Supplemental Materials and Methods

RNA-seq of liver organoids in hepatic differentiation

Total RNA was isolated from cells using the miRNeasy Kit (Qiagen) according to the manufacturer's protocol. An additional DNase1 digestion step was performed to ensure that the samples were not contaminated with genomic DNA. RNA purity was assessed using the Agilent 2100 Bioanalyzer. Briefly, total RNA (500 ng) was converted to cDNA using the ABI System according to the manufacturer's protocol (High-Capacity cDNA Reverse Transcrition Kit). The cDNA was then used for Illumina sequencing library preparation by TruSeq Stranded mRNA and Total RNA Library Prep Kit. DNA fragments were then end-repaired to generate blunt ends with 5′ phosphatase and 3′ hydroxyls and adapters were ligated for paired end sequencing on Illumina HiSeq 2000. The purified cDNA library products were evaluated using the AATI Fragment Analyzer and diluted to 8-10 pM for cluster generation in situ on the HiSeq paired-end flow cell using the Rapid mode cluster generation system followed by massively-parallel sequencing (2×75 bp) on HiSeq 2000. We obtained 75-bp mate-paired reads from DNA fragments of average length of 150-bp.

Liver organoid implantation into mice

Immunodeficient NOD scid gamma (NSG) mice (8-12 weeks old, male; Jackson Laboratories) were kept according to protocols approved by Institutional Animal Care and Use Committee standards at Cincinnati Children's Hospital Medical Center. NSG mice were fed Bactrim Chow (Test Diet). The liver organoids at day-2 of culture were implanted under the kidney capsule, as previously described (Takebe et al., 2013). Briefly, mice were anesthetized with 2% inhaled Isoflurane (Butler Schein) and a small incision was made to expose the kidney. A subcapsular pocket was created and 5-7 liver organoids were placed into the pocket. Beginning at 2 weeks after implantation, the mice were briefly anesthetized and a few drops of blood were collected weekly from tail veins. The blood was then spun and the serum was subjected to protein quantification of

human specific albumin and alpha1 antitrypsin (A1AT), measured with ELISA kit (Bethyl Laboratories). At 8 weeks after implantation, the mice were euthanized and liver organoids were excised and examined.

Primary mouse hepatocyte isolation

Mouse primary hepatocytes were isolated from male wild-type by collagenase perfusion through the portal vein. Livers were perfused with Gibco Liver perfusion Media (Invitrogen) followed by Gibco Liver Digestion Media. The liver was excised, minced and strained through a steel mesh sieve. The dispersed hepatocytes were collected by centrifugation at 50*g* for 2 minutes and washed twice with Williams media (Invitrogen). Hepatocytes were isolated via Percoll separation and washed twice with Williams media. The final pellet was re-suspended with Williams media. Hepatocytes were counted and viability was checked by trypan blue exclusion. Hepatocytes cells were distributed onto Transwell membrane and incubated overnight to allow cell adherence.

Measurement of albumin, A1AT and urea

Unless specified, LO-medium of upper and lower chamber was collected together 24 hours after the last medium exchange. Human albumin and A1AT in the collected culture supernatant were quantified with ELISA kits (Bethyl Laboratories). The protein concentrations were monitored from day-2 to day-12 of culture. Of note, all of the day-2 supernatant contained no albumin nor A1AT. To quantify urea production, cells were incubated with LO-medium containing 2mmol L^{-1} of NH_4Cl for 48 hours. The supernatant was collected and urea was measured using the QuantiChrom Urea Assay Kit (BioAssay Systems). Because serum in the culture medium contained urea, the serum urea level was subtracted from the amount of urea measured in the media alone.

Quantitative PCR

Total RNA was extracted from cells by the TriAzol method as previously described (Shivakumar et al., 2004). After measuring total RNA concentration,

500ng of RNA were subjected to reverse transcription reactions. The real-time PCR by Brilliant III SYBR Green QPCR Master Mix Gene Expression Assay Kit and the Mx3005p system (Stratagene) quantified mRNA of target genes, with specific primers (Table S3) and quantification protocol as described previously (Bessho et al., 2014). After normalized with a housekeeping gene (*GAPDH*), each gene expression level was described relative to primary hepatocytes or baseline controls.

Immunostaining of cultured cells

Protocols for immunostaining in monolayer cells on the Transwell membrane and formalin fixed paraffin embedded liver organoids were modified from previous reports (Shivakumar et al., 2004). In brief, cells were fixed with 4% paraformaldehyde (or methanol for CPS1 detection) at 4°C for 30 minutes, permeabilized with 0.5% Triton X100, and blocked with 5% donkey serum, then incubated with primary antibodies at 4°C overnight. The liver organoids were sectioned at 5μm and placed on the glass slides. The list of antibodies and dilution factors are described in the Table S4. For immunofluorescent histology, secondary antibodies with fluorescent probes were incubated at room temperature for 1 hour. For immunohistochemistry, we used Vectastain ABC kit (Vector Laboratories) per manufacturer's instruction and DAB (3,3'-Diaminobenzidine) as a chromogen, followed by counter staining of hematoxylin. The monolayer cells remained on the Transwell membrane throughout the process and cover glasses were mounted onto the cell directly with mounting medium. Imaging were performed using an Olympus microscope and DP71 camera (Olympus) or a Zeiss LSM710 confocal microscope.

Periodic Acid Schiff staining

For glycogen detection, differentiated hepatocyte-like cells were fixed using 4% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100 in PBS. Control cells were incubated with Diastase (1mg/ml in PBS; Sigma). Cells were then incubated with Periodic acid for 5 min, washed with distilled water, and

incubated with freshly prepared Schiff's solution for 15 min. Finally, cells were rinsed and nuclei were stained with Hematoxylin.

Whole mount staining of the liver organoids

At 6 hours, 24 hours, 48 hours, 4 days, and 6 days of co-culturing, liver organoids (n=3 in each group) were fixed in 4% paraformaldehyde at 4°C for 1 hour. The protocol for the whole mount immunostaining of the liver organoids was modified from a previous study (Dipaola et al., 2013). Briefly, specimens were permeabilized in Dent's fixative (80% methanol and 20% dimethyl sulfoxide) for 30 min, followed by rehydration through a series of methanol dilutions. The liver organoid was incubated with 10% normal donkey serum (1 % Triton X in PBS) for 2 hours, and incubated with anti-AFP antibody and anti-CD31 antibody overnight at 4°C. Liver organoids were then incubated in Alexa Fluor 647 donkey anti-rabbit Ab (diluted 1:500) and Alexa Fluor 488 donkey anti-mouse Ab (diluted 1:500) overnight at 4°C. Nuclear staining was performed by incubation with Hoechst 33342 (Invitrogen) at 10 μg ml^{-1} for 60 min. The liver organoid was dehydrated in 100% methanol and clarified with Murray's clear (2:1 benzyl benzoate/benzyl alcohol). Imaging was performed using a Zeiss LSM710 confocal microscope. 3D image reconstruction of z-stack confocal images was generated using Imaris Version 7.7 software (Bitplane).

Transmission electron microscopy

The monolayer hepatocyte-like cells on the Transwell membrane were fixed with 2% paraformaldehyde, 2.5% glutaraldehyde and 1% Tannic acid in 0.1 mol L^{-1} cacodylate, pH 7.2 for 1 hour at 4°C. Specimens were then post-fixed with 1% OsO4 for 1 hour, dehydrated in an ethanol series (25, 50, 75, 95, and 100%), and infiltrated with dilutions of ETOH/LX-112 and then embedded in LX-112 (Ladd Research Industries) while still on the culture membrane surface. Blocks were polymerized for 3 days at 60°C. The monolayer was ultra-thin sectioned on Reichert EM UC7 ultra-microtome, perpendicular to the plane of the Transwell membrane and mounted on grids, which were post-stained with uranyl acetate and lead citrate. The sections were viewed using a Hitachi H7650 electron microscope.

Reference

Bessho, K., Mourya, R., Shivakumar, P., Walters, S., Magee, J. C., Rao, M., Jegga, A. G. and Bezerra, J. A. (2014). Gene expression signature for biliary atresia and a role for interleukin-8 in pathogenesis of experimental disease. *Hepatology* **60**, 211-223.

Dipaola, F., Shivakumar, P., Pfister, J., Walters, S., Sabla, G. and Bezerra, J. A. (2013). Identification of intramural epithelial networks linked to peribiliary glands that express progenitor cell markers and proliferate after injury in mice. *Hepatology* **58**, 1486-1496.

Shivakumar, P., Campbell, K. M., Sabla, G. E., Miethke, A., Tiao, G., McNeal, M. M., Ward, R. L. and Bezerra, J. A. (2004). Obstruction of extrahepatic bile ducts by lymphocytes is regulated by IFN-γ in experimental biliary atresia. *Journal of Clinical Investigation* **114**, 322-329.

Takebe, T., Sekine, K., Enomura, M., Koike, H., Kimura, M., Ogaeri, T., Zhang, R. R., Ueno, Y., Zheng, Y. W., Koike, N., et al. (2013). Vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature* **499**, 481-484.

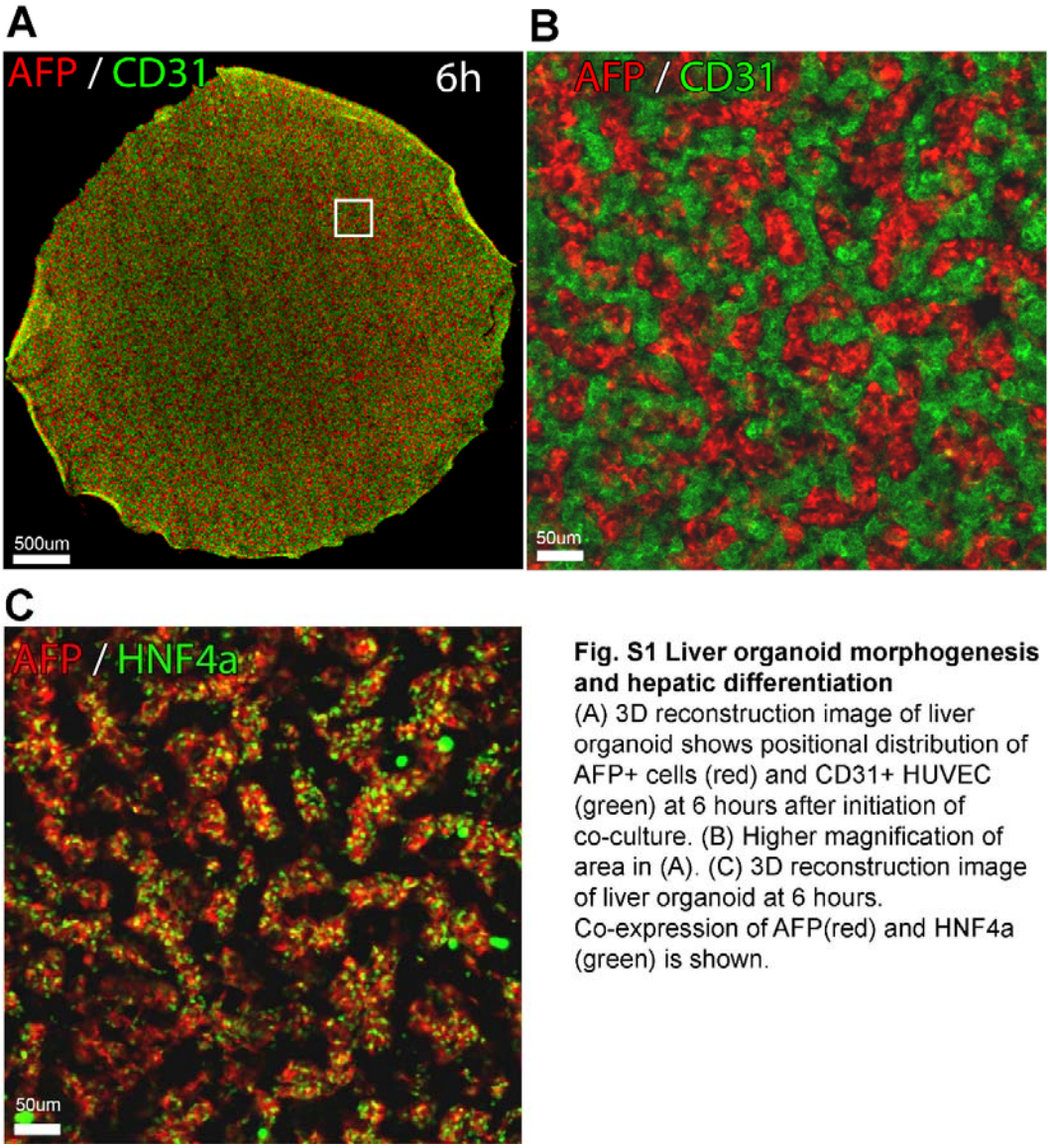

Fig. S1 Liver organoid morphogenesis and hepatic differentiation
(A) 3D reconstruction image of liver organoid shows positional distribution of AFP+ cells (red) and CD31+ HUVEC (green) at 6 hours after initiation of co-culture. (B) Higher magnification of area in (A). (C) 3D reconstruction image of liver organoid at 6 hours. Co-expression of AFP(red) and HNF4a (green) is shown.

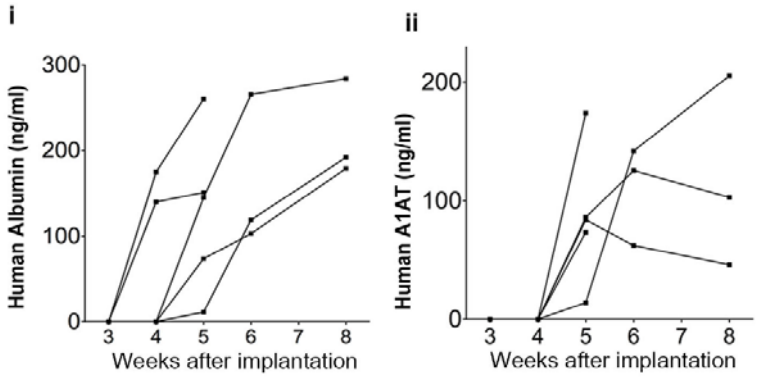

Fig. S2 Human albumin and alpha1 antitrypsin production by implanted liver organoids in vivo. Increasing concentrations of human specific albumin (i) and alpha1 antitrypsin (A1AT) (ii) were detected in mouse serum following implantation of the liver organoids into kidney capsules of immunodeficient mice; each line represents one mouse monitored by weekly blood sampling.

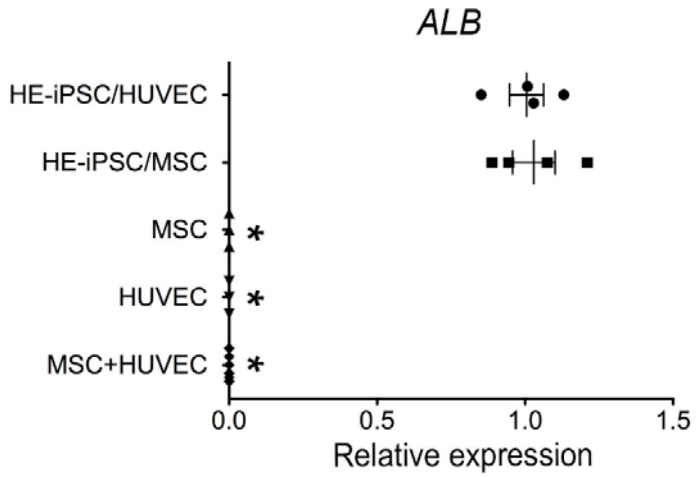

Fig. S3 The Albumin mRNA expression in the MSC, HUVEC, and MSC+HUVEC
The MSC, HUVEC, and MSC+HUVEC, co-cultured with HE-iPSC for 12 days showed no gene expression of *ALB*. (n=3 or more in each group. *:p<0.01).

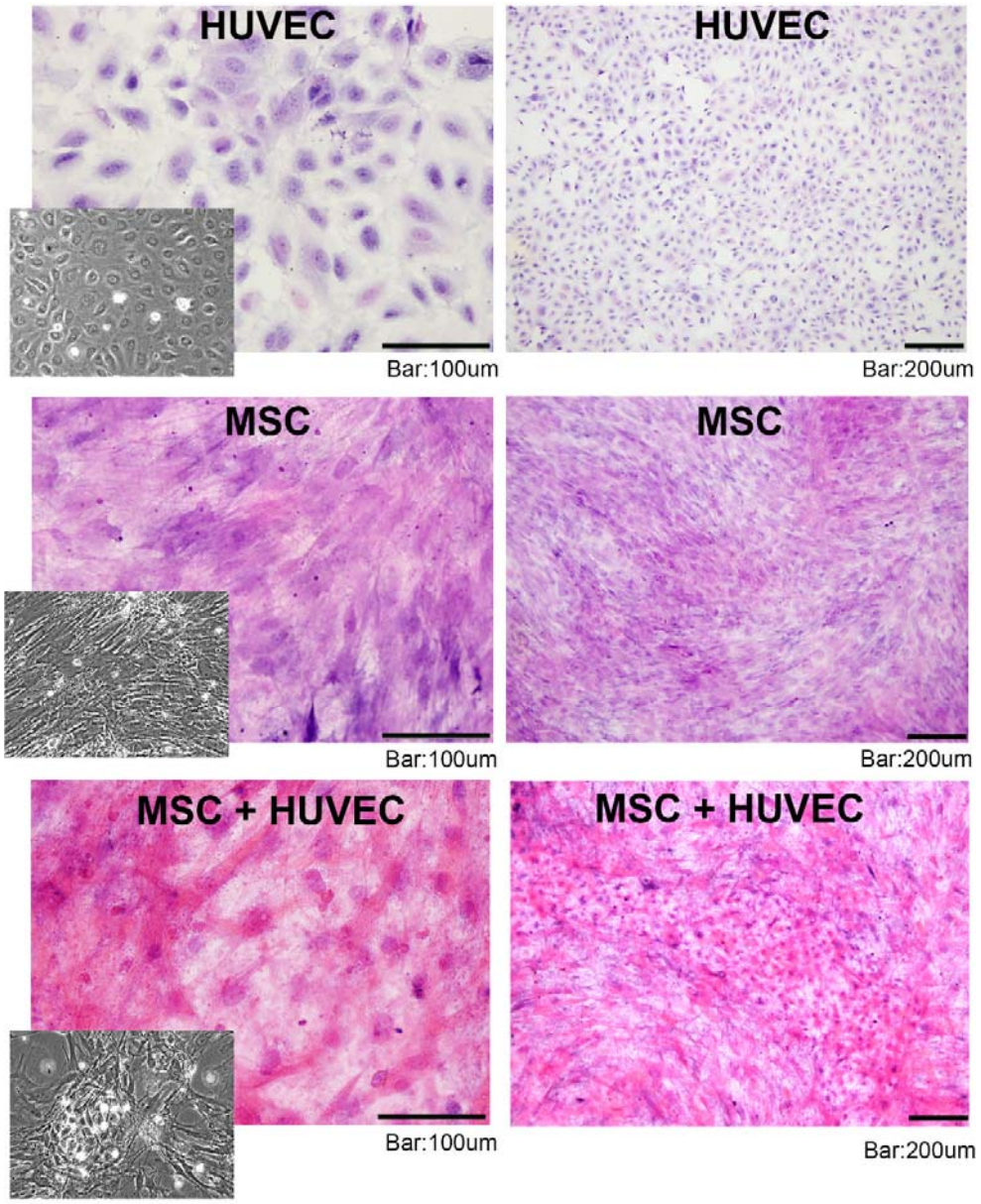

Fig. S4 Hematoxylin and Eosin staining revealed morphological change of the MSC and HUVEC 4 days after mix-culture in the lower chamber. The mix-culture of MSC and HUVEC, compared to solo-culture of MSC or HUVEC, formed cell clusters and showed distinct cellular morphology by day-4. Inset images are bright field capture of cells in the culture dish.

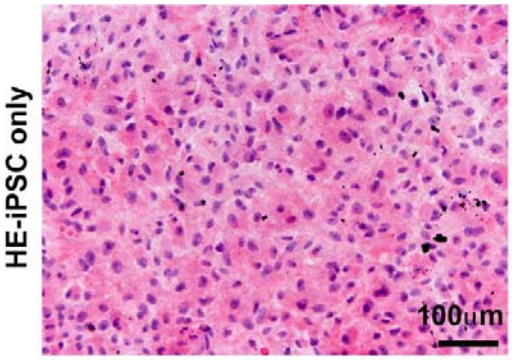

Fig.S5 Hematoxylin and Eosin staining of cells in the upper chamber and no cells in the lower at day-8. When cultured without any cells in the lower chamber (iPSC only), HE-iPSCs showed cellular morphology similar to immature endoderm cells.

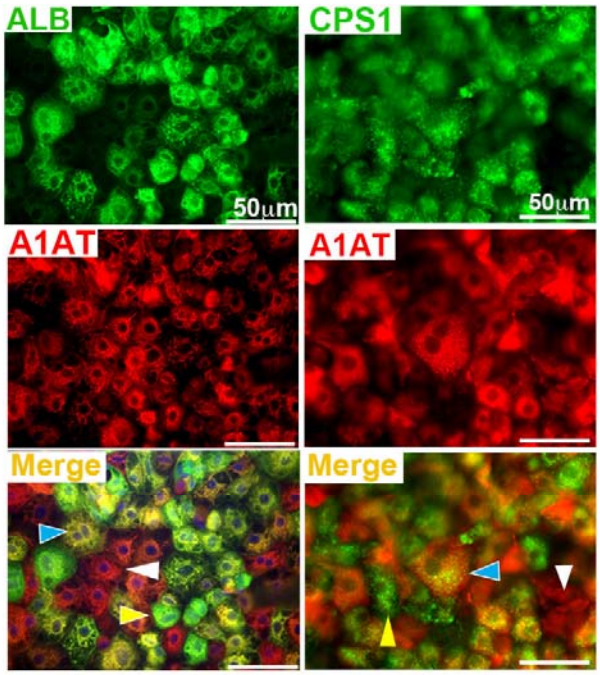

Fig. S6 The expression pattern of hepatic markers. The hepatocyte-like monolayer cells showed ALB+ and A1AT+ cells with single expression (ALB+: yellow arrowhead and A1AT+: white arrowhead) and double expression (blue arrowhead). The liver specific marker, CPS1, was also expressed in the hepatocyte-like cells in both single and double expression with A1AT.

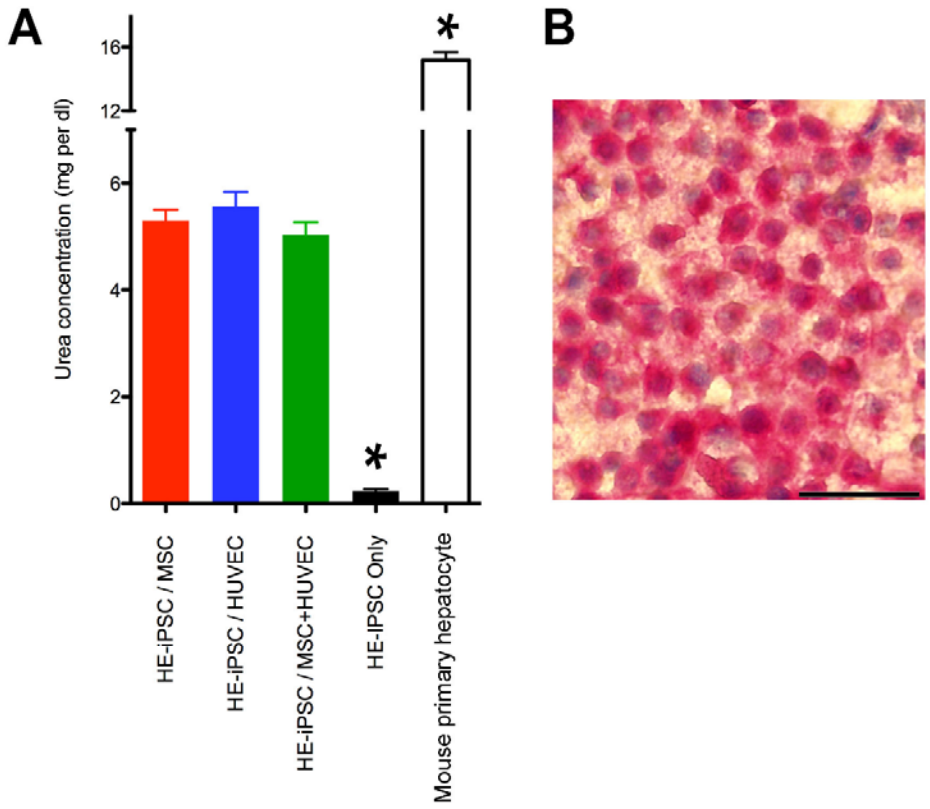

Fig. S7 Hepatic functions of iPSC-derived monolayer cells in 2-chamber culture

(A) Urea production of the hepatocyte-like cells at day-8. To quantify urea production, cells were incubated with LO-medium containing 2mmol L^{-1} of NH_4Cl for 48 hours. The supernatant was collected and urea was measured using the QuantiChrom Urea Assay Kit (n=4 of each group). Because serum in the culture medium contained urea, the serum urea level was subtracted from the amount of urea measured in the cultured media alone. HE-iPSC only showed significantly low urea production (*: $p<0.05$).

(B) Periodic Acid Schiff staining detected glycogen in the cytoplasm of hepatocyte-like cells after 8 days of culture with HUVEC. A similar staining pattern was also seen in cells cultured with MSC and MSC+HUVEC. The nuclei were stained purple with Hematoxylin. (Scale bar = 50μm)

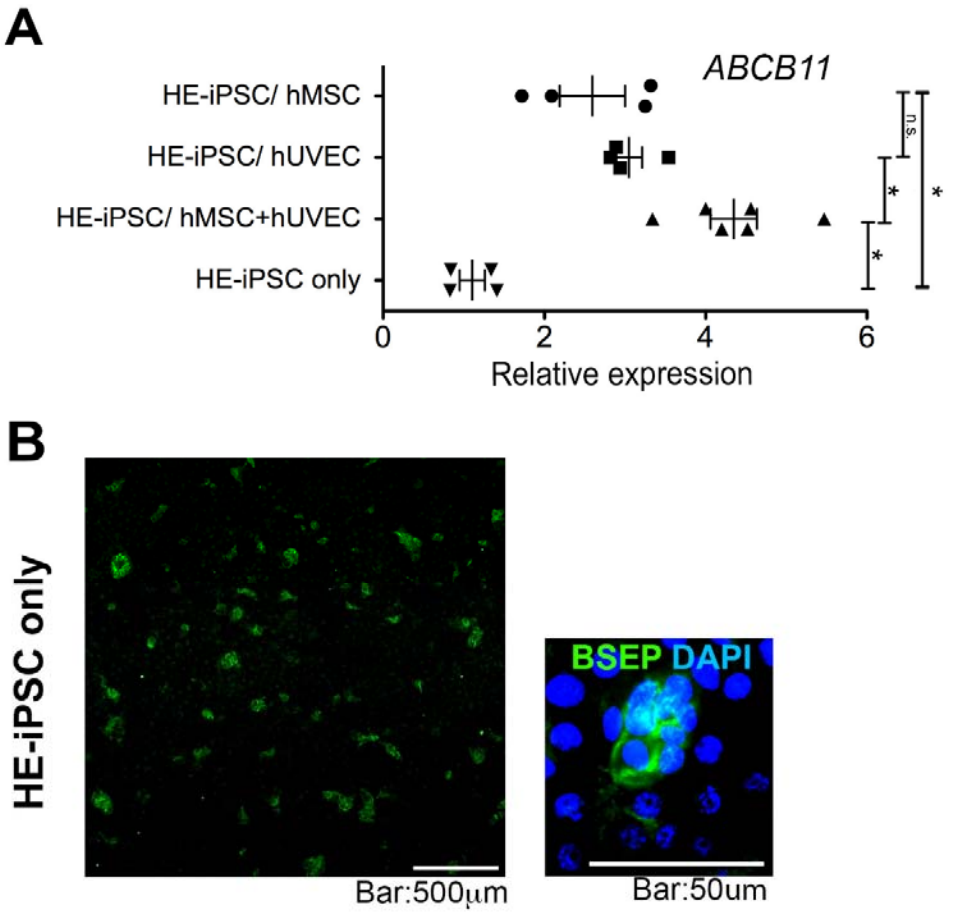

Fig. S8 BSEP expression in the hepatocyte-like cells
(A) The hepatocyte-like monolayer cells, co-cultured with MSC, HUVEC and MSC+HUVEC, showed higher gene expression of ABCB11 (encoding BSEP) than cells in HE-iPSC only. (n=4 or more in each group. *:$p<0.05$).
(B) BSEP in cells cultured in a condition of HE-iPSC only was visualized by immunofluorescent staining. In a low magnification image (left panel), a few cells expressed BSEP. In a high magnification image (right panel), BSEP is localized on the membrane.

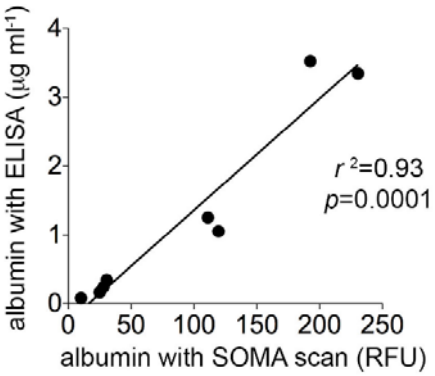

Fig. S9 Data validation of the SOMAscan results
An albumin concentration of the same culture supernatant was measured by ELISA and SOMAscan. The results by ELISA are described with μg ml^{-1} and by SOMAscan with RFU (relative fluorescent unit).

Supplemental Table

Table S1, Gene list of 3146 genes over-expressed in primary hepatocyte

Click here to Download Table S1

Table S2, Gene list of 442 genes over-expressed in day-2 liver organoid

Click here to Download Table S2

Table S3, Primer list for RT-PCR

Gene name	Forward primer	Reverse primer
GAPDH	TGACATCAAGAAGGTGGTGAAGC	TCAAAGGTGGAGGAGTGGGTG
ALB	GGATGAAGGGAAGGCTTCGT	GAAATCTCTGGCTCAGGCGA
SERPINA1	GCATAAGGCTGTGCTGACCATC	TTGTTGAACTTGACCTCGGGG
TTR	ACCGGTGAATCCAAGTGTCC	AATGGCTCCCAGGTGTCATC
HNF4a	TGCGACTCTCCAAAACCCTC	ATTGCCCATCGTCAACACCT
PYGL	AATGGGATCACTCCAAGGCG	AGCTGGCTCAGGTCTTTCAC
CPS1	GTGGCTTGCTTTGGTGAAGG	CTTGGCCGGAATGATTGCTG
CP	TGGTACTTATTCAGCGCCGG	AGGTTTGCTGTGTCTCTCCG
ABCB11	TGTTGGGATTTTCAGGGGTTG	CCGTAAACTTGGACACACTCAGACC

Table S4, Primary and secondary antibodies for immunostaining

Target protein	Origin	Manufacturer	Dilution
AFP	Rabbit	DAKO (A0008)	1/200
CD31	Mouse	Cell Marque (131M-94)	1/200 (Fluorescent) 1/1000 (Immunohistochemistry: IHC)
Albumin	Rabbit	Sigma (A3293)	1/1000 (Fluorescent) 1/3000 (IHC)
Alpha1 antitrypsin	Goat	Gene Tex (GTX77515)	1/1000 (Fluorescent) 1/3000 (IHC)
CPS1	Rabbit	Abcam (ab45956)	1/500 (Fluorescent) 1/1500 (IHC)
ZO-1	Mouse	Thermo (33-9100)	1/100
BSEP	Rabbit	Sigma (HPA019035)	1/500 (Fluorescent) 1/1500 (IHC)

Secondary antibody list

	Manufacturer
Alexa Fluor 647 Goat anti-Rabbit	Thermo/Life Technologies
Alexa Fluor 488 Donkey anti-Rabbit	Jackson Immunoresearch
Alexa Fluor 594 Donkey anti-Goat	Jackson Immunoresearch
Alexa Fluor 488 Goat anti-Mouse	Jackson Immunoresearch
Alexa Fluor 647 Goat anti-Mouse	Thermo/Life Technologies

Table S5, Full result of SOMAscan protein analysis of culture supernatant

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Table S6, List of over-expressed proteins (3 or higher than HE-iPSC only culture condition) in HE-iPSC/MSC or HE-iPSC/HUVEC or HE-iPSC/MSC+HUVEC

A2M	CFI	GPNMB	NAAA	SFTPD
ACP5	CFP	GZMA	NAGK	SHBG
ADAM12	CGA CGB	H2AFZ	NAMPT	SHC1
ADSL	CHRDL1	HGF	NAPA	SHH
AGT	CHST6	HIST1H1C	NID1	SLPI
AHSG	CLEC11A	HNRNPAB	NID2	SNRPF
AK1	CLEC11A	HRG	NME1	SPARC
AKR7A2	CLU	IDUA	NRP1	SPHK1
ALB	CPB2	IGF2R	NTN4	SPOCK1
ANG	CRK	IGFBP1	NXPH1	SSRP1
ANGPT1	CRLF1 CLCF1	IGFBP3	OMD	STC1
ANGPT2	CSF1	IGFBP4	PA2G4	STIP1
ANGPTL4	CSK	IGFBP5	PARK7	SYNCRIP
ANP32B	CTGF	IGFBP6	PCSK9	TFF3
ANXA1	CTSD	IL12RB2	PDGFB	TGFBI
ANXA2	CXCL1	IL18R1	PDGFC	TGFBR3
APOA1	CXCL11	IL1R1	PDIA3	THBS2
APOB	CXCL12	IL1RL1	PF4	TIE1
APOE	CXCL6	IL25	PGAM1	TNFAIP6
BGN	DCN	IL6	PGD	TNFRSF12A
BMP6	DDR1	IL6R	PGF	TNFRSF13C
BMP7	DIABLO	IL6ST	PI3	TNFRSF1B
BMPER	DYNLRB1	INHBA	PKM2	TNFSF15
BOC	ECE1	ITIH4	PLAT	UBE2I
C1R	EIF5A	KIR2DL4	PLAUR	UFC1
C2	ENTPD5	KLK7	PLCG1	UNC5C
C3	EPB41	KNG1	PLG	VEGFA
C3	EPO	KPNA2	PLG	VEGFA
C3	ESAM	KPNB1	POSTN	VEGFC
C4A C4B	ESM1	LBP	PTPN11	VTA1

C4A C4B	F10	LEPR	RAC1	VWF
C5	F10	LGALS3BP	RAN	WFIKKN1
C5	F5	LYZ	RBM39	XPNPEP1
C5 C6	F7	MAP2K4	RGMB	XRCC6
C6	F9	MAPK1	RPS3	
CAMK2B	FETUB	MAPK12	RPS7	
CCDC80	FGA FGB FGG	MAPK3	SAA1	
CCL13	FGA FGB FGG	MET	SBDS	
CCL14	FGF7	METAP2	SCARF1	
CCL15	FGF9	MFGE8	SELL	
CCL16	FGG	METAP2	SELP	
CCL20	FLT4	MFGE8	SERPINA6	
CCL23	FRZB	MICB	SERPINA7	
CCL23	FYN	MMP1	SERPINC1	
CCL7	GAPDH	MMP10	SERPINE1	
CD55	GDF5	MMP13	SERPINE2	
CFB	GFRA1	MMP2	SERPINF2	
CFC1	GNS	MRC2	SET	
CFH	GPI	MST1	SFRP1	

Table S7, Lists of all proteins in cluster A and B in figure 6

Cluster A (44 proteins)	Cluster B (50 proteins)
A2M	ANGPT2
AGT	ANP32B
AHSG	ANXA1
ALB	ANXA2
ANG	CCL23
APOA1	CCL23
APOB	CHST6
APOE	CRK
C2	CRLF1 CLCF1
C5	CXCL11
C5	DIABLO
C5 C6	DYNLRB1
C6	EIF5A
CAMK2B	GPI
CCL15	IGF2R
CCL16	IGFBP1
CCL7	IGFBP3
CPB2	IGFBP4
ENTPD5	IGFBP5
EPO	IL6
F10	IL6ST
F10	INHBA
F7	KIR2DL4
F9	LEPR
FETUB	MAP2K4
FGA FGB FGG	MET
FGA FGB FGG	MMP13
FGG	MRC2
HRG	NAPA
IL25	NRP1
IL6R	PARK7
KLK7	PDGFC
KNG1	PDIA3
MST1	PGAM1
PCSK9	PGD
PI3	PKM2
PLG	PLAUR
PLG	POSTN
SERPINA7	PTPN11
SERPINC1	RGMB
SERPINF2	SBDS
SHBG	SHC1
SLPI	SPARC
TFF3	SPHK1
	TGFBI
	TNFAIP6
	TNFRSF13C
	UFC1
	VEGFC
	VTA1

Table S8, Lists of enrichment analysis of each cluster in figure 6

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