and hepatic endoderm/progenitor marker EpCAM expression (Panel iii and iv) respectively. Scale bars = 100 μ m.

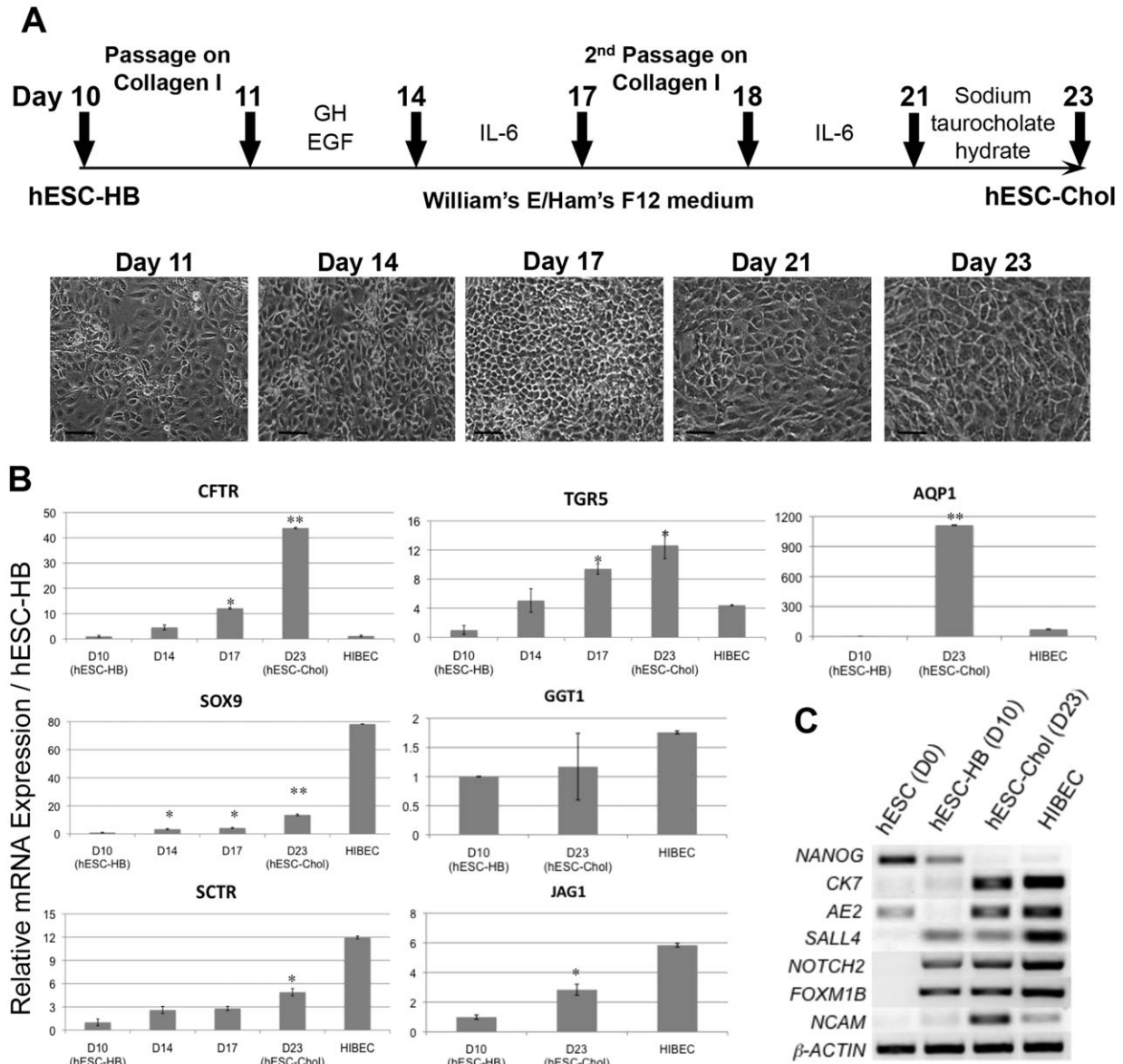


Fig. 2. hESC-derived hepatoblasts differentiate into cholangiocytes. (A) Diagram summarizing our cholangiocytic differentiation protocol. hESC that had been maintained in a feeder-free condition were differentiated into hepatoblasts before passaging onto collagen I-treated wells, then induced into cholangiocytic differentiation. Cells were grown 3 days in GH and EGF then IL-6. At day 17, cells reached confluency and were replated onto collagen I-treated wells. Cells were further differentiated for 3 days in IL-6, then for 2 additional days in sodium taurocholate hydrate. (B) Cholangiocytic marker gene expression level was quantified at different timepoints of the differentiation procedure by qRT-PCR. Human intrahepatic biliary epithelial cells (HIBEC) cDNA was used as a positive control. In all histograms, the value of hESC-HB was arbitrarily set to 1. * $P < 0.05$; ** $P < 0.01$. (C) RT-PCR analysis of gene expression of pluripotency marker NANOG and of the biliary markers CK7, anion exchanger 2, SALL4, NOTCH2, FOXM1B, and NCAM in hESCs, hESC-derived hepatoblasts (hESC-HB), and hESC-derived cholangiocytes (hESC-Chol). Results represent the mean \pm SD of three independent experiments.

differentiation of precursors toward biliary epithelial cells.¹⁵ To further define the biliary commitment potential of our hepatoblasts we analyzed the expression of different biliary markers. We found that hESC-HBs expressed FOXM1B, NOTCH2, and SALL4 (Fig. 2C).

To then trigger cholangiocyte differentiation and proliferation of committed hepatoblasts, we tested factors in diverse combinations: epidermal growth factor

(EGF) and interleukin 6 (IL-6)¹⁶ present in fetal liver and growth hormone (GH), a regulator of the insulin-like growth factor-1 (IGF1) pathway,¹⁷ the receptor of which is expressed in early periportal hepatoblasts.¹⁸ We also tested the effect of sodium taurocholate hydrate, which not only stimulates proliferation and differentiation of cholangiocytes but also displays anti-apoptotic activity on these cells in rat.¹⁹ Our final differentiation strategy is illustrated in Fig. 2A.

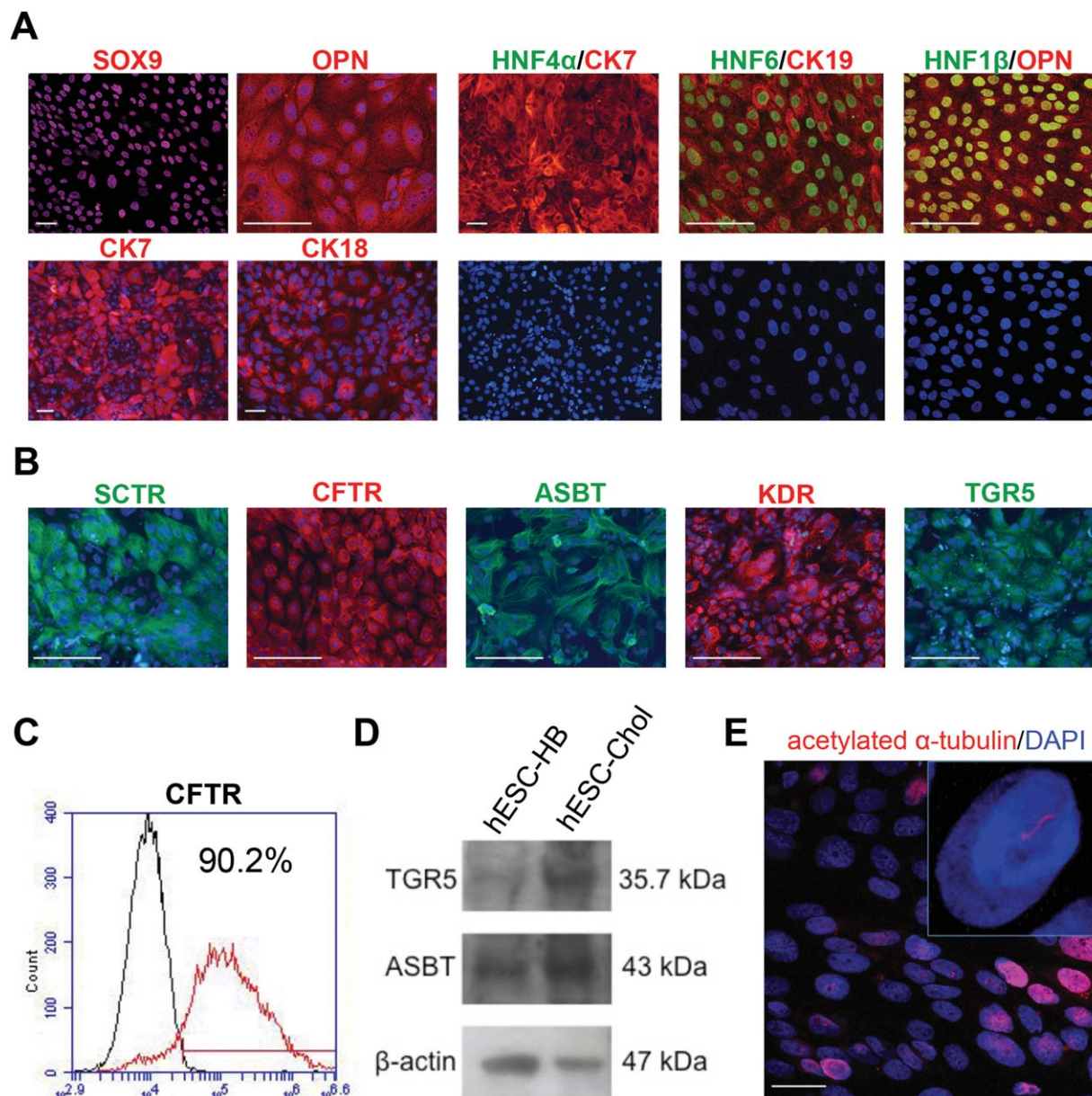


Fig. 3. Hepatic cells generated from hESCs display characteristics specific to cholangiocytes. (A) Immunocytochemical analysis at day 23 of differentiation shows the expression of cholangiocyte markers SOX9, OPN, CK7, CK19, CK18, HNF1 β , HNF6. CK7-positive cells were negative for hepatocytic marker HNF4 α . (B) Expression of cholangiocyte-specific transporters (SCTR, CFTR, ASBT, TGR5) and of VEGF receptor 2 (KDR). Scale bars = 50 μ m. (C) Flow cytometry analysis illustrates that more than 90% of cells express CFTR receptor. (D) Western blot analysis confirmed the expression of cholangiocyte transporters TGR5 and ASBT in differentiated cells. (E) Immunocytochemical analysis shows the expression of acetylated α -tubulin localized on primary cilia of cholangiocytes. Cholangiocyte nuclei were visualized by staining with 4',6-diamidino-2-phenylindole (DAPI, shown in blue). Scale bar = 20 μ m.

At the hepatoblast stage (day 10), cells were passaged on collagen I-coated plates and maintained for 1 day in William's E/Ham's F12 medium as described in the Materials and Methods. Cell cycle analysis showed that after 3 days of GH/EGF the cells were actively proliferating. The percentage of cells in S and G2/M phase was 36% and 8%, respectively (Supporting Fig. 2A). Differentiating cells remained in proliferation

after addition of IL-6, yielding up to 68% Ki67-positive cycling cells (Supporting Fig. 2B). This was confirmed by expression of Cyclin A, Cyclin E, CDK1, and CDK2 (Supporting Fig. 2C).

Gene expression of differentiating cells was analyzed after addition of GH/EGF and IL-6 by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). A combination of these factors increased the

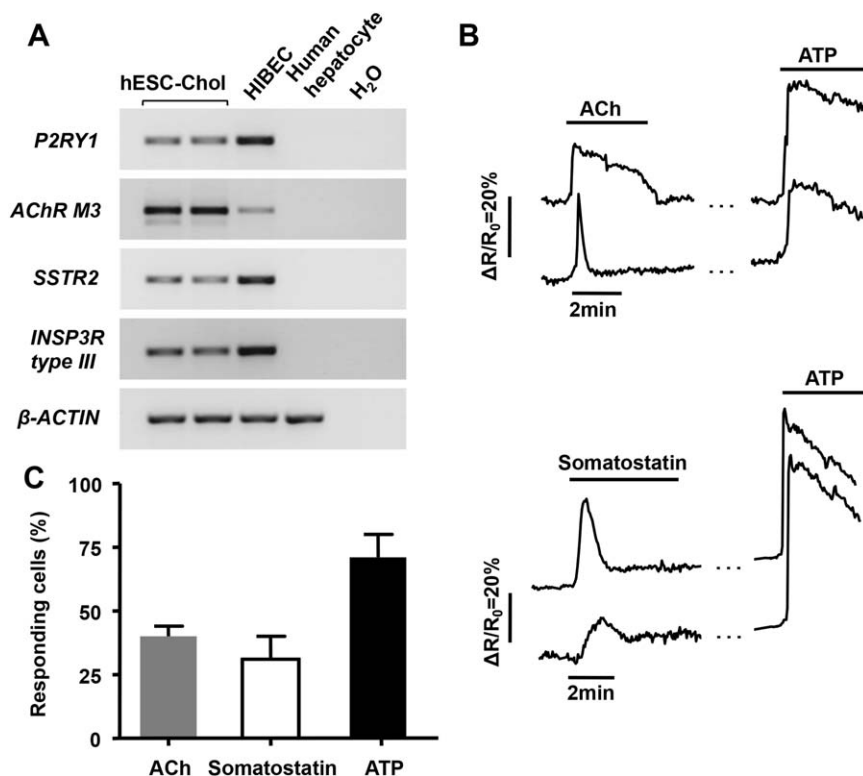


Fig. 4. Agonists induce Ca^{2+} increase in hESC-Chol. (A) RT-PCR analysis of gene expression of receptors involved in intracellular Ca^{2+} signaling: P2RY1, AChR M3, SSTR2, INSP3R type III. (B,C) Fura-2-loaded hESC-Chol were stimulated either with acetylcholine (ACh, 1 μM), somatostatin (1 nM), or ATP (30 μM) for the times indicated by the horizontal bars. Traces have been shifted arbitrarily along the y-axis for clarity. For technical convenience, traces were interrupted during the washes (each gap lasted 5 minutes). The traces shown are representative of the Ca^{2+} signal observed in the presence of these different agonists in responding cells in four independent experiments. (D) Summary of the Ca^{2+} induction data (mean \pm SEM).

expression of cholangiocyte markers such as CFTR, G protein-coupled bile acid receptor 1 (TGR5), SOX9, and secretin receptor (SCTR) (Fig. 2B).

hESC-Derived Hepatoblasts Differentiate Into Functional Cholangiocyte-Like Cells. Treatment of hepatoblasts with GH/EGF then IL-6 allowed the cells to reach confluency around day 17. This population of proliferating biliary-committed cells was then passaged on collagen I. The cells were treated with IL-6 for 3 days and then with sodium taurocholate hydrate for 2 days. In the course of differentiation the cell population progressively acquired a cuboidal morphology (Fig. 2A). qRT-PCR analysis revealed a significant increase in gene expression level of cholangiocyte markers such as CFTR, TGR5, aquaporin-1 (AQP1), SOX9, SCTR, and JAG1 in hESC-derived cholangiocytes (hESC-Chol) compared to hESC-HB (Fig. 2B). The expression level of γ -glutamyltransferase 1 (GGT1) was equivalent to that of hESC-HB and control cells.

In addition, RT-PCR analysis on hESC-Chol revealed that these cells expressed CK7, and also biliary markers including $\text{Cl}^-/\text{HCO}_3^-$ anion exchanger 2 (AE2), and NCAM, a marker of proliferating cholangiocytes.²⁰ We

observed that SALL4, NOTCH2, and FOXM1B were also expressed in hESC-Chol, whereas the stemness marker NANOG was extinguished (Fig. 2C).

We also evaluated hepatocytic marker expression by qRT-PCR in hESC-Chol. The AFP and albumin (ALB) expression level was 10^4 and 10^7 less than in fetal and adult hepatocytes, respectively. Aldolase B transcripts were not detectable (Supporting Fig. 3A).

Cholangiocyte differentiation was confirmed by immunostaining analysis, showing that hESC-derived cholangiocytes expressed CK7, CK19, CK18, osteopontin (OPN), a downstream target of SOX9 during normal development,²¹ SOX9, HNF6, and HNF1 β , whereas HNF4 α expression was not detected, as shown by costaining experiments with CK7/HNF4 α (Fig. 3A).

In addition, to further confirm the identity of these cholangiocyte-like cells, we tested whether these cells expressed specific biliary receptors. Immunostaining analysis revealed that these cells expressed CFTR, SCTR, apical Na^+ -dependent bile acid transporter (ASBT), TGR5, and KDR (VEGFR-2) (Fig. 3B). Flow cytometry analysis revealed that 90% of the cells

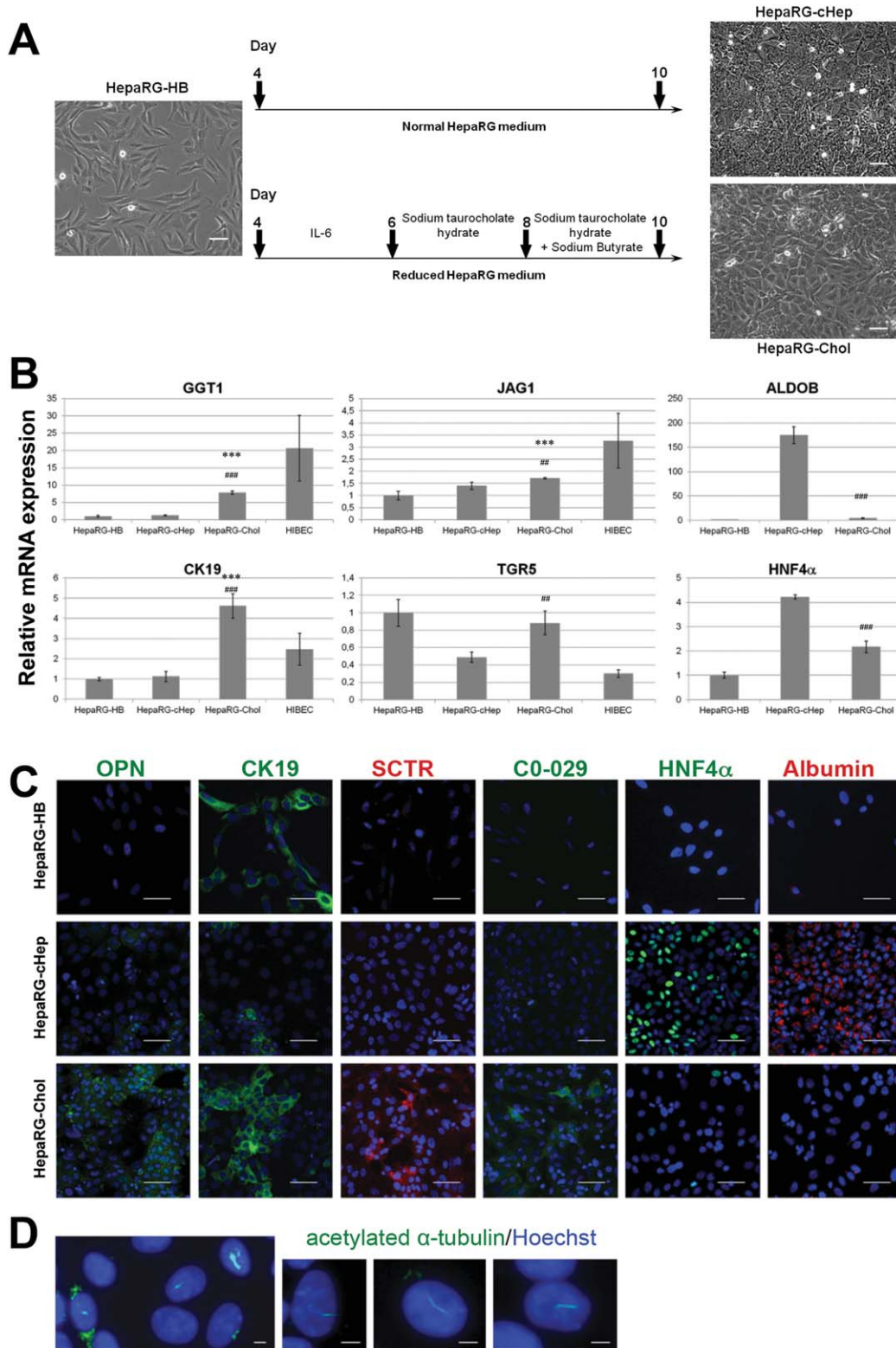


Fig. 5. Differentiation of HepaRG-hepatoblasts into cholangiocytes. (A) Diagram of both protocols for HepaRG cell differentiation from the progenitor stage into hepatocytes or cholangiocytes. Scale bars = 50 μ m. (B) QRT-PCR analysis of the cholangiocyte marker expression GGT1, JAG1, CK19, and TGR5 and of the hepatocytic marker expression ALDOB and HNF4 α , compared to HIBEC (positive control), during cholangiocyte and hepatocytic differentiation, respectively. *** P < 0.001, HepaRG-Chol versus HepaRG-HB; ## P < 0.01, HepaRG-Chol versus HepaRG-cHep; ### P < 0.001, HepaRG-Chol versus HepaRG-cHep. (C) Immunocytochemical analysis shows the expression of OPN, CK19, SCTR, CO-029, HNF4 α , and albumin in HepaRG-HB, HepaRG-cHep, and HepaRG-Chol. Scale bars = 50 μ m. (D) Immunocytochemical analysis shows the expression of acetylated α -tubulin localized on primary cilia of cholangiocytes. Cholangiocyte nuclei were visualized by staining with Hoechst (shown in blue). Scale bars = 5 μ m.

expressed CFTR (Fig. 3C). We also examined expression of cholangiocyte transporters by western blot analysis, which confirmed expression of ASBT and TGR5 in hESC-derived cholangiocytes (Fig. 3D).

By contrast to hepatocytes, cholangiocytes bear primary cilia, which play an important role in modulating the secretory and proliferative functions of fully differentiated cells.²² The presence of cilia (one cilium per cell) was detected using an antibody to a ciliary marker, acetylated α -tubulin (Fig. 3E).

To assess the functionality of our hESC-Chol, we analyzed their response to adenosine triphosphate (ATP), acetylcholine, and somatostatin, known to induce a Ca^{2+} increase in biliary cells by way of P2Y1 receptors,⁵ M3 muscarinic receptor (AChR M3),²³ and somatostatin receptor type 2 (SSTR2),²⁴ respectively, as well as type III Inositol 1,4,5-triphosphate (InsP3) receptor (InsP3R), an intracellular Ca^{2+} channel, which stimulates the release of Ca^{2+} from intracellular stores into the cytosol.²⁵

RT-PCR on hESC-Chol revealed that the four different receptors were specifically expressed in these cells compared to human hepatocytes (Fig. 4A). ATP, acetylcholine, or somatostatin stimulation resulted in a Ca^{2+} increase (Fig. 4B). The percentage of responsive cells was quantified to be $70\% \pm 5\%$, $40\% \pm 4\%$, and $31\% \pm 9\%$, respectively (Fig. 4C).

HepaRG-Progenitor Cells Can Be Driven to Differentiate Into Cholangiocytes. We then assessed whether our protocol could be used to drive differentiation of HepaRG to a cholangiocyte lineage.^{13,14} In our conditions, addition of GH did not improve proliferation and differentiation of HepaRG-hepatoblasts (HepaRG-HB) (data not shown) since they expressed HNF3 β (FOXA2), required for normal bile duct development²⁶ and a very low level of C/EBP α , known to play a role in hepatocyte differentiation.²⁷ However, 2 days of IL-6 followed by 2 days of sodium taurocholate hydrate treatment was sufficient to drive commitment toward cholangiocyte differentiation (Fig. 5A). Then, to prevent spontaneous differentiation of HepaRG-HB into hepatocytes, Na butyrate (NaBut) was added after 2 days of sodium taurocholate hydrate treatment.²⁸

At the end of the differentiation protocol (day 10), HepaRG-derived cholangiocytes (HepaRG-Chol) expressed high levels of GGT1, CK19, JAGGED1, and TGR5 compared to hepatocyte-committed HepaRG cells (HepaRG-cHep) and HepaRG-HB (Fig. 5B).

Immunostaining revealed that HepaRG-Chol expressed OPN, CK19, SCTR, and tetraspanin-8 (CO-029), whereas they were not expressed in our regular

HepaRG culture conditions. Hepatocytic markers such as ALB and HNF4 α were repressed (Fig. 5C).

Immunostaining of acetylated α -tubulin revealed the presence of primary cilia in HepaRG-Chol (Fig. 5D). In addition, HepaRG-Chol expressed the receptors P2RY1, AChR M3, SSTR2, and InsP3R at levels similar to that of hESC-Chol (Supporting Fig. 3B).

Finally, since cholangiocytes proliferate in response to VEGF (vascular endothelial growth factor), we stimulated HepaRG-Chol with VEGF, which induced a significant 2-fold increase in the number of cells in G2/M phases (Supporting Fig. 2D).

Transcriptomic Profiles of Cholangiocytes Derived From Both hESCs and HepaRG. To analyze gene expression profiling in cholangiocytes derived from both hESCs and HepaRG under similar conditions, we first verified that addition of NaBut on hESC-Chol did not significantly change expression of cholangiocyte markers (Supporting Fig. 3C,D). Microarrays were conducted at day 23 and day 10 of differentiation for hESC-Chol and HepaRG-Chol, respectively, and compared to hESC-HB and HepaRG-HB. Integrative genomics was first used to determine the level of differentiation of the cells. The 2920 genes differentially expressed in cholangiocytes (analysis of variance [ANOVA]) were integrated with gene expression profiles of normal human biliary epithelial cells²⁹ and of an immortalized human intrahepatic biliary epithelial cell line (H69).³⁰ Hierarchical clustering analysis revealed two main branches dividing the samples with regard to the lineage: hESC/hepatoblasts/cholangiocytes in one branch and hepatocytes in the other (Fig. 6A). Cluster 1 included both hESC-Chol and HepaRG-Chol as well as H69 and normal human biliary epithelial cells. Cluster 2 included HepaRG-Hep and HepaRG-cHep. Cluster 1 was divided into two branches driven by hESC- or HepaRG-Chol differentiation. HepaRG-Chol clustered with both H69 cell line and human normal biliary epithelial cells. This subdivision probably reflects the origin of the cells. HepaRG cells derive from differentiated hepatocellular carcinoma; normal human biliary epithelial cells were microdissected from six metastatic liver tumors,³⁰ and H69 cells are SV40-transformed cells.²⁹

During hESC-HB differentiation we found that up to 3,429 genes were deregulated ($P < 0.05$; FC > 2) (Fig. 6B; Supporting Table 1). In HepaRG-Chol only 197 genes were significantly deregulated ($P < 0.05$; FC > 2) (Supporting Table 2). Interestingly, ingenuity pathway analysis (IPA) revealed that the 489 strongest up-regulated genes ($P < 0.001$; FC > 7) in hESC-Chol and the 126 up-regulated genes in HepaRG-Chol were

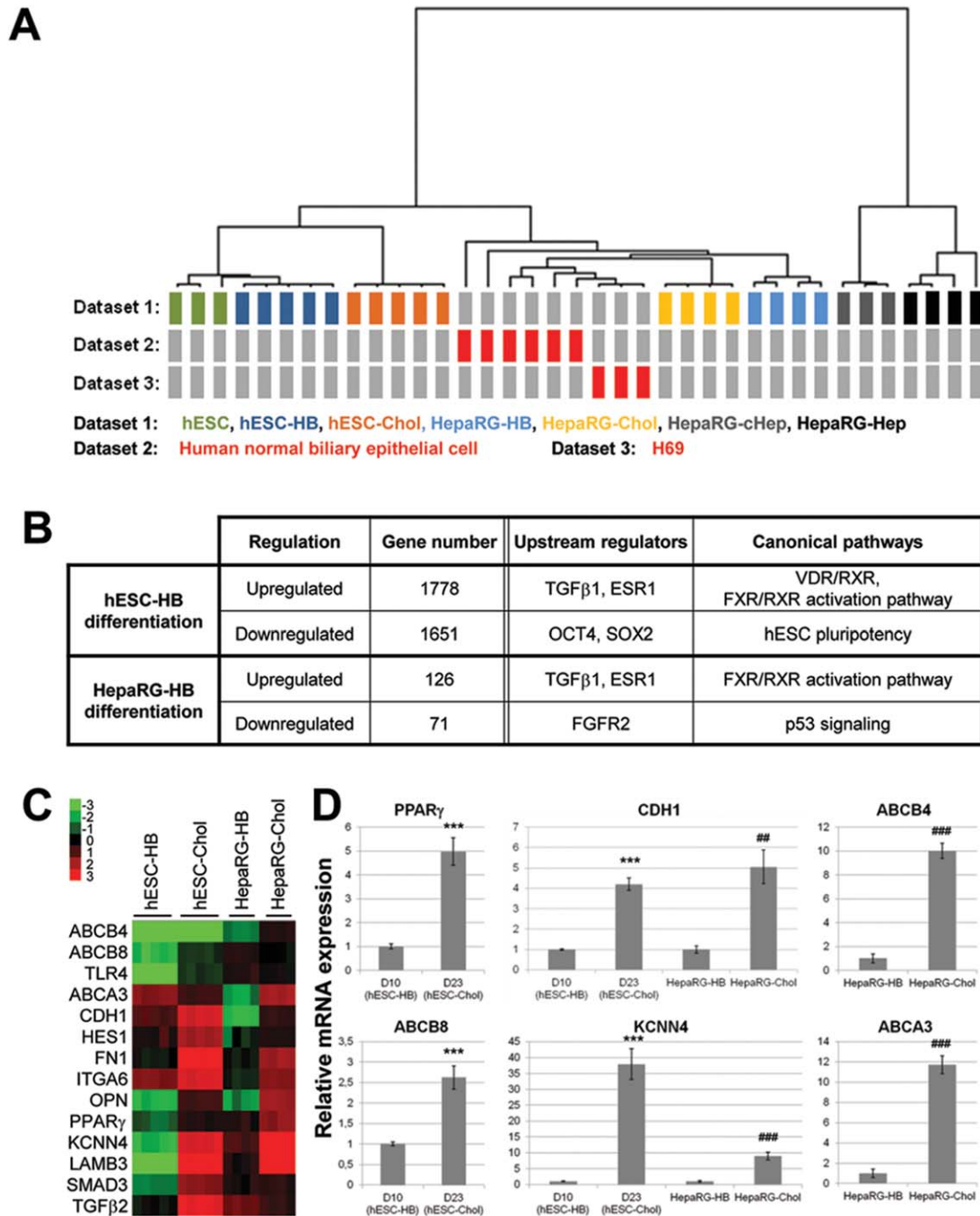


Fig. 6. Transcriptomic profiles of cholangiocytes derived from both hESCs and HepaRG. (A) Integrative genomic analysis of hESC-Chol and HepaRG-Chol gene signature (ANOVA $P < 0.001$; FC > 3) (dataset 1) with normal human biliary epithelial cells (dataset 2) and immortalized human intrahepatic biliary epithelial cell line H69 (dataset 3). Dataset 2 corresponds to six samples of human normal biliary epithelial cells. Dataset 3 corresponds to three samples of the H69 cell line. The dendrogram shows a significant separation of the hepatocytic lineage from hESC/hepatoblast/cholangiocyte lineages. (B) Number of genes significantly deregulated in hESC-Chol and HepaRG-Chol ($P < 0.05$, FC > 2). The top upstream regulators and canonical pathways were highlighted by IPA. (C) Heat map showing the expression of key genes in hESC-Chol and/or HepaRG-Chol. Up-regulation is represented by red shading; down-regulation is represented by green shading. (D) mRNA levels of common deregulated genes (middle panel), hESC-Chol specific genes (left panel), and HepaRG-Chol specific genes (right panel) were validated by qRT-PCR. A high correlation was obtained between microarray data and qRT-PCR analysis for all the specific genes validated by qRT-PCR. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

involved in common pathways including the farnesoid X receptor / retinoid X receptor (FXR/RXR) activation pathway. In addition, vitamin D receptor (VDR)/RXR was strongly up-regulated in hESC-Chol. Two of the

main upstream regulators of up-regulated genes were transforming growth factor beta 1 (TGFβ1) and estrogen receptor 1 (ESR1), known to play a role in modulating rat cholangiocyte proliferation.³¹

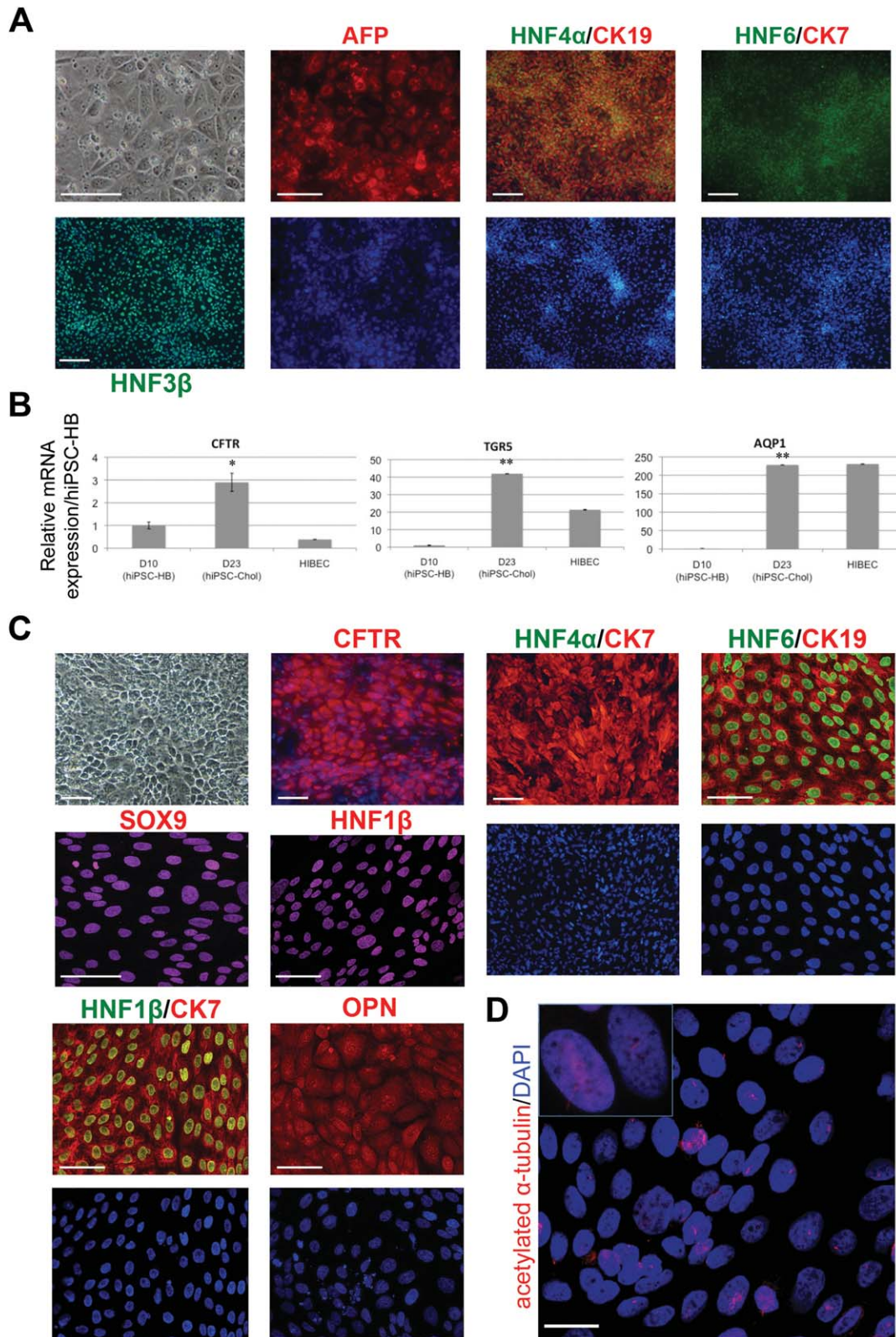


Fig. 7. Generation of cholangiocytes from hiPSCs. (A) Immunocytochemical analysis of hiPSC-derived hepatoblasts showing the expression of hepatoblast markers AFP, HNF4 α , HNF6, HNF3 β , and CK19. (B) qRT-PCR analysis of the cholangiocyte markers CFTR, TGR5, and AQP1 showing a significant increase in hiPSC-Chol compared to hiPSC-HB. * $P < 0.05$; ** $P < 0.01$. (C) hiPSC-Chol acquire an epithelial morphology similar to that of hESC-Chol and express cholangiocyte markers SOX9, HNF6, CK19, CK7, and CFTR, whereas HNF4 α expression is repressed in CK7-positive cells. Scale bars = 50 μ m. (D) Immunocytochemical analysis showing the expression of acetylated α -tubulin localized in primary cilia of cholangiocytes. Cholangiocyte nuclei were visualized by staining with DAPI (shown in blue). Scale bar = 20 μ m.

Comparison of both expression profiles of hESC-Chol and HepaRG-Chol highlighted up-regulation of common and distinct genes (Fig. 6C). For instance, OPN, E-cadherin (CDH1), and KCNN4, a potassium intermediate-conductance Ca^{2+} -activated K^{+} channel, were up-regulated in both cell types. By contrast, peroxisome proliferator activated receptor gamma (PPAR γ) or the ABCB8 transporter were specifically up-regulated only in hESC-Chol. Other transporters such as ABCA3 and ABCB4 were induced specifically in HepaRG-Chol. Expression of these genes was confirmed by qRT-PCR (Fig. 6D).

Generation of Cholangiocytes From hiPSCs. Finally, we investigated whether the culture conditions developed to differentiate hESCs into cholangiocytes could also efficiently drive differentiation of hiPSCs. To this end, we used the iPS P2 cell line, which was reprogrammed using a polycistronic retroviral vector encoding OCT4, SOX2, KLF4, and C-Myc.³² iPSCs were characterized for pluripotency markers such as OCT4, NANOG, TRA-1-60, and SSEA4 by immunostaining and flow cytometry (Supporting Fig. 4A-C). iPSC P2 cells were differentiated into hepatoblasts as described for hESCs. Immunostaining confirmed that hiPSC-derived hepatoblasts (hiPSC-HB) expressed hepatic progenitor markers AFP, HNF4 α , CK19, HNF6, and HNF3 β , whereas they were negative for biliary marker CK7 (Fig. 7A). hiPSC-HB were then differentiated into cholangiocytes (hiPSC-Chol). Analysis of biliary markers expressed in hiPSC-Chol by qRT-PCR revealed a significant increase in CFTR, TGR5, and AQP1 expression compared to that of hiPSC-HB (Fig. 7B). In addition, hiPSC-Chol expressed biliary markers CK7, CK19, HNF6, SOX9, and CFTR, whereas expression of HNF4 α was not detected (Fig. 7C).

As in hESC-Chol, AFP, ALB, and aldolase B expression level was 10^4 , 10^6 , and 10^6 less than in fetal and adult hepatocytes, respectively (Supporting Fig. 3A). Expression of cholangiocyte-specific calcium signaling receptors SSTR2, P2RY1, InsP3R type III, and AChR M3 was confirmed in hiPSC-Chol (Supporting Fig. 5). Finally, the presence of primary cilia was visualized by immunostaining (Fig. 7D). Taken together, these data suggest that our approach, developed initially for hESCs, can be used to generate cholangiocyte-like cells from hiPSCs as well as from HepaRG cells.

Functionality of Cholangiocyte-Like Cells Generated From Pluripotent Stem Cells and HepaRG. To assess the potential of cholangiocytes derived from hESC/hiPSC and HepaRG to form cysts and tubules,

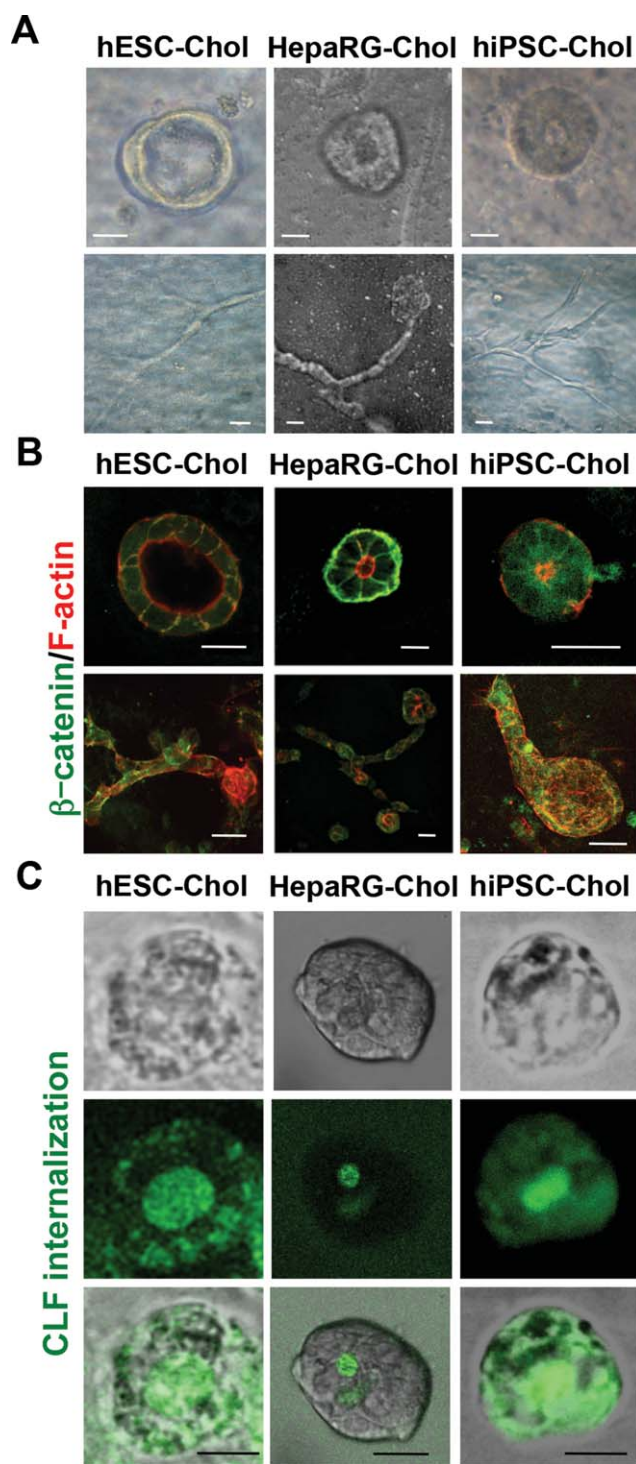


Fig. 8. Hepatic cells generated from hESC/hiPSC/HepaRG display functional properties specific to cholangiocytes. (A) Images showing the morphology of cysts and tubules after 2 weeks in the 3D culture system. (B) Immunocytochemical analysis showing epithelial polarity by the expression of β -catenin on basolateral membrane of the cysts and of F-actin bundles on the apical side of the cells in the lumen. (C) Confocal microscopy images show transport of cholyl-L-lysyl-fluorescein into the central lumen of a cyst. Scale bars = 20 μm .

we used a 3D culture system. After 7 days of culture, cholangiocytes formed round cysts with luminal space. Moreover, when the cysts were kept in culture for 1 more week, they were able to bud, and formed branching tubular structures (Fig. 8A). Of note, HepaRG-cHep cultured in 3D did not form cysts and tubules. Cyst polarity was verified by basolateral and apical localization of β -catenin and F-actin, respectively (Fig. 8B). A physiological function of cholangiocytes is the secretion of small substances by various transmembrane channel proteins such as multidrug resistance protein 1 (MDR1). After incubation with rhodamine 123, MDR1 substrate, fluorescence accumulation was detected inside the central lumen of hESC/hiPSC/HepaRG-cysts. Furthermore, when cysts were incubated in verapamil, an MDR1 inhibitor, rhodamine 123 transport was abolished (Supporting Fig. 6). Since MDR1 is also expressed by progenitor cells³³ we further investigated the transport of fluorescent bile salt, a property of functional cholangiocytes. When cysts were incubated with cholyl-lysyl-fluorescein (CLF), fluorescence accumulation was detected inside the central lumen (Fig. 8C).

Discussion

This study provides the first evidence of the differentiation of functional cholangiocyte-like cells from hepatic progenitors generated from human pluripotent stem cells and HepaRG cells. Our approach is based on fully defined culture conditions devoid of serum and of feeder cells.

In vivo maturation of hepatoblasts to cholangiocytes is regulated by several factors secreted in the microenvironment around the portal vein, which consists of mesenchymal cells, other cells, and extracellular matrix. After liver specification, HNF3 β , HNF6, and HNF1 β are required for normal bile duct development.³⁴ HNF1 β , a direct target of HNF6, is also required for duct morphogenesis. We found that these transcription factors were expressed in hepatoblasts and in differentiating cholangiocytes. SOX9, which is also regulated by HNF6, was expressed in our cholangiocytes and, interestingly, it is known to negatively regulate C/EBP α governing transcription of mature hepatocyte marker genes.^{1,27,35}

As hepatoblasts forming primitive ductal structures respond to periportal mesenchymal cues such as bone morphogenetic protein 4 (BMP4), TGF β 1, and VEGF, we first analyzed the effect of these factors on hepatic progenitors. We found that addition of these factors did not induce significant cell commitment

toward cholangiocytes in our conditions (data not shown). Therefore, since it seemed difficult to find a signal pathway truly specific for cholangiocyte differentiation, we reasoned that addition of GH on hepatoblasts, followed by IL-6, might efficiently trigger these cells toward the cholangiocyte lineage and induce their proliferation. Indeed, GH is present abundantly in fetal serum and GH receptor is expressed in fetal liver mainly in periportal hepatoblasts as early as 8.5 weeks of gestation in human, when ductal plate is formed.^{18,36} GH controls IGF production and its signaling pathway, and IGF1 and its receptor are expressed in ductal plate cells.³⁷ In addition, IL-6 is also expressed in developing cholangiocytes.¹⁶

Interestingly, transcriptome analysis revealed that factors important for ductal plate commitment and/or their downstream targets were activated in both hESC/HepaRG-cholangiocytes. For instance, downstream target genes of TGF β , including those coding for matrix proteins such as fibronectin 1, integrin α 6 and laminin 3, were expressed by both hESC/HepaRG-Chol. Of note, we confirmed by immunostaining that the cells also expressed OPN and VEGFR-2. NOTCH2 and downstream effectors of transcription factor HES1, a target of NOTCH signaling, were also up-regulated. Altogether, these data suggest that our differentiation protocol allows activation of appropriate signaling pathways known to be involved in biliary differentiation.

Gene expression profile analysis allowed us to identify a panel of genes and pathways characteristic of cholangiocytes. It is known that cholangiocytes express a subset of nuclear receptors including VDR, FXR, PPAR delta/gamma, ESR1, and ABCA.^{38,39} Our data show that most of them were induced in hESC-Chol and most of their downstream targets were activated in both hESC/HepaRG-Chol. Human cholangiocytes also express a variety of Toll-like-receptors (TLRs) which mediate (via TLR2 and 4) host epithelial defense responses to microbial infection.⁴⁰ Interestingly, TLR4 was significantly induced in hESC-Chol.

Genomic integration of our dataset with gene expression profiles of normal human biliary epithelial cells and H69 cell line confirmed the commitment of progenitors into cholangiocytes. Importantly, both hESC/HepaRG-Chol as well as H69 and normal human biliary epithelial cells clustered in the same branch.

Morphogenesis of the biliary tree is tightly linked to the differentiation of the cholangiocytes that line the lumen of the biliary tree. According to the position of the cholangiocytes along the intrahepatic

biliary tree, their size, morphology, proliferation activity, and function differ.⁴¹ Small cholangiocytes are thought to be committed biliary progenitors lining the interlobular bile ducts, ductules, and the canal of Hering expressing CK7, CK19, NCAM, CFTR, and AQP1, while large cholangiocytes line large ducts and express SCTR, GGT1, TGR5, and AE2.⁴² Cholangiocytes also express ASBT, providing a mechanism to mediate bile acid uptake¹⁹ and TGR5 that may function as a bile sensor coupling biliary bile acid concentration to ductular bile formation and bile flow.⁴³ hESC/hiPSC-Chol and HepaRG-Chol expressed various levels of these markers, suggesting a mixed population containing both types of cholangiocytes. Of note CFTR, ASBT, and EpCAM are mutated in HepaRG (Dubois-Pot-Schneider, pers. commun.).

Our data show that differentiated cholangiocytes express SSTR2, P2RY1, AChR M3, and InsP3R type III receptors, known to be restricted to bile ducts in human liver,^{5,23-25} and that their stimulation resulted in an increase of intracellular Ca^{2+} . Interestingly, hESC-, HepaRG-, and hiPSC-Chol have primary cilia, the sensory organelle present on cholangiocyte apical surface, and form polarized cysts able to transport a fluorescent bile acid. Altogether, our data reveal the functionality of our cholangiocyte-like cells and highlight the importance of our *in vitro* model.

Pathogenic aspects of the most important primary cholangiopathies (polycystic and fibropolycystic liver diseases, Alagille syndrome) are related to altered biliary development, which in some cases (notably, polycystic disease), can be due to defects in cilia. Thus, cholangiocytes generated by our approach may represent a useful model for studying not only the molecular mechanisms of bile duct development but also the pathogenic mechanisms leading to liver fibrosis or cholangiopathies. It may contribute to the development of therapeutic strategies, including those with bioengineered livers.

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Supporting Information

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