

Evidence of adult features and functions of hepatocytes differentiated from human induced pluripotent stem cells and self-organized as organoids

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Supplemental Materials and Methods

Human induced pluripotent stem cells

Human iPSC lines were generated and characterized as described in Steichen et al 2014 (1) (Fig. S1). Briefly, human foreskin fibroblast (ATCC CRL2097 – passage 6) were reprogrammed by the daily transfection with mRNAs produced in the laboratory, for 16 days.

Human iPSC differentiation into hepatoblasts

Human iPSC-derived hepatoblasts (iHBs) were obtained as follows. Briefly, hiPSC colonies, cultured on Geltrex™ (Gibco) coated petri dishes, were detached enzymatically using 0.5% Trypsin-EDTA 1X solution (Gibco), seeded at a density of 4.2×10^4 cells/cm² on gelatin-coated plates and incubated at 37°C under 5% CO₂ in StemMACS™ iPS-Brew XF medium with 10 nM Y-27632. Once 70% of confluence was reached, the differentiation protocol was started using RPMI-1640 medium (Gibco) with B-27 serum-free supplement (Life technologies), 1X MEM non-essential amino acid solution (NEAA, Gibco) and 1X penicillin-streptomycin (Gibco). To induce endoderm differentiation, the cells were treated with 5 nM CHIR99021 (Stemcell™ Technologies) for one day and 100 ng/ml Activin A (Miltenyi Biotech) and 10 nM LY294002 (Sigma-Aldrich) for the next four days. From day 5 to day 7, 50 ng/ml Activin A, 10 ng/mL BMP4 (R&D Systems) and 20 ng/mL FGF2 (Miltenyi Biotech) were supplied to specify the hepatic endoderm. From day 8 to day 10, which generated a homogeneous monolayer of differentiated iHBs, 20 ng/ml HGF (Miltenyi Biotech) and 30 ng/ml FGF4 (Miltenyi Biotech) were added to produce a homogeneous monolayer of differentiated iHBs.

Organoid viability assay

iHep-Orgs were recovered on day 38 of culture from the non-adherent agarose μ -cylinders (μ wells) using DURAN® disposable micropipettes (Hirschmann Laborgeräte GmbH & Co. KG). They were then washed with 1X DPBS (Gibco) and tested for viability with the Ready Probes® cell viability imaging kit (Molecular Probes®) according to the manufacturer's instructions.

Primary human hepatocytes

Cryopreserved PHHs (Biopredic international) have been thawed according to the provider's instructions, and processed as for self-assembling of iHBs. Spheroids were obtained after 48h, the medium was refreshed every other day and PHH organoids (PHH-Orgs) were maintained in culture for 8 days to be used as control for the functional assessment of iHep-Orgs.

Nucleic acid extraction and gene expression assay

At established time points throughout the culture period, the total RNA/DNA content of iHep-Orgs, fetal human hepatocytes (FHHs) and PHHs was extracted using TRIzol™ Reagent (Sigma-Aldrich Aldrich) and purified with the Direct-zol™ DNA/RNA MiniPrep Kit (Zymo Research) following the manufacturer's instructions. The quantification of RNA and DNA in the samples was performed by UV-visible Nanodrop Lite (ThermoFisher). The DNA contents were used to define the number of cells per sample, so that the

quantification assay results could be shown as value/ $10^6/24$ h if not otherwise specified. Reverse transcriptase-PCR (RT-PCR) was performed using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) with oligoDT primer and Platinum Taq DNA Polymerase (Invitrogen), according to the manufacturer's instructions. For quantitative PCR analysis, the mRNA extraction and RTqPCR conditions were set in line with the manufacturer's instructions. cDNAs were obtained using the SuperScript III kit (Invitrogen) with random hexamers. Three replicates per sample were analyzed for differential gene expression using the Mx3000P qPCR thermocycler system (Agilent) with brilliant III ultrafast SYBR Green (Agilent). Relative levels of expression were determined using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the reference gene, and expression levels were described relative to FHHs. A list of primer sequences is shown in Supplementary Table ST2.

H&E and immunofluorescence staining

For histochemical staining, slices of iHep-Orgs (d38) were fixed in 4% PFA for 15 minutes then stained with Hematoxylin and Eosin (Sigma-Aldrich). For immunofluorescence labelling, whole iHep-Orgs and their cryosections (7 μ m) were treated according to the same protocol with appropriate adaptations. The slices were permeabilized with 0.1% Triton-X solution supplemented with 1% BSA and 0.2 μ g/ml EDTA for 5 minutes on ice before being saturated with 3% BSA solution and treated with primary antibodies overnight. Secondary antibodies were incubated for 1h at RT and the nuclei were stained with DAPI. Whole 3D-iHeps were fixed for 4h in 4% PFA, washed extensively and permeabilized for 45 minutes. A 3-hour saturation step with 5% BSA was carried out before incubation with primary and secondary antibodies for 48 h (4°C) and 3h (RT), respectively; DAPI was used for nuclear staining. Images were captured using the EVOS™ FL Auto Imaging System and a confocal microscope. A list of primary antibodies is shown in Supplementary Table ST3.

Indocyanine green (ICG) uptake and release

To monitor Indocyanine green (ICG) uptake and excretion, iHep-Orgs were treated with 5 μ M Cardiogreen (Sigma-Aldrich) solution for 15 minutes at 37°C. The Cardiogreen solution was removed and images were captured under an inverted light microscope (EVOS™ FL Auto Imaging System) immediately and then after 30, 60, and 180 minutes, following rapid washing to remove the excreted ICG.

Measurement of cytochromes P450 activity

Luminescence-based assays (Promega, Madison, WI) for cytochromes P450 (CYP450) 1A1, 1A2, 3A7, 3A4, and 2B6 were used to determine CYP450 activity. Briefly, about 50 iHep-Orgs were incubated at 37°C with luminogenic (luciferin) cytochrome P450 substrate for 4h. The supernatants were collected and luciferase was quantified according to the manufacturer's instructions. Further, in order to assess the inducibility of CYP450 isoforms in iHep-Orgs, 25 μ M rifampicin (RMP) (an inducer of CYP3A4, CYP3A7 and CYP2B6) or 100 μ M omeprazole (OMP) (an inducer of CYP1A1 and CYP1A2) were added to the culture medium 48h before the test. The supernatants were collected and processed as indicated above. This analysis produced mean luciferase values obtained during three independent experiments.

To enable specific measurement of the activities of CYP1A2 and CYP3A4, iHep-Orgs and PHH-Orgs were treated with 8 μ M 5-ethoxyresorufin (EROD test) or 8 μ M 7-benzyloxyresorufin (BROD test), which are respectively the substrates for the two isoforms. To inhibit phase II CYP450 enzyme interference, 3mM salicylamide (Sigma-Aldrich) and 10 μ M dicoumarol (Sigma-Aldrich) were also added. The supernatants were collected and the metabolite, resorufin, was quantified using a fluorescence microplate reader at 595 nm (Spectafluor Plus, TECAN). 10 μ M rifampicin (RMP) (Sigma-Aldrich) was also used to assess the inhibition and inducibility of the CYP1A2 and CYP3A4, respectively.

Morphological analysis of bile canaliculi

During treatment with DCFA (5(6)-Carboxy-2',7'-dichlorofluorescein (Abcam), images were captured using the EVOS™ FL Auto Imaging System in the GFP channel. The resulting image dataset, together with the images obtained after immunofluorescence staining of BSEP, were processed using FIJI software to threshold the background signals. Gaussian smooth treatment and the skeleton plug-in were then used to highlight the bile canalicular network, and then Volume viewer and 3D viewer were used to generate 3D reconstructions. Analysis of the skeleton datasets, produced for all the surfaces and sections, enabled quantification of the average lengths of the bile canaliculi.

Supplemental Tables

Table S1: Protocol for hiPSC differentiation into hepatoblasts, related to figure 1

Hepatoblast differentiation	Day	Medium	Growth factors and cytokines
	0	RPMI/B27	CHIR (5 nM)
	1-4	RPMI/B27	Activin A (100 ng/ml) + LY294002 (10 nM)
	5-7	RPMI/B27	BMP4 (10 ng/ml) + FGF2 (20 ng/ml) + Activin A (50 ng/ml)
	8-9	RPMI/B27 ^{-met}	HGF (20 ng/ml) + FGF4 (30 ng/ml)
	10	RPMI/B27 : HCM™ (50:50)	HGF (20 ng/ml) + OSM (10 ng/ml) + Dex (0.1 nM)

Table S2: List of primer sequences used for gene expression analysis, related to figure 1 and figure S1

Gene	Primer 5' sequence 3'	Primer 3' sequence 5'
<i>A1AT</i>	CCA ACA GCA CCA ATA TCC ATC TTC	GTC CTC TTC CTC GGT GTC CTT G
<i>ACTIN</i>	GCA CTC TTC CAG CCT TCC TTC C (exon4)	CTG CTG TCA CCT TCA CCG TTC C (exon6)
<i>AFP</i>	TTT TGG GAC CCG AAC TTT CC	CTC CTG GTA TCC TTT AGC AAC TCT
<i>ALB</i>	CCT TTG GCA CAA TGA AGT GGG TAA CC	CAG CAG TCA GCC ATT CAC CAT AGG
<i>APOA2</i>	GGA GAA GGT CAA GAG CCC AGA G (exon 3)	AGC AAA GAG TGG GTA GGG ACA G (exon 4)
<i>ASGR</i>	TAG GAG CCA AGC TGG AGA AA	ACC TGC AGG CAG AAG TCA TC
<i>CYP2B6</i>	GAT TGA AGG CGT CTG GTT T	AGG GAG ATT GAA CAG GTG ATT
<i>CYP3A4</i>	GAT TTG GCT CCT CTG CTT CT	GCC TCC TGT GTA GTG AGA TTA C
<i>CYP3A7</i>	ATT CCA AGC TAT GTT CTT CAT CAT (exon 11)	AAT CTA CTT CCC CAG CAC TGA (exon 13)
<i>GAPDH</i>	CGG GAA ACT GTG GCG TGA TG (exon 1)	TGT GCT CTT GCT GGG GCT GGT G (exon8)
<i>GATA4</i>	CTA GAC CGT GGG TTT TGC AT	TGG GTT AAG TGC CCC TGT AG
<i>HNF1α</i>	AGA CTT CAC GCC ACC CAT CCT C	TTC CTC CGC CCC TTC TTG GTT G
<i>HNF3β</i>	AAC ACC ACT ACG CCT TCA ACC A (exon2)	GCA ACA CCG TCT CCC CAA AGT C (exon2)
<i>HNF4α</i>	CGG GTG TCC ATA CGC ATC CTT G (exon 7)	GAC CCT CCC AGC AGC ATC TCC T (exon8)
<i>HTERT</i>	TGT GCA CCA ACA TCT TGA AGC C	GCG TTC TTG GCT TTC AGG AT
<i>LDL-R</i>	GGG CTG GAA ATT GCG CTG GAC CGT C	TCA CAG ACG AAC TGC CGA GAG ATG C
<i>OCT4</i>	GTG GAG GAA GCT GAC AAC AA	CAG GTT TTC TTT CCC TAG CT
<i>Bil-UGT</i>	ATG ACC CGT GCC TTT ATC AC (exon4)	TCT TGG ATT TGT GGG CTT TC (exon5)

Table S3: List of primary antibodies used for immunofluorescence staining, related to figure 2

Protein	Ref.	Specifics
A1AT	DAKO A0012	rabbit polyclonal IgG
AFP	SC 8399	mouse monoclonal IgG
ALB	Sigma A6684	mouse monoclonal IgG
BSEP (ABCB11)	Sigma HPA019035	rabbit polyclonal IgG
CK-19	DAKO M0888	mouse monoclonal IgG
CK-8	SC 8020	mouse monoclonal IgG
CLDN1	SC 166338	mouse monoclonal IgG
CX26	SC 7261	goat polyclonal IgG
CX32	SC 7258	goat polyclonal IgG
CYP3A4	SC 53850	mouse monoclonal IgG
ECADH	DAKO M3612	mouse monoclonal IgG
EPCAM	SC 66020	mouse monoclonal IgG
HNF1 α	SC 135939	mouse monoclonal IgG
HNF4 α	SC 8987	rabbit polyclonal IgG
Ki67	Abcam ab80827	rabbit polyclonal IgG
MDR1	Fisher 4MA-26528	mouse monoclonal IgG
Phalloidin (Alexa Fluor 488)	Life Technologies A12381	
Alexa Fluor 488-conjugated	Life Technologies	

Table S4: Summary table of the hepatocyte functional features, related to figure 2-6. Table recapitulates the functional data recorded for iHep-Orgs and data from literature concerning 3D hPSC-derived hepatocytes, FHHs and PHHs. Data presented have been extracted from references selected on the similarity between the reported protocols and that of the present work. Data are shown as range of published values (when given in units allowing comparison).

	Published data						
	iHep-Orgs	3D iPSC-derived Heps		3D FHHs		3D PHHs	
	Experimental outcome	Ref.	Experimental outcome	Ref.	Experimental outcome	Ref.	Experimental outcome
AFP secretion	Not detected	(2-6)	Not quantified	(7)	Not quantified	(3,5,7,8)	Not quantified
Albumin secretion	7 µg/ml/24h/10 ⁶	(2-6,8)	0.05-5 µg/ml/24h/10 ⁶	(7)	25-100 µg/ml/24h/10 ⁶	(3,5,7,8)	10-80 µg/ml/24h/10 ⁶
Glycogen synthesis (PAS staining)	+	(2-4,6)	+			(3,9)	+
Glycogenolysis (Glucose-6-Phosphatase activity)	350 µg/ml/24h						
Gluconeogenesis	500 µg/ml/24h						
Urea production	45 µg/ml/24h/10 ⁶	(6,8,9)	20-72 µg/24h/10 ⁶			(8)	20 µg/ml/24h/10 ⁶
Production and secretion of bile acids	76 µM/24h/10 ⁶	(5,8)	4-25 µM/24h/10 ⁶				
Uptake and secretion of bile acids into the canalicular network (CDFA)	+	(5,6,10)	+				
Phase 0-III metabolism: ICG uptake/secretion	+	(2,4)	+				
CYP1A2 activity	6 µM/ml/4h/10 ⁶	(5)	1-15 RLU/GTC/10 ⁶				
CYP3A4 activity	9 µM/ml/4h/10 ⁶ 65 RLU/ml/4h/10 ⁶	(3,5,6,8-10)	≈ 50 RLU/ml/10 ⁶	(7)	2.5 RLU/ml/per cell	(7,10)	≈ 60-80 RLU/ml/10 ⁶
UGT1A1 activity	9 µM/ml/4h/10 ⁶						
ADH activity	6 mU/ml/4h/10 ⁶						

Supplemental references

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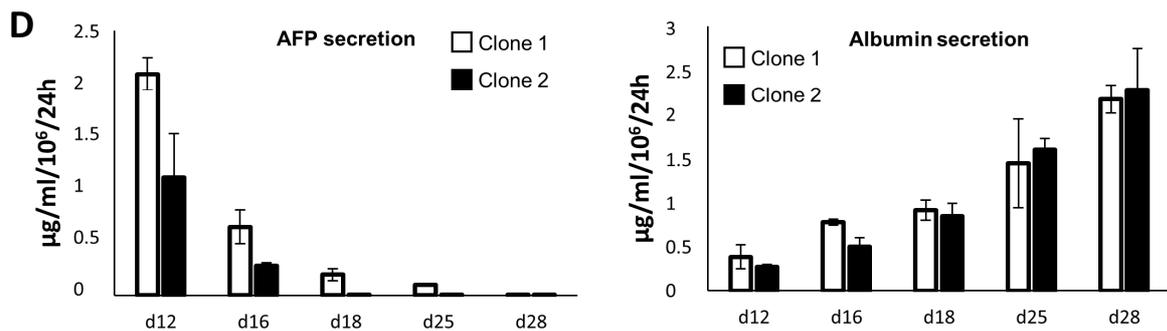
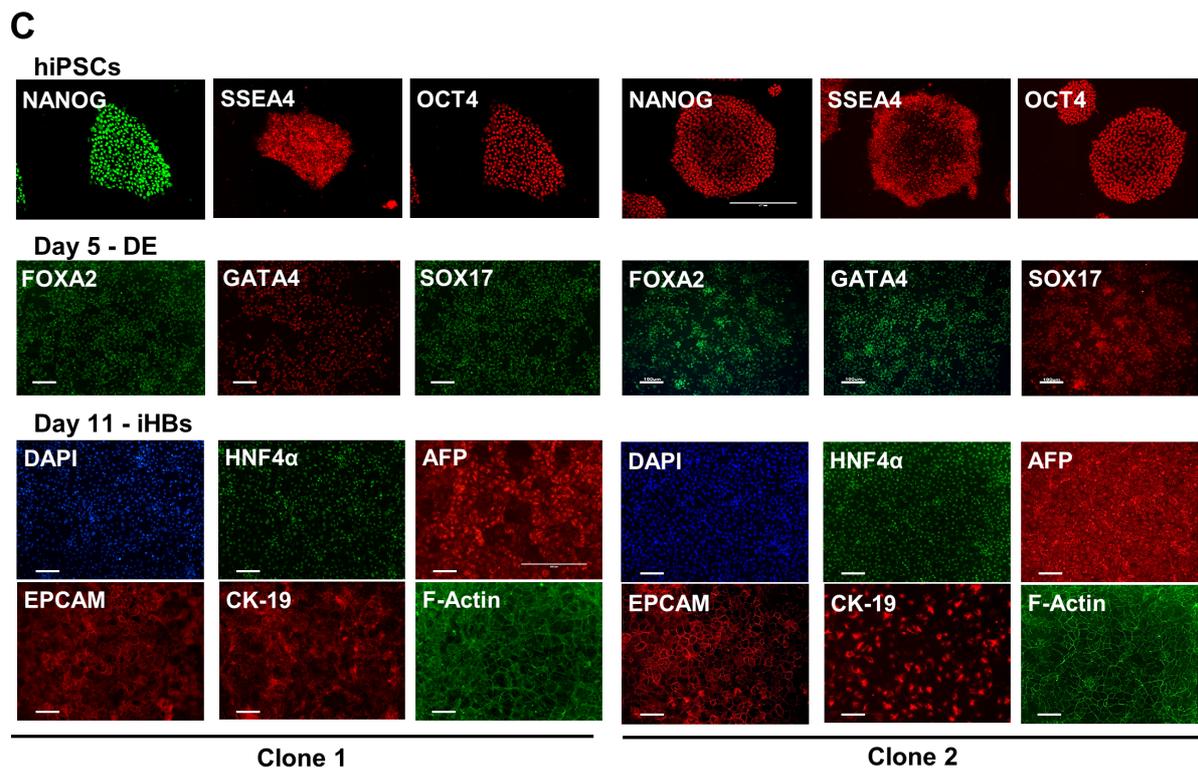
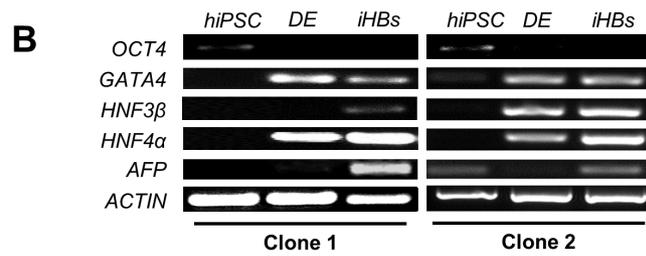
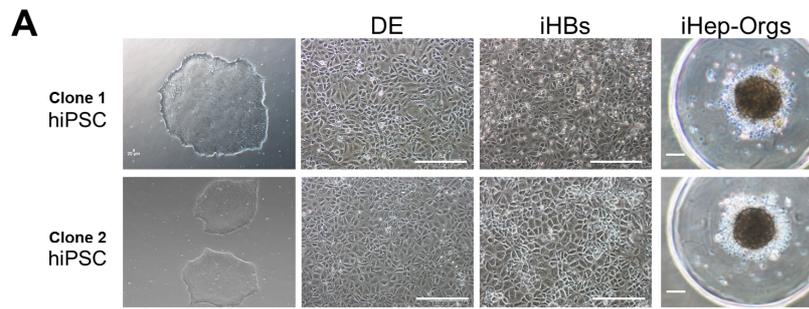


Figure S1. Assessment of hiPSC differentiation into iHBs, related to figure 1.

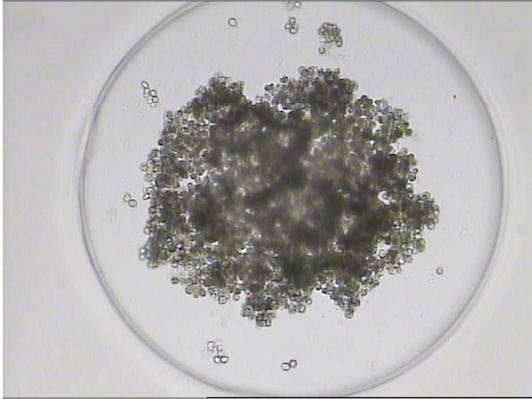
(A) Brightfield images of hiPSC clone 1 (upper panel) and clone 2 (left panel). From left to right: hiPSC colonies before differentiation, at the definitive endoderm stage and at hepatoblastic stage. Scale bars for all brightfield images = 100 μ m.

(B) RT-PCR analysis of the gene expression of stemness and hepatoblastic markers over time for the hiPSCs, the definitive endoderm (DE) and iHBs. Clone 1 (left panel) and clone 2 (right panel).

(C) Immunofluorescence staining of stemness, endoderm and hepatoblast markers from day 0 to day 11 of the differentiation protocol. Clone 1 (left panel) and clone 2 (right panel). Scale bar = 500 μ m for day 0 - hiPSCs. Scale bars = 100 μ m for day 5 – DE and day 11 – iHBs.

(D) ELISA quantification of AFP and ALB secretion of clone 1 (white bars) and clone 2 iHep-Orgs (black bars). Histograms represent mean \pm SD (n=16).

PHH 24 h after seeding



PHH-Orgs at 48h



Figure S2. PHH self-assembling into organoids in agarose μ wells, related to figure 1. Left panel: PHHs 24h after seeding into agarose μ wells. Right panel: PHH-Orgs formed after 48h of culture

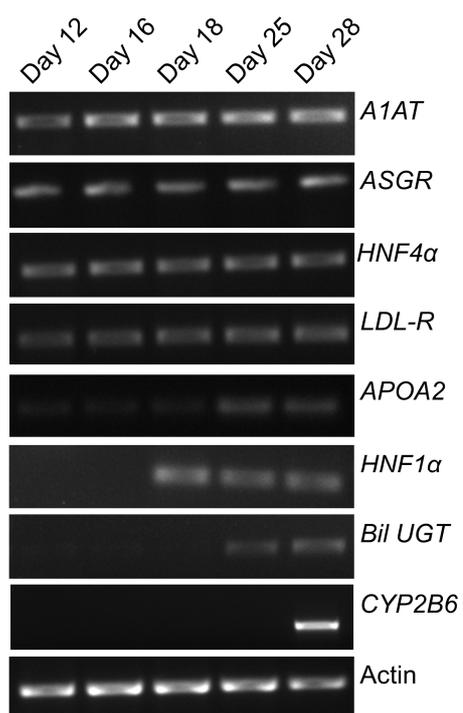


Figure S3. Gene expression of hepatocyte markers, related to figure 1. RT-PCR analysis of hepatic gene expression in iHep-Orgs over time.

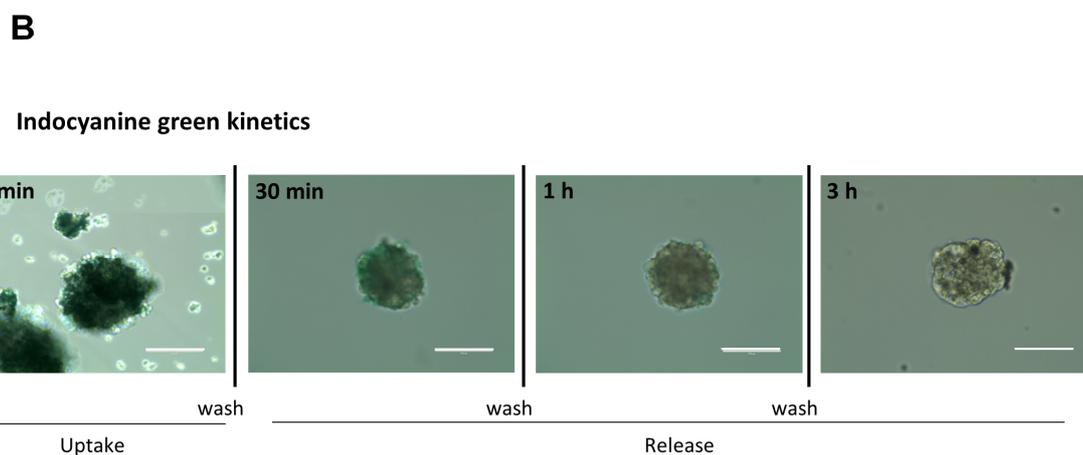
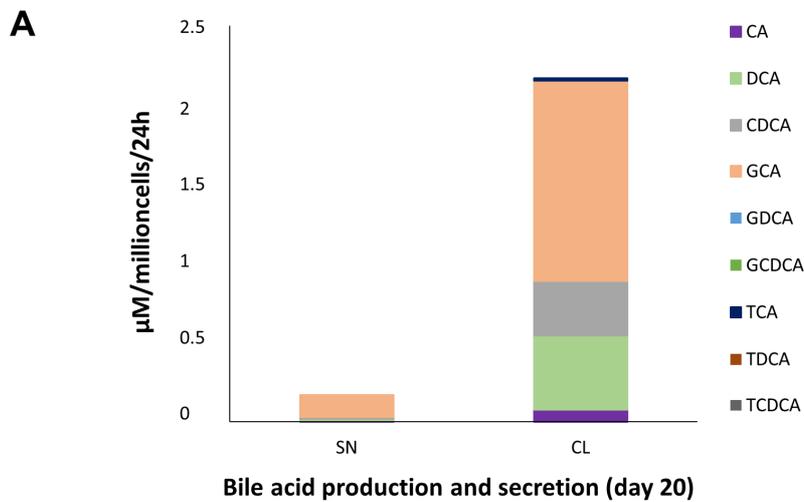


Figure S4. Assessment of iHep-Org functions associated with polarization, related to figure 3.

(A) Quantification of bile acid (BA) production in cell lysates and secretion into culture supernatants from iHep-Orgs at day 20 of culture. SN = supernatant; CL= cell lysate. The higher values in BA concentration detected in the CL compared to the SN could be due to i) the quantities excreted are insufficient to be quantified (excessive dilution) ii) the incomplete maturation of the bile canalicular network in iHep-Orgs at day 20.

(B) Indocyanine Green (ICG) uptake and release assay in iHep-Orgs. Scale bar = 200 μ m. The assay allows the assessment of OATP1B3, NTCP and MRP2 functions associated with polarization and transport.