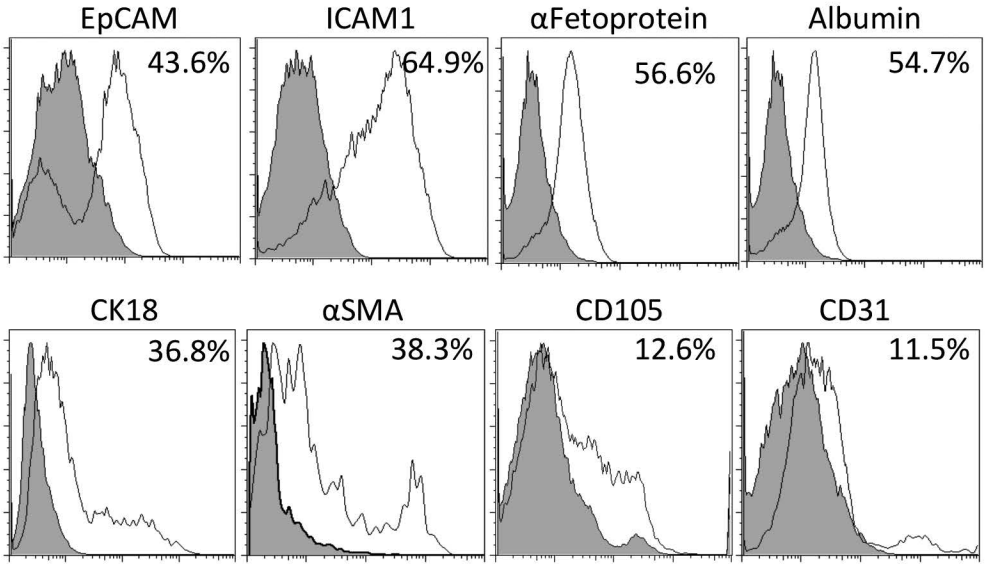
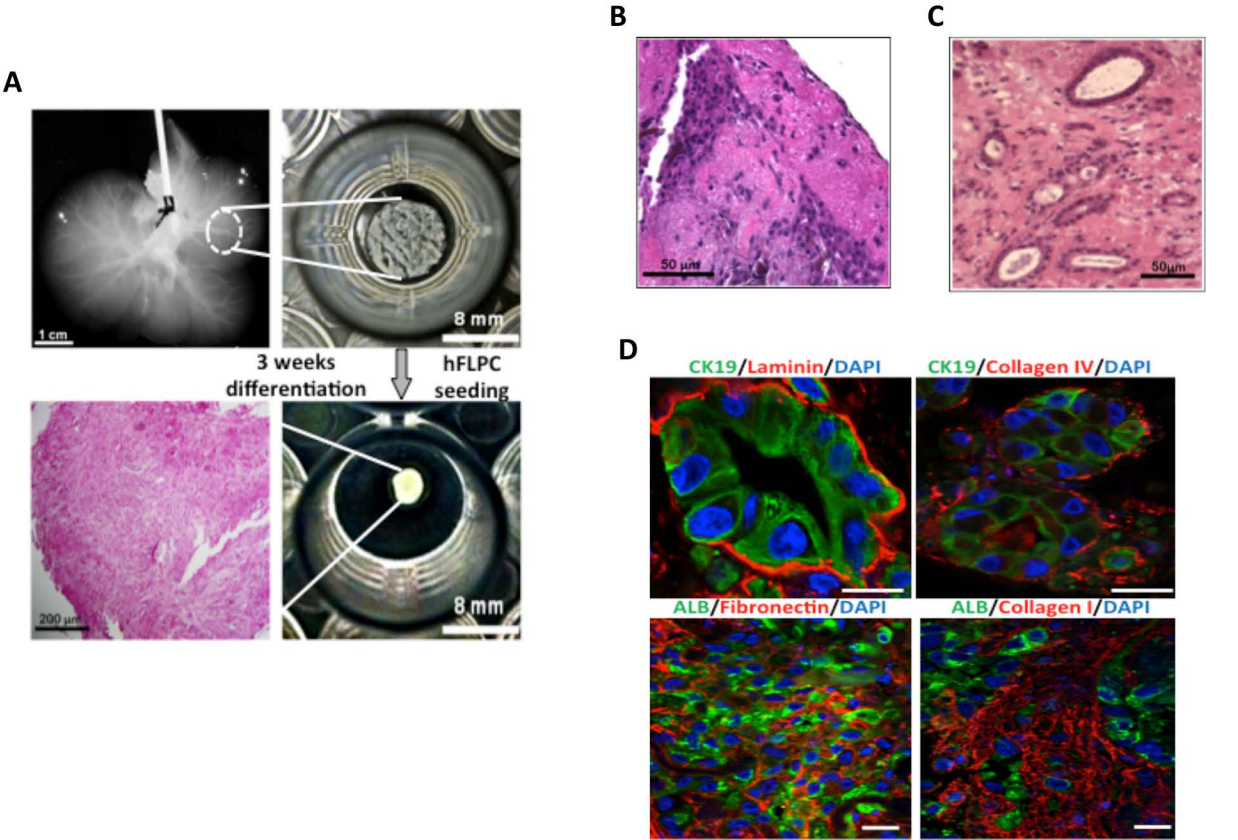
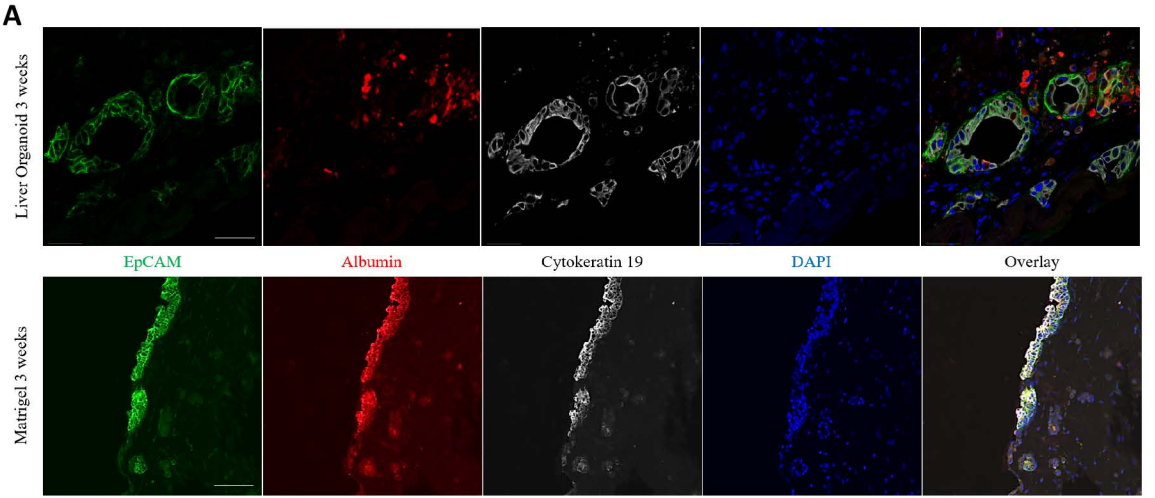
Supplemental Figure. 1

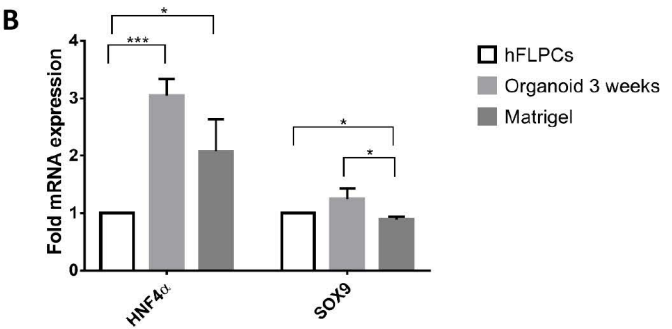


Supplemental Figure. 2

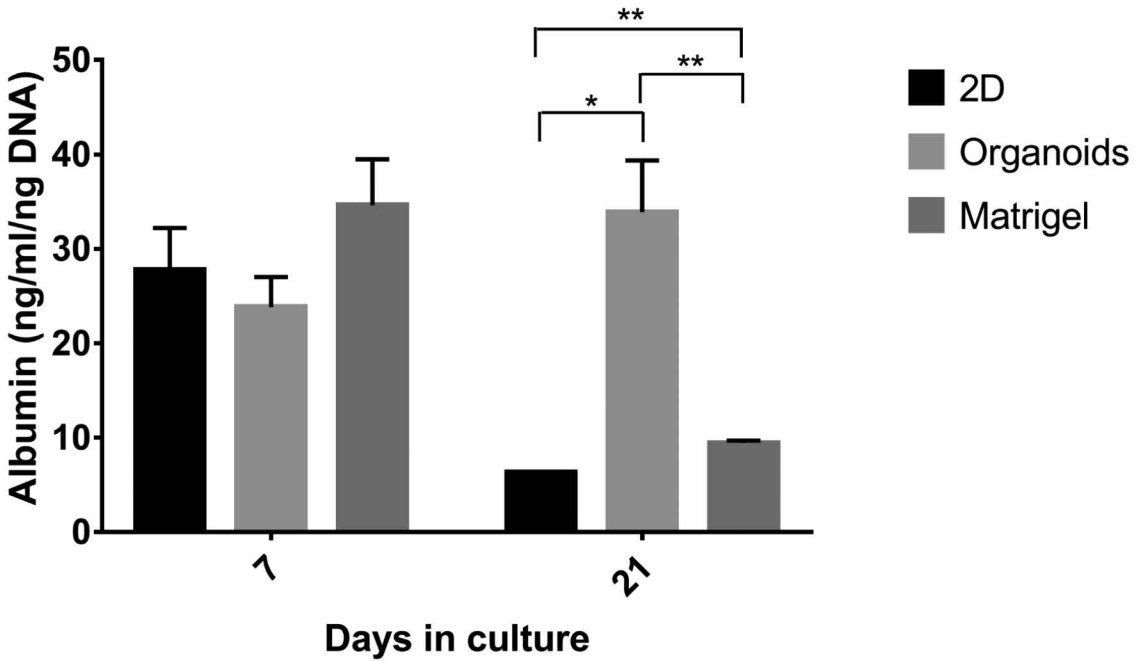


Supplemental Figure. 3





Supplemental Figure. 4



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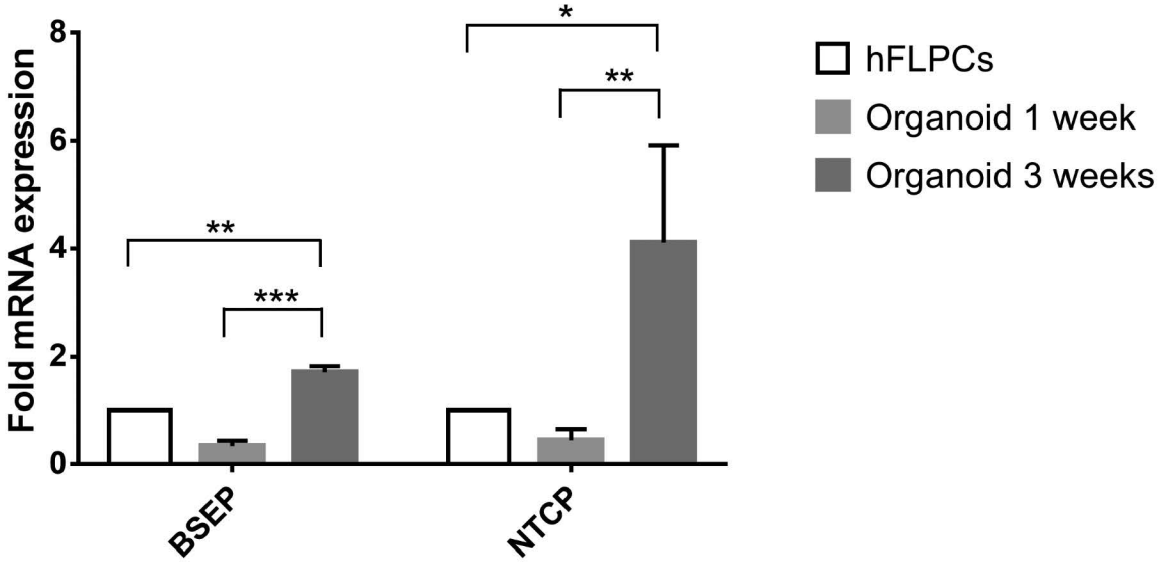


Supplemental Figure. 6

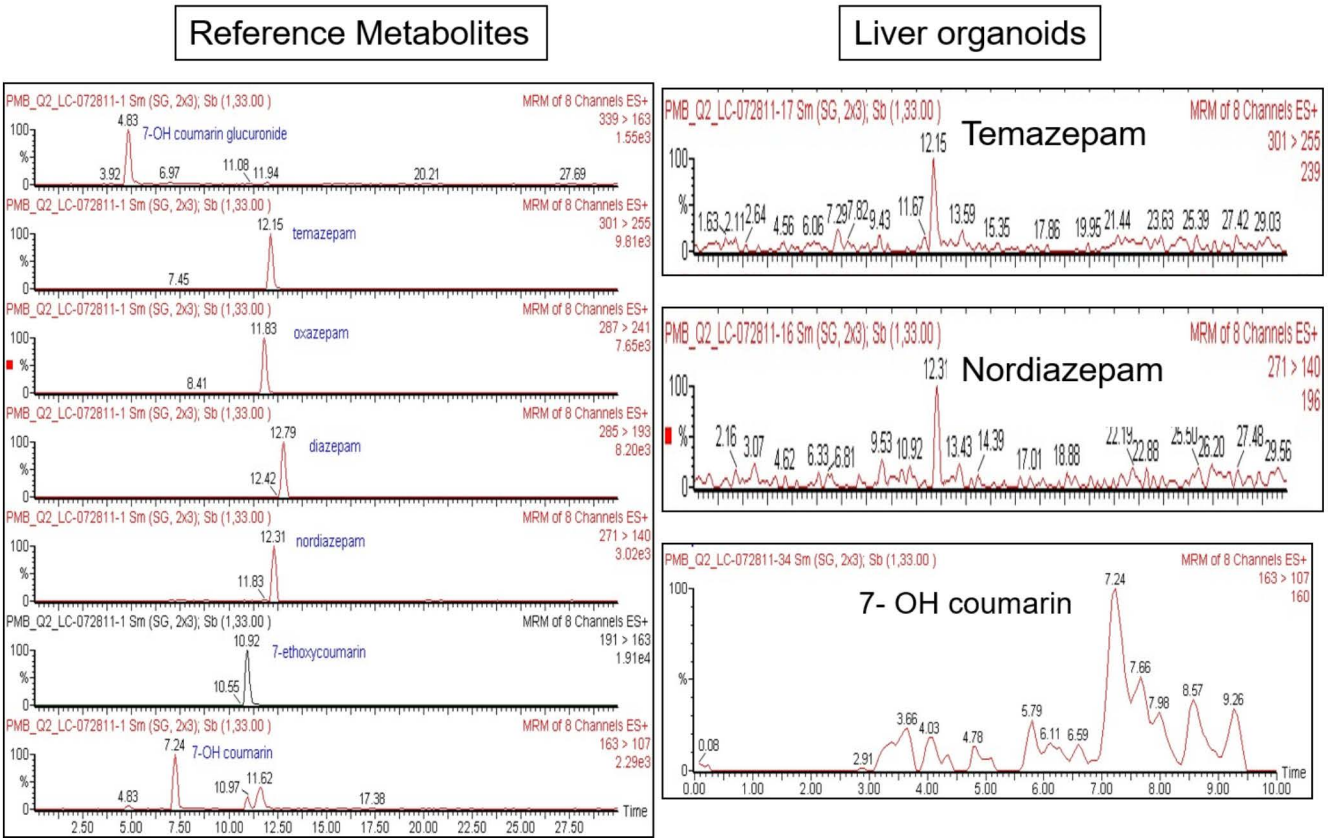
Liver organoids

Substrate	Metabolite	1 week differentiation				3 week differentiation			
		Time after substrate addition (Hours)				Time after substrate addition (Hours)			
		3	6	12	24	3	6	12	24
Diazepam	Temazepam	-	+	+	+	+	+	+	+
	Nordiazepam	-	-	+	+	-	+	+	+
7-OCH coumarin	7-OH coumarin	-	-	+	+	-	-	-	+

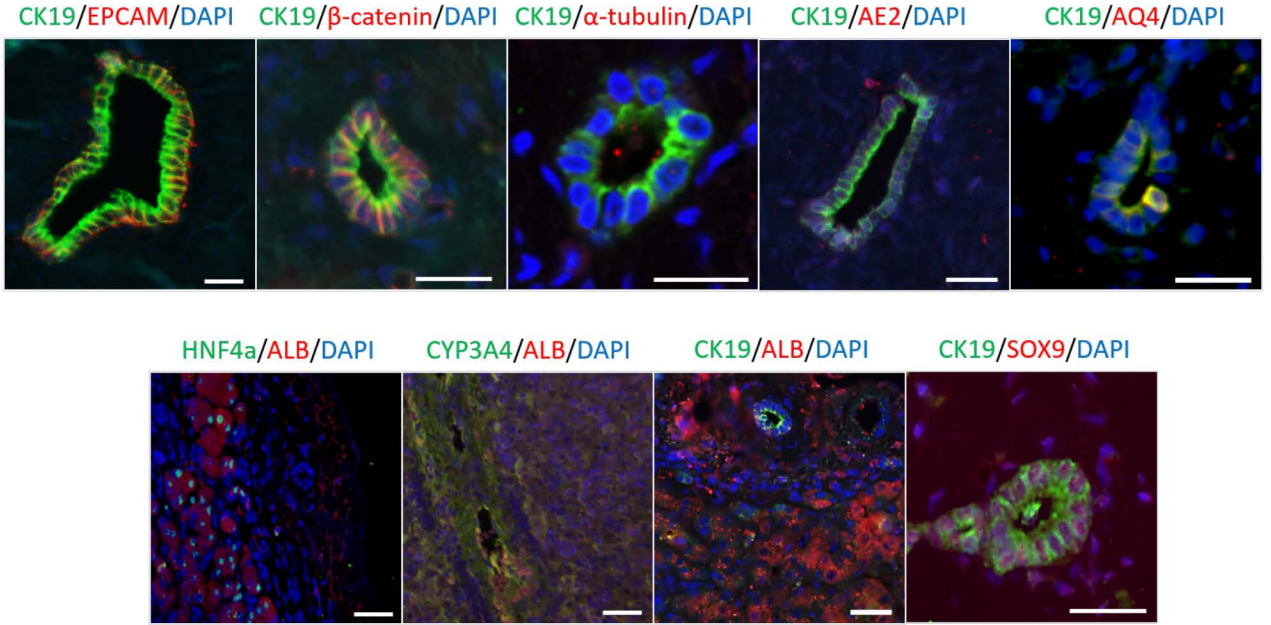
Sandwich cultured Hepatocytes

Substrate	Metabolite	Time after substrate addition (Hours)			
		3	6	12	24
Diazepam	Temazepam	+	+	+	+
	Nordiazepam	+	+	+	+
7-OCH coumarin	7-OH coumarin	+	+	+	+

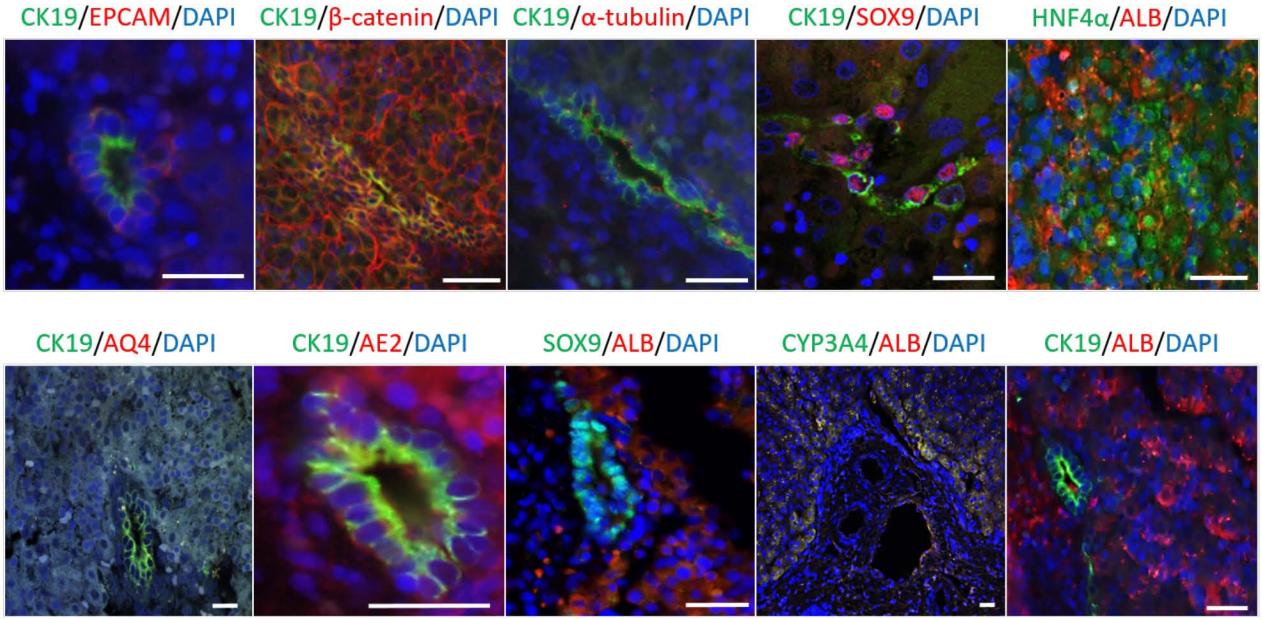
Supplemental Figure. 7



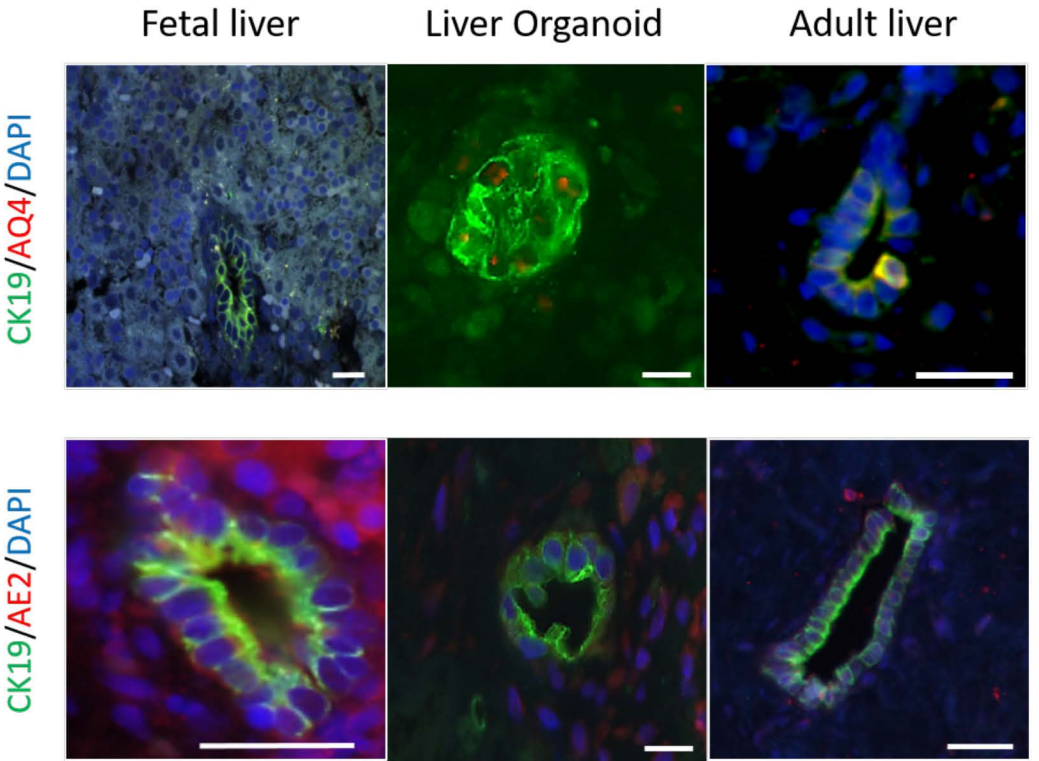
Supplemental Figure. 8



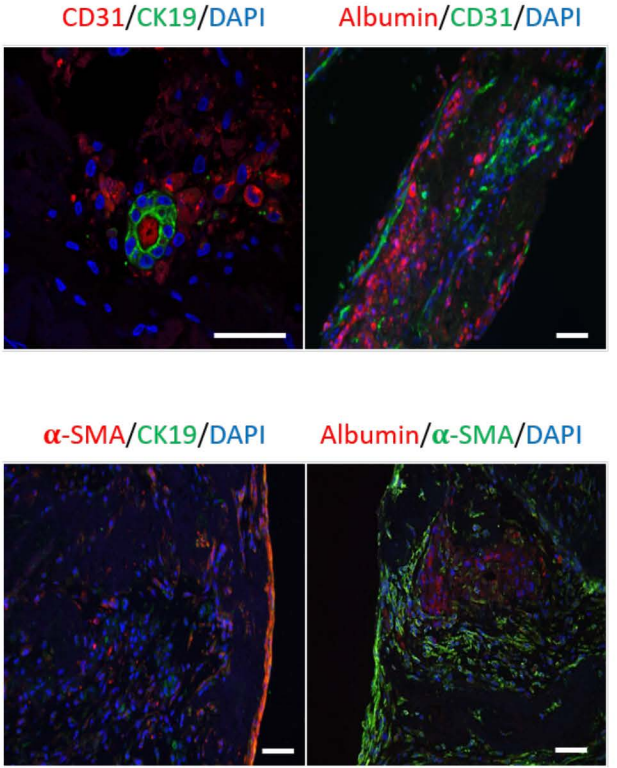
Supplemental Figure. 9



Supplemental Figure. 10

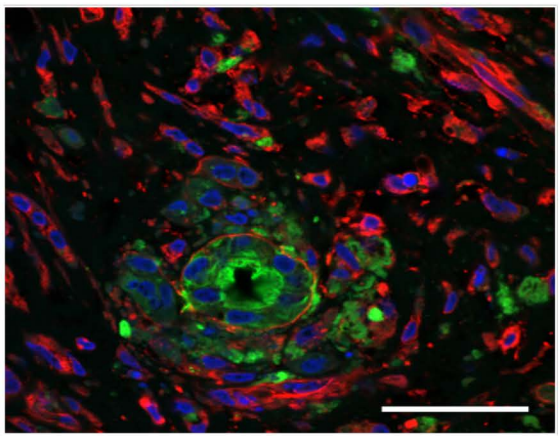


Supplemental Figure. 11

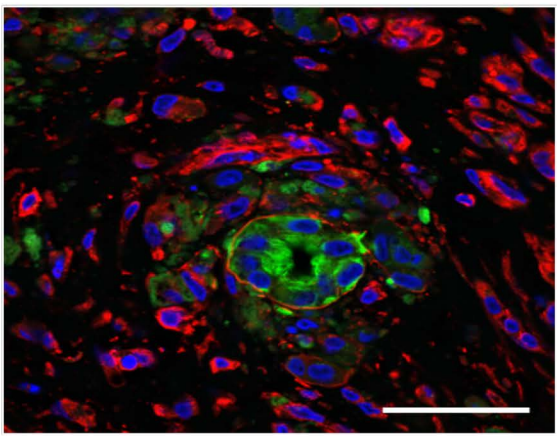


Supplemental Figure. 12

Collagen IV/CK19/DAPI



Laminin/CK19/DAPI



Supplemental Table 1. List of antibodies used in immunofluorescent analysis

Antibody	Species	Vendor & Catalogue Number	Concentration
AFP	Rabbit	DAKO, A0008	1:400
Albumin	Goat	Bethyl Laboratories, A80-129A	1:200-1:400
CK19	Mouse	Novocastra, NCL-CK19	1:100
CK19	Goat	Santa Cruz, sc-33120	1:100
SOX9	Rabbit	Santa Cruz, sc-20095	1:100
Laminin	Rabbit	Sigma, L-9393	1:50
HNF4α	Rabbit	Santa Cruz, sc8987	1:50
EpCAM	Rabbit	Abcam, ab124825	1:50
A1AT	Rabbit	Abcam, ab922	Ready to use
ASBT	Rabbit	Gift from Dr. Paul Dawson	1:400 – 1:1000
α-acetylated tubulin	Mouse	Invitrogen, 322700	1:300
β-catenin	Rabbit	Santa Cruz, sc-7199	1:1000
CYP3A4	Rabbit	Novus Biologicals, NBP1-95969	1:100
AE2	Rabbit	Abcam, Ab42687	1:100
Aquaporin 4	Goat	Santa Cruz, sc-9888	1:100
Collagen I	Goat	Southern Biotech, 1310-01	1:50
Collagen IV	Goat	Southern Biotech, 1340-01	1:50
Fibronectin	Rabbit	Santa Cruz, sc-9068	1:100
CD31	Mouse	Abcam, ab9498	1:25
α-SMA	Rabbit	Abcam, ab5694	1:100

Supplemental Table 2. List of Primers used in RT-PCR studies

Gene Name	Gene Symbol	Forward /reverse	Sequence	PCR product (bp)
α-fetoprotein	AFP	F	ACCATGAAGTGGGTGGAATC	148
		R	TGGTAGCCAGGTCAGGTCAGCTAAA	
Albumin	ALB	F	GATGCTGTGAAGGGGATGTT	357
		R	TGTTGCAGCAATTTCTCAGG	
Hepatocyte Nuclear Factor 4 alpha	HNF4α	F	TCAACCCGAGAAAACAAACC	131
		R	ACCTGCTCTACCAGCCAGAA	
Aspartate Aminotransferase	AST	F	TCCAAGAACTTCGGGCTCTA	119
		R	GACCAAGTAATCCGCACGAT	
Transferrin	TF	F	CTACACAGGCGCTTTCAGGT	152
		R	TACCATCAAGGCACAGCAAC	
Glucose -6- Phosphatase	G6PC	F	TCAGGGAAAGATAAAGCCGACC	105
		R	AGGTAGATTCGTGACAGACAGAC	
Tryosine Aminotransferase	TAT	F	TTTGGGACCCTGTACCATTGT	102
		R	GCATTGGACTTGAGGAAGCTC	
Cytochrome P450, Family 3, Subfamily A, Polypeptide 7	CYP3A7	F	AAGTCTGGGGTATTTATGACT	210
		R	CGCTGGTGAATGTTGGAGAC	
Cytochrome P450, Family 3, Subfamily A, Polypeptide 4	CYP3A4	F	GCCTGGTGCTCCTCTATCTA	187
		R	GGCTGTTGACCATCATAAAAGC	
Cytochrome p450, Family 2, Subfamily B, Polypeptide 6	CYP2B6	F	GGCCATACGGGAGGCCCTTG	243
		R	AGGGCCCCTTGGATTTCCG	
Cytochrome p450, Family 2, Subfamily C, Polypeptide 9	CYP2C9	F	TCCTATCATTGATTACTTCCCG	217
		R	AACTGCAGTGTTTTCCAAGC	
Cytochrome p450, Family 2, Subfamily E, Polypeptide 1	CYP2E1	F	GACTGTGGCCGACCTGTT	297
		R	ACTACGACTGTGCCCTTGG	
Hepatocyte Nuclear Factor 6	HNF6	F	CAGCACCTCACGCCCACCTC	369
		R	CAGCCACTTCCACATCCTCCG	
Hepatocyte Nuclear Factor 1-beta	HNF1β	F	GAAAGCAACGGGAGATCCTC	279
		R	CCTCCACTAAGGCCTCCCTC	
Cytokeratin 19	CK19	F	TTTGAGACGGAACAGGCTCT	279
		R	CTCGGCCATGACCTCATATT	
Anion Exchange Protein 2	AE2	F	GCCAAGGGCGCAGATTCTT	103
		R	CCAGGGTGCGGTGAAGTTC	
Gamma-glutamyltransferase 1	GGT1	F	GGGGAGATCGAGGGCTATGAG	150
		R	GATGACGGTCCGCTTGTTTTC	
Glyceraldehyde-3-phosphate Dehydrogenase	GAPDH	F	AGAAGGCTGGGGCTCATTTG	258
		R	AGGGGCCATCCACAGTCTTC	

Supplemental Figure Legends

Supplemental Figure 1. Flow cytometric (FACS) analysis of hFLPCs after 7 days in culture.

FACS analysis shows a large population (indicated by percent of total analyzed cells) of cells positive for hepatoblast markers such as EpCAM, ICAM1, αFP, ALB and CK18 and a smaller proportion of stromal cells expressing αSMA and CD105 and endothelial cells expressing CD31 positive.

Supplemental Figure 2. Liver organoid formation inside the liver ECM discs.

A) Schematic representation of the experimental approach for creating liver ECM discs from decellularized liver scaffold and seeding of hFLPCs. 3D liver organoids repopulated with liver cells are formed in culture over a 3-week period (H&E staining). B, C) H&E staining of section made form liver organoids after 3 weeks of differentiation shows clusters of hepatocytes (B) and ductal structures resembling bile ducts (C). D) ECM molecules organization around bile duct structures and hepatocytic clusters within the liver organoids. The organoids were stained for cytokeratin19 (CK19), albumin (ALB), laminin, fibronectin, collagen I and IV (Col I, Col IV), and for cell nuclei (DAPI).

Supplemental Figure 3. hFLPC differentiation in Matrigel®.

A) Distribution and phenotypic characteristics of hFLPCs in liver ECM organoids (top panel) and Matrigel® (bottom panel) after 3 weeks of differentiation in culture. Cells were stained for epithelial cell adhesion molecule (EpCAM), albumin (ALB), cytokeratin19

(CK19) and for cell nuclei (DAPI). Scale bar is 20 µm for top panel and 50 µm for bottom panel. B) RT-PCR analysis of the expression of HNF4α and SOX9 in liver organoids and matrigel (* = $p<0.05$, *** = $p<0.0005$).

Supplemental Figure 4. Albumin secretion in liver organoids and Matrigel®.

Albumin levels were measured by ELISA from culture medium collected after 1 week and 3 weeks of differentiation of hFLPCs in liver organoids and Matrigel® (* = $p<0.05$, ** = $p<0.005$).

Supplemental Figure 5. Bile acid transporter expression in liver organoids.

RT-PCR analysis of the expression of bile acid transporters BSEP and NTCP (* = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$).

Supplemental Figure 6. Metabolic activity of liver organoids.

Metabolic activity of liver organoids after 1 and 3 weeks of differentiation and sandwich cultured hepatocytes, as measured by the conversion of Diazepam to Temazepam and Nordiazepam and of 7-ethoxy (OCH) coumarin to 7- hydroxyl (OH) coumarin.

Supplemental Figure 7. Mass Spectrometry profile of metabolites formed by liver organoids.

Liver organoids were incubated with Diazepam or 7-ethoxy coumarin and the presence of Temazepam and Nordiazepam or 7- hydroxyl (OH) coumarin, respectively, was measured

in the conditioned media by mass spectrometry (right panels). The profile of reference metabolites is shown in the left panels.

Supplemental Figure 8.

Immunostaining of adult human liver showing biliary ducts expressing CK19, EpCAM, acetylated α-tubulin, anion exchange protein 2 (AE2), Aquaporin4 and SOX9 along with clusters of hepatocytes expressing Albumin, HNF4α and CYP3A4. Scale bar is 25 μM.

Supplemental Figure 9.

Immunostaining of fetal human liver showing biliary ducts expressing CK19, EpCAM, β-catenin, acetylated α-tubulin, anion exchange protein 2 (AE2), Aquaporin4 (AQP4) and SOX9 along with clusters of hepatocytes expressing Albumin, HNF4α and CYP3A4. Scale bar is 20 μM.

Supplemental Figure 10.

Immunostaining panel showing comparative expression of AE2 and AQ4 in human fetal liver, liver organoids and adult liver. Scale bar is 20 μM.

Supplemental Figure 11.

Immunostaining of liver organoids showing CD31 expressing endothelial cell population and α-smooth muscle actin expressing stromal cell population surrounding bile ducts and hepatocyte clusters. Scale bar is 50 μM.

Supplemental Figure 12.

Immunostaining of liver organoids showing Collagen IV and Laminin deposition surrounding CK19 positive bile duct structures. Scale bar is 50 μM.