

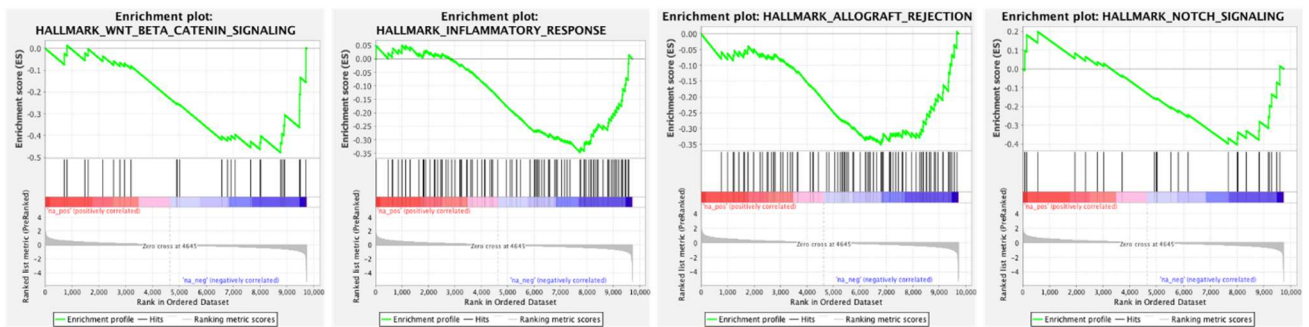
## Supplemental Information

### **Human biliary epithelial cells from discarded donor livers rescue bile duct structure and function in a mouse model of biliary disease**

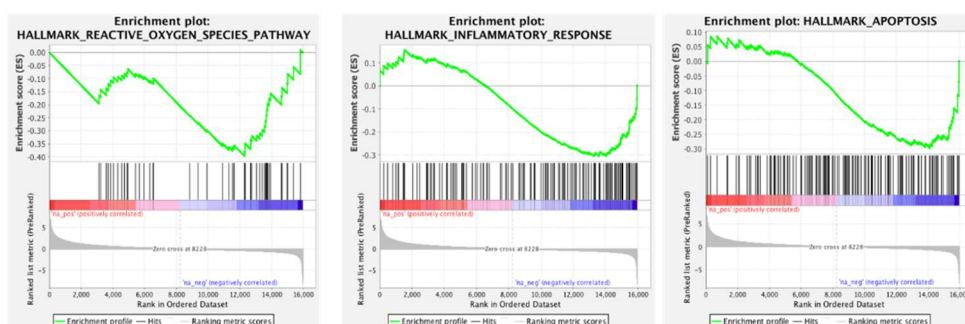
**John M. Hallett, Sofia Ferreira-Gonzalez, Tak Yung Man, Alastair M. Kilpatrick, Hannah Esser, Kayleigh Thirlwell, Mark T. Macmillan, Daniel Rodrigo-Torres, Benjamin J. Dwyer, Victoria L. Gadd, Candice Ashmore-Harris, Wei-Yu Lu, John P. Thomson, Maurits A. Jansen, Eoghan O'Duibhir, Philip J. Starkey Lewis, Lara Campana, Rhona E. Aird, Thomas S.R. Bate, Alasdair R. Fraser, John D.M. Campbell, Gabriel C. Oniscu, David C. Hay, Anthony Callanan, and Stuart J. Forbes**

**A****GSEA SIGNIFICANTLY DOWNREGULATED IN CD133+ VS CD133- hBEC IN HEALTHY LIVERS**

GENE SET	SIZE	ES	NES	NOM p-val	FDR q-val
APICAL JUNCTION	125	-0.45	-1.90	< 0.001	0.0159
EPITHELIAL-MESENCHYMAL TRANSITION	104	-0.45	-1.82	0.0018	0.0118
ESTROGEN RESPONSE LATE	132	-0.43	-1.78	< 0.001	0.0126
COAGULATION	75	-0.42	-1.61	0.0035	0.0527
ESTROGEN EARLY RESPONSE	143	-0.37	-1.61	< 0.001	0.0443
MYOGENESIS	91	-0.40	-1.61	0.0036	0.0371
ANGIOGENESIS	22	-0.53	-1.57	0.0205	0.0521
MITOTIC SPINDLE	160	-0.35	-1.51	0.0068	0.0716
WNT/BETA-CATENIN SIGNALING	28	-0.48	-1.47	0.0466	0.0872
CHOLESTEROL HOMEOSTASIS	60	-0.38	-1.40	0.0635	0.1398
INFLAMMATORY RESPONSE	97	-0.35	-1.40	0.0309	0.1345
PI3K/AKT/MTOR SIGNALING	77	-0.36	-1.38	0.0342	0.1341
ALLOGRAFT REJECTION	86	-0.35	-1.36	0.0489	0.1441
IL2/STATS SIGNALING	125	-0.33	-1.36	0.0420	0.1356
IL6/JAK/STAT3 SIGNALING	43	-0.39	-1.32	0.1000	0.1679
GLYCOLYSIS	140	-0.31	-1.32	0.0504	0.1575
NOTCH SIGNALING	26	-0.40	-1.25	0.1653	0.2423

**B****C****GSEA SIGNIFICANTLY DOWNREGULATED IN STEATOTIC VS HEALTHY CD133+ hBEC**

GENE SET	SIZE	ES	NES	NOM p-val	FDR q-val
INTERFERON-GAMMA RESPONSE	176	-0.40	-1.86	< 0.001	0.0267
INTERFERON-ALPHA RESPONSE	96	-0.42	-1.75	< 0.001	0.0422
MYC TARGETS V1	199	-0.39	-1.66	< 0.001	0.0550
OXIDATIVE PHOSPHORYLATION	200	-0.35	-1.61	< 0.001	0.0620
COAGULATION	109	-0.37	-1.58	< 0.001	0.0564
COMPLEMENT	162	-0.34	-1.52	< 0.001	0.0684
MTORC1 SIGNALING	194	-0.32	-1.50	< 0.001	0.0765
ANDROGEN RESPONSE	97	-0.36	-1.47	< 0.001	0.0853
IL6/JAK/STAT3 SIGNALING	65	-0.38	-1.46	0.0172	0.0780
PROTEIN SECRETION	95	-0.35	-1.45	< 0.001	0.0738
REACTIVE OXYGEN SPECIES PATHWAY	47	-0.40	-1.41	0.0719	0.0915
XENOBIOTIC METABOLISM	174	-0.31	-1.41	< 0.001	0.0839
PEROXISOME	92	-0.33	-1.38	0.0390	0.0919
INFLAMMATORY RESPONSE	140	-0.31	-1.32	< 0.001	0.1282
TNFA SIGNALING VIA NFKB	187	-0.30	-1.32	< 0.001	0.1256
BILE ACID METABOLISM	92	-0.32	-1.30	0.0290	0.1349
APOPTOSIS	146	-0.30	-1.28	0.0303	0.1374
FATTY ACID METABOLISM	143	-0.29	-1.25	< 0.001	0.1546
UNFOLDED PROTEIN RESPONSE	112	-0.29	-1.25	0.0678	0.1501
CHOLESTEROL HOMEOSTASIS	71	-0.32	-1.23	0.0957	0.1702

**D**

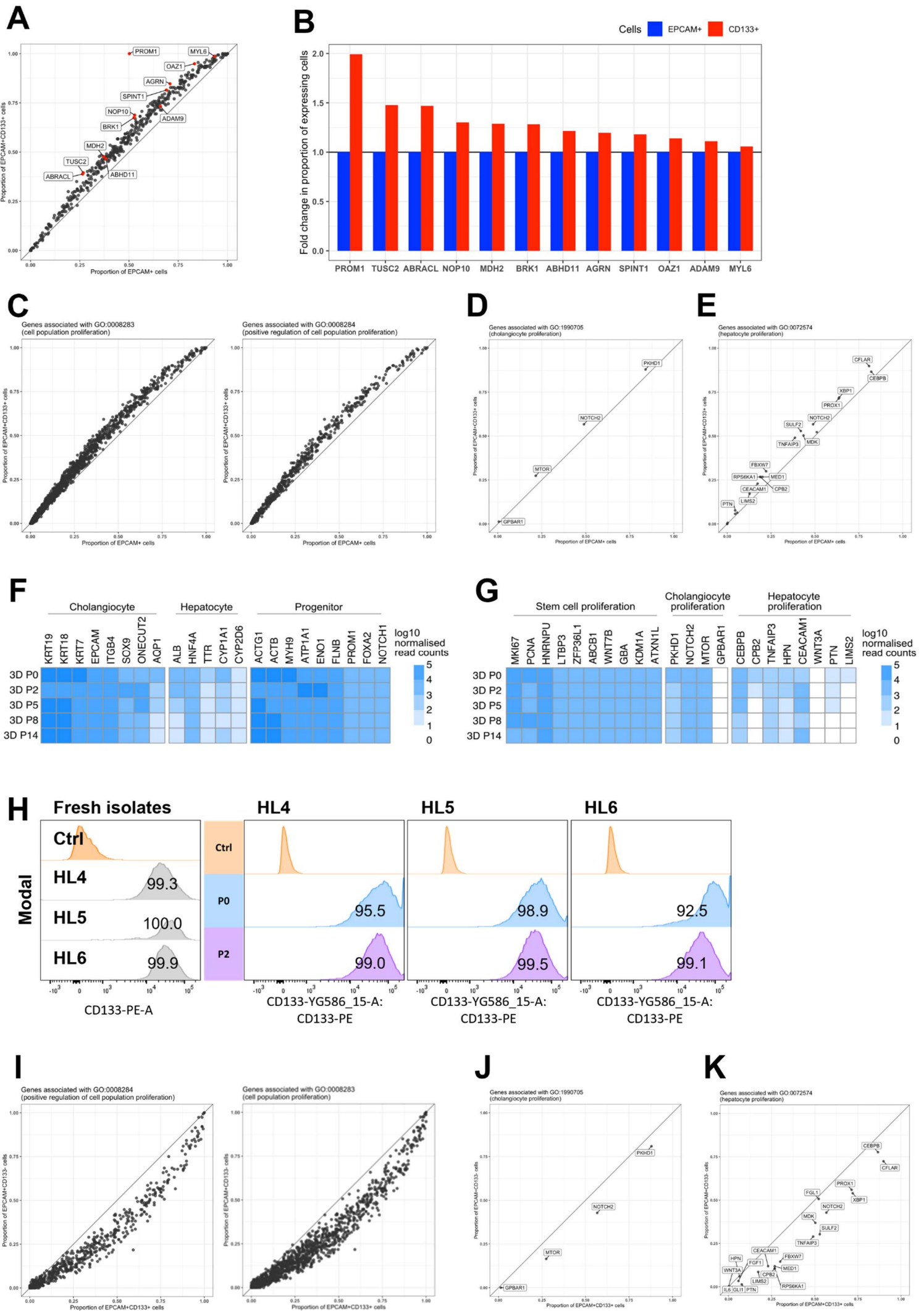
**Supplemental Figure S1. Gene set enrichment analysis of CD133+ and CD133- hBEC isolated from healthy and steatotic livers** (related to Figure 2).

**(A)** Table of MSigDB Hallmark gene sets found to be significantly depleted in CD133+ hBEC isolated from healthy livers (associated with genes downregulated in CD133+ hBEC) by gene set enrichment analysis (GSEA). Table presents gene set size, enrichment score (ES), normalized enrichment score (NES), nominal  $p$ -value and FDR ( $q$ -value). Gene sets with FDR less than the default GSEA FDR threshold of 0.25 are deemed significant.

**(B)** Enrichment plots of GSEA results for selected significant Hallmark gene sets. Genes associated with inflammatory response are downregulated in healthy CD133+ hBEC compared to healthy CD133- hBEC.

**(C)** Table of MSigDB Hallmark gene sets found to be significantly depleted in CD133+ hBEC isolated from steatotic livers (associated with genes downregulated in CD133+ hBEC isolated from steatotic livers) by gene set enrichment analysis (GSEA). Table presents gene set size, enrichment score (ES), normalized enrichment score (NES), nominal  $p$ -value and FDR ( $q$ -value). Gene sets with FDR less than the default GSEA FDR threshold of 0.25 are deemed significant.

**(D)** Enrichment plots of GSEA results for selected significant Hallmark gene sets. Genes associated with inflammatory response are downregulated in steatotic CD133+ hBEC compared to healthy CD133+ hBEC.



**Supplemental Figure S2. Stability analysis: hBEC phenotype and CD133+ expression in culture and comparative analysis of EpCAM+ hBEC (Sampaziotis et al., 2021) and EpCAM+CD133+ hBEC (related to Figure 2).**

**(A)** Proportion of EpCAM+CD133+ cells vs EpCAM+ cells that have detectable expression of markers associated with progenitor cell response. The straight line at  $y=x$  represents equal proportions of EpCAM+ and EpCAM+CD133+ hBEC.

**(B)** Fold change in proportion of expressing cells for EpCAM+ hBEC (blue) and EpCAM+CD133+ hBEC (red).

**(C)** Proportion of EpCAM+CD133+ cells vs EpCAM+ cells that have detectable expression of markers associated with proliferation and positive regulation of proliferation.

**(D)** Proportion of EpCAM+CD133+ cells vs EpCAM+ cells that have detectable expression of markers associated with cholangiocyte proliferation.

**(E)** Proportion of EpCAM+CD133+ cells vs EpCAM+ cells that have detectable expression of markers associated with hepatocyte proliferation.

**(F)** Heatmap of normalised expression values across genes associated with cholangiocytes, hepatocytes or progenitor cell populations. These markers were analysed in hBEC isolated from two donor livers maintained in 3D *in vitro* culture conditions. P0 represents freshly isolated hBECs. Darker blue represents higher normalised gene expression.

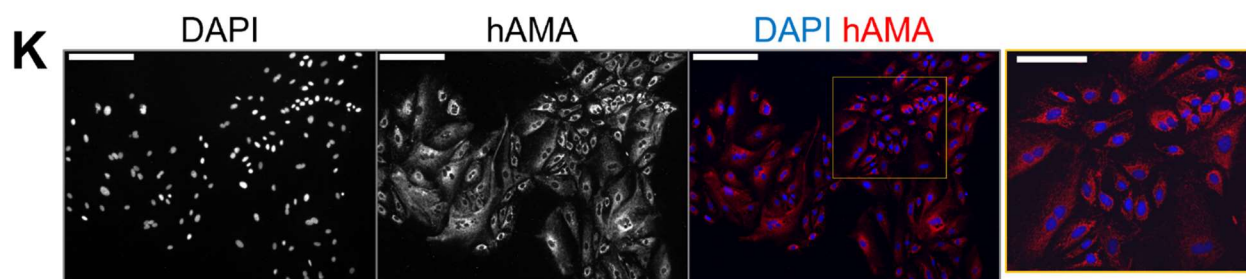
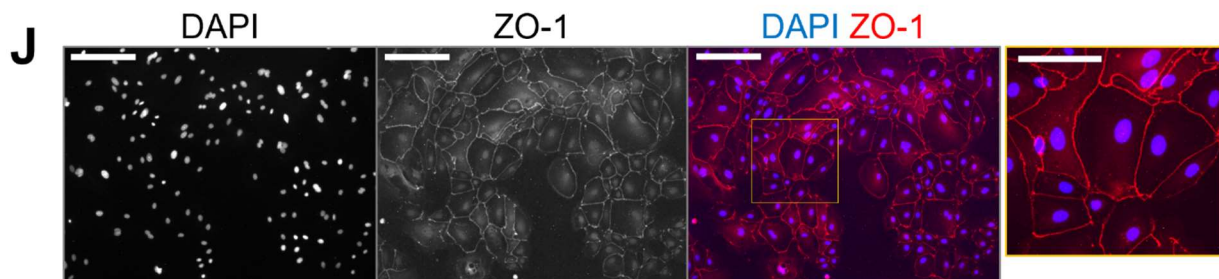
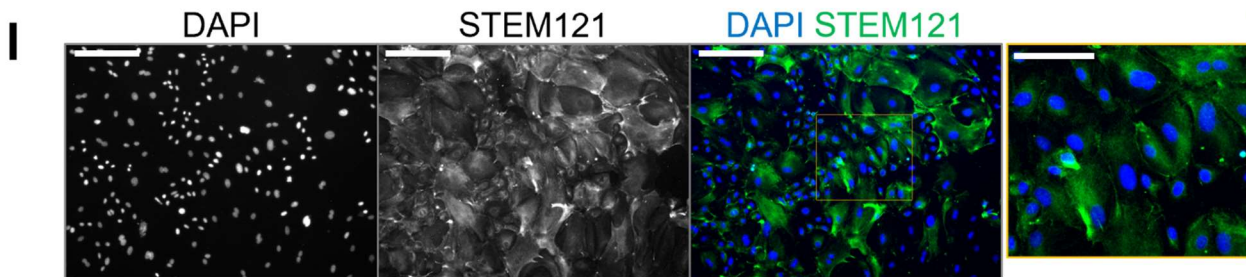
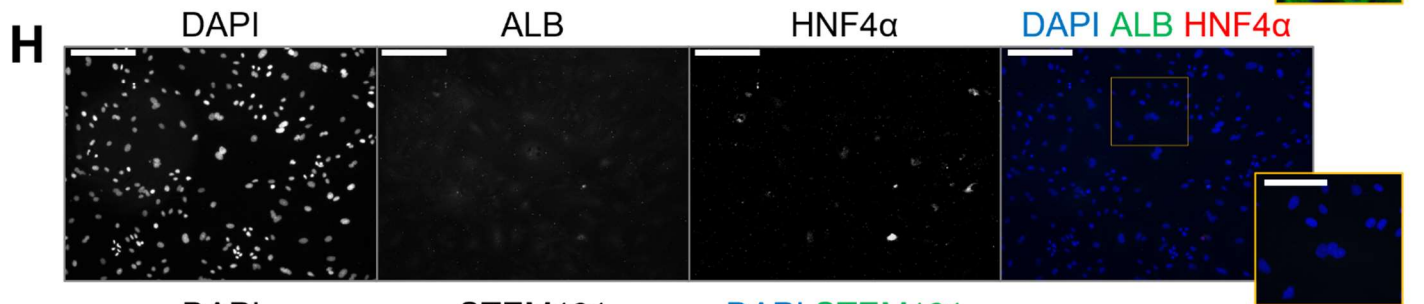
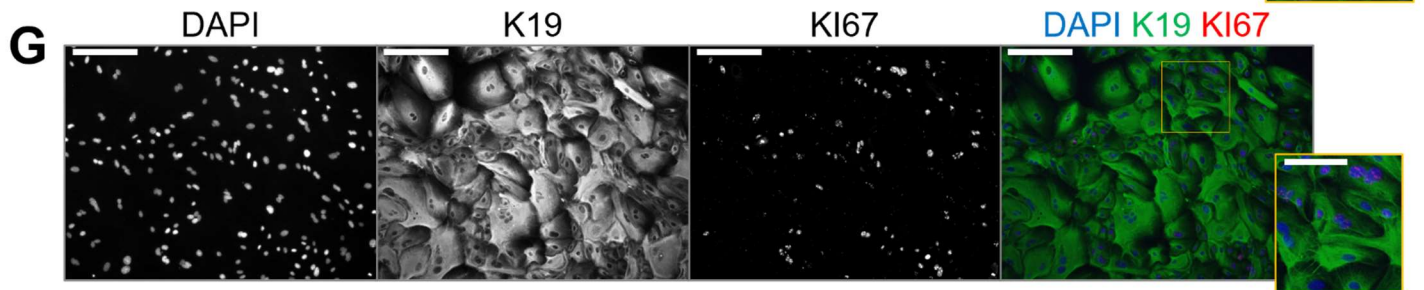
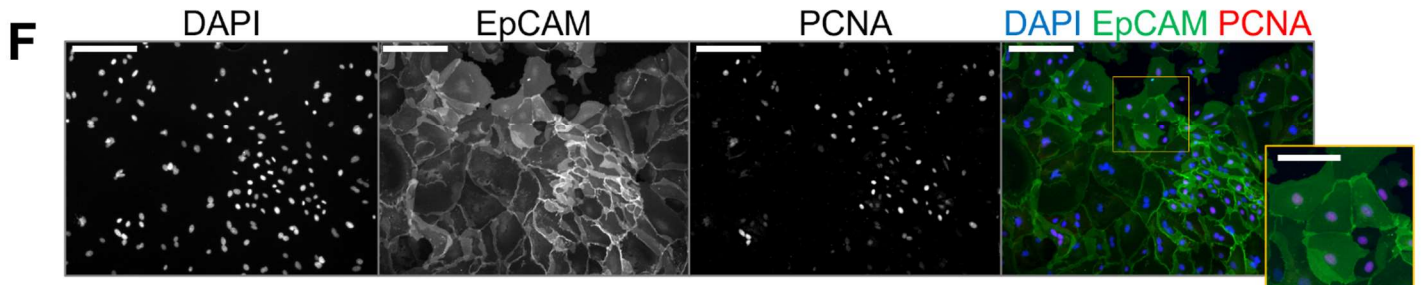
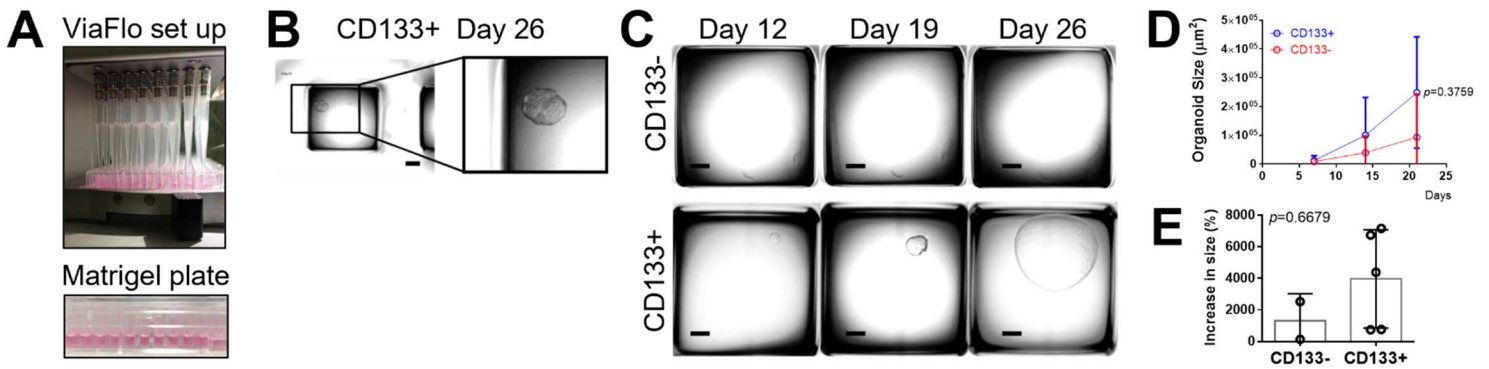
**(G)** Heatmap of normalised expression values across genes associated with stem cell, cholangiocyte and hepatocyte proliferation.

**(H)** Flow cytometry analysis of CD133 expression in BEC freshly isolated from three livers (HL4, HL5 and HL6) and subsequently cultured in 2D. The histograms indicate that the high levels of CD133 expression seen in freshly isolated EpCAM+CD24+ cells are retained following *in vitro* culture with analyses at passage 0 and passage 2 for each liver.

**(I)** Analysis of EpCAM+CD133+ vs EpCAM+CD133- hBEC profile using the publicly available single cell transcriptomic data from Sampaziotis, *et al.* (Science, 2021). Proportion of EpCAM+CD133+ cells vs EpCAM+CD133- cells that have detectable expression of markers associated with positive regulation of cell population proliferation (GO:0008284) and general cell population proliferation (GO:0008283). The straight line at  $y=x$  represents equal proportions of EpCAM+CD133+ and EpCAM+CD133- BEC.

**(J)** Proportion of EpCAM+CD133+ cells vs EpCAM+ cells that have detectable expression of markers associated with cholangiocyte proliferation (GO:1990705).

**(K)** Proportion of EpCAM+CD133+ cells vs EpCAM+ cells that have detectable expression of markers associated with hepatocyte proliferation (GO:0072574).



**Supplemental Figure S3. Clonal density analysis in CD133- and CD133+ hBEC. Immunohistological characterisation of CD133+ hBEC (related to Figure 2).**

**(A)** ViaFlo 96/384 Electronic Channel Pipette set up. Below, lateral view of a 96 well plate after plating the Matrigel that encapsulates single hBEC.

**(B)** Close view of one well at day 26 after plating one single CD133+ hBEC. Scale bar=100  $\mu\text{m}$ . Right, digital magnification of the organoid formed.

**(C)** Bright field of CD133- and CD133+ clonal density assay at different time points (day 12, 19 and 26) after plating one single hBEC. Scale bars=100  $\mu\text{m}$ .

**(D)** Organoid size (in  $\mu\text{m}^2$ ) over the course of days for the CD133- (red) and CD133+ (blue) hBEC populations.  $p=0.3759$  (Mean $\pm$ SEM), Student's t-test (N=3 technical replicates per group).

**(E)** Increase in size (expressed as percentage) of the CD133- and CD133+ hBEC organoids over the course of the experiment (26 days).  $p=0.6679$  (Mean $\pm$  SEM), Student's t-test. (N=2 for CD133- and N=5 for CD133+).

**(F)** CD133+ cells expanded in Matrigel culture and immunostained for cholangiocyte marker EpCAM (green) and proliferative marker PCNA (red).

**(G)** Immunostaining for cholangiocyte marker K19 (green) and proliferative marker Ki67 (red).

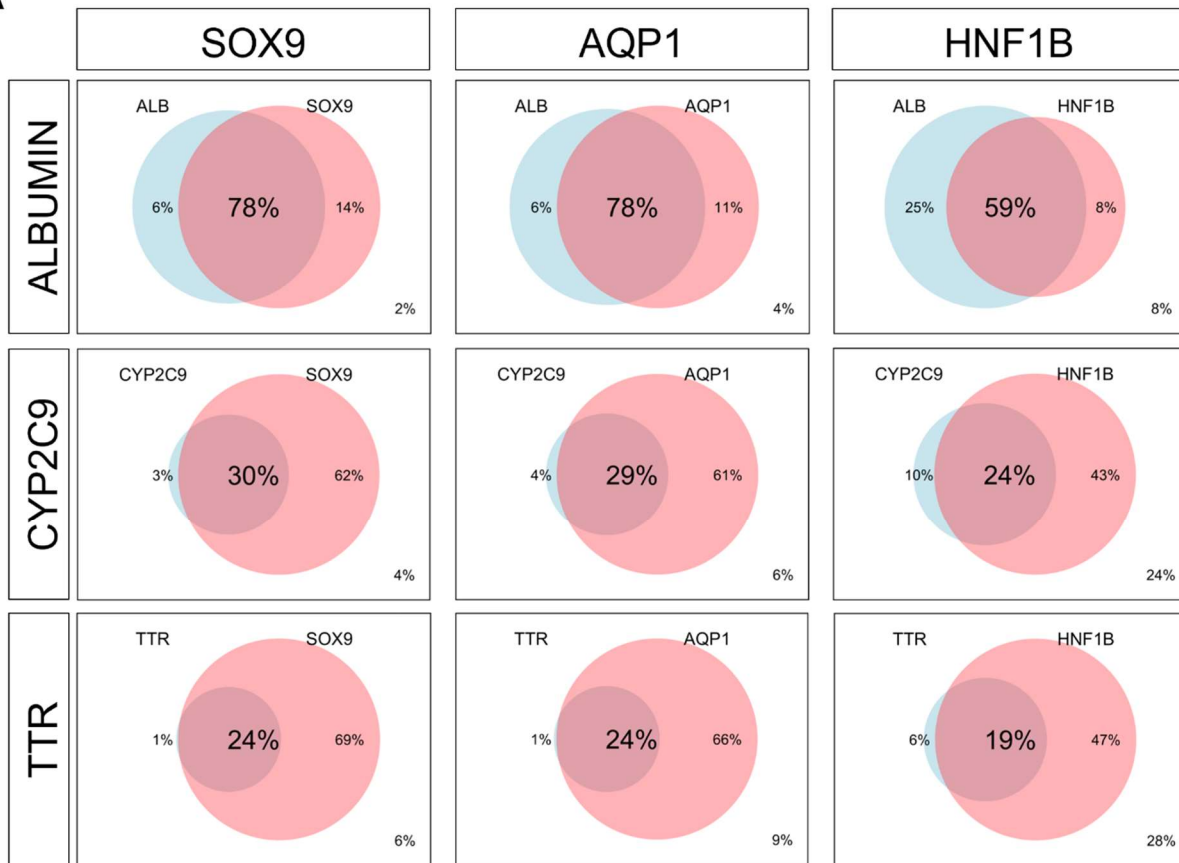
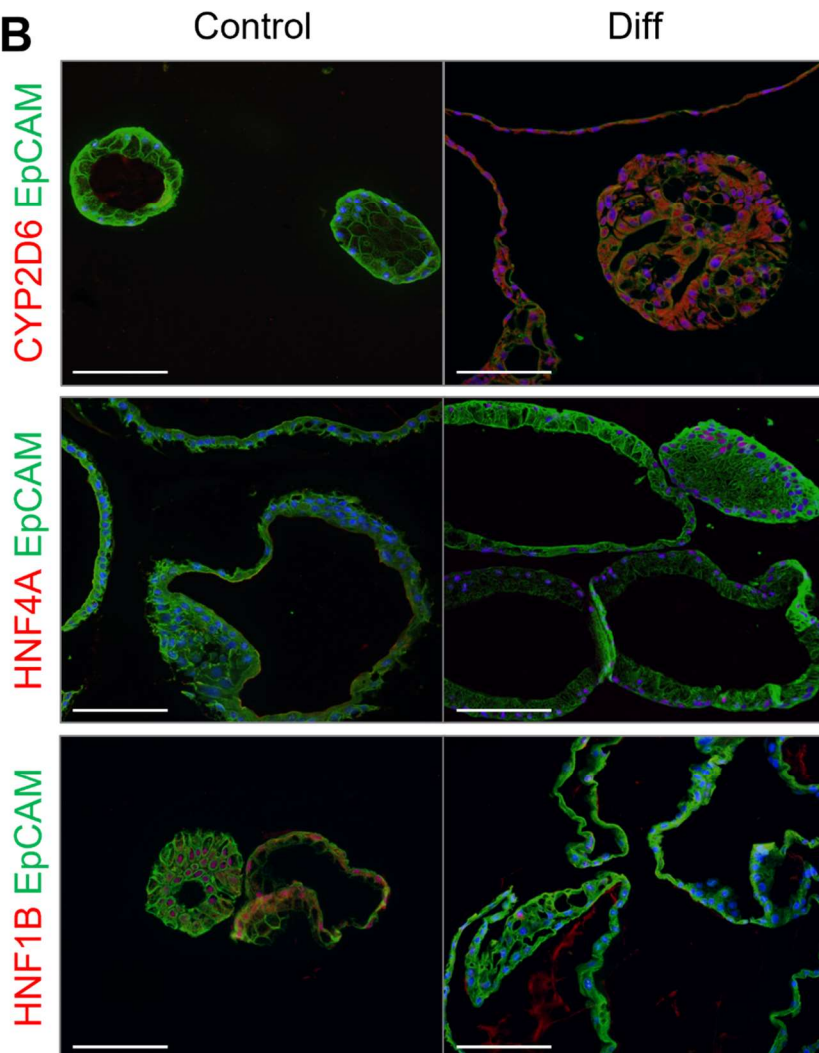
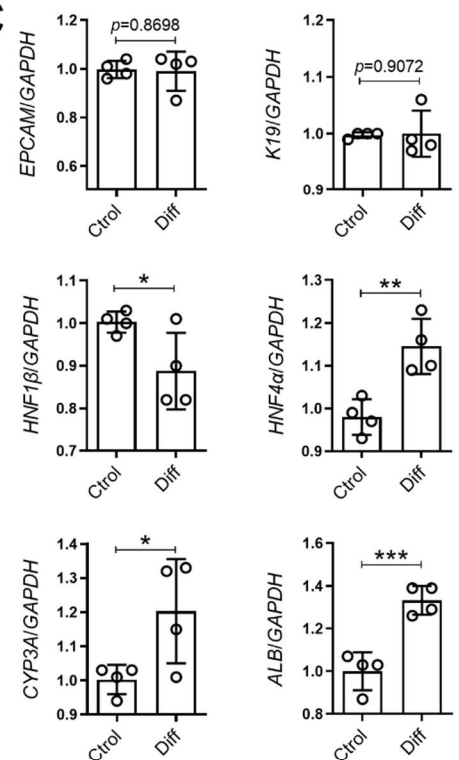
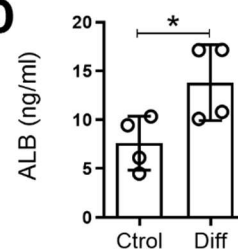
**(H)** Immunostaining for hepatocyte markers Albumin (green) and HNF4 $\alpha$  (red).

**(I)** Immunostaining for stem cell marker STEM121 (green).

**(J)** Immunostaining for tight junction protein ZO1 (red).

**(K)** Immunostaining for human antimitochondrial antibody (hAMA, red).

For F-K, scale bars=60  $\mu\text{m}$ .

**A****B****C****D**



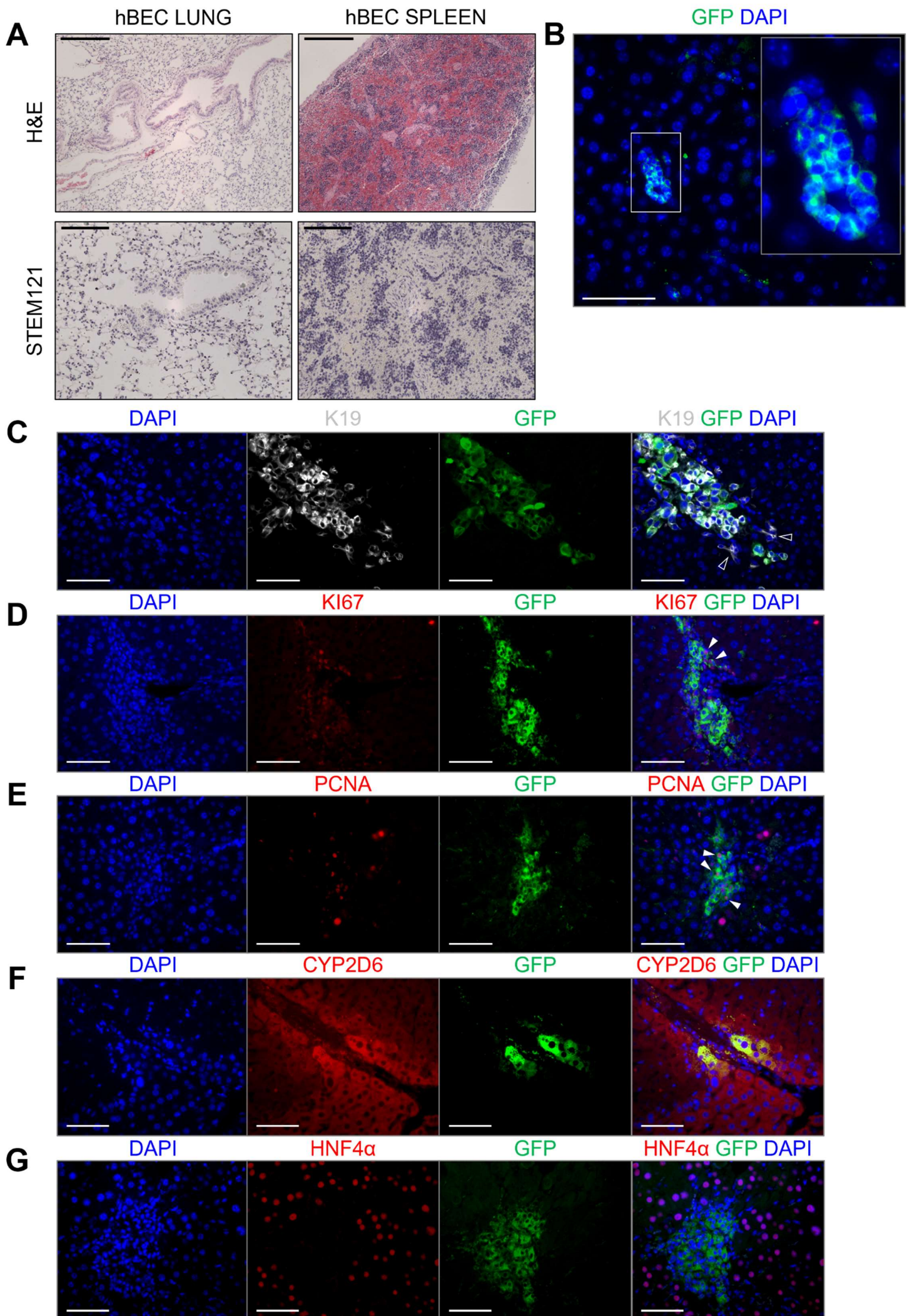
**Supplemental Figure S4. Assessing the bipotential capacity of hBEC in vitro** (related to Figure 2).

**(A)** Venn diagram indicating the proportion of hBEC that show detectable expression of markers of mature cholangiocytes (SOX9, Aquaporin1 [AQP1], HNF1B), hepatocytes (Albumin, CYP2C9 and TTR) or both. Transcriptomic data extracted from the single cell data of Sampaziotis et al., (Science 2021) filtered to EpCAM+CD24+CD133+ population.

**(B)** hBEC organoids cultured in 3D-Matrigel spheres in standard expansion media (Control) and differentiation media (Diff), immunostained for markers of cholangiocytes (EpCAM, HNF1 $\beta$ ) and hepatocytes (CYP2D6, HNF4 $\alpha$ ). Scale bars = 120  $\mu$ m.

**(C)** Gene expression of genes associated with mature cholangiocytes and hepatocytes in hBEC cultured in 3D-Matrigel spheres in standard expansion media (Ctrl) and differentiation media (Diff). All results displayed as relative fold increase compared to controls and normalised to *GAPDH*. \* denotes  $p < 0.05$ , \*\*  $p < 0.005$ , \*\*\*  $p < 0.001$  (Mean $\pm$ SEM), Student's *t*-test. (N=4 donor livers).

**(D)** Albumin (ALB) ELISA for hBEC in standard expansion media (Ctrl) and differentiation media (Diff). \* denotes  $p < 0.05$ , (Mean $\pm$ SEM), Student's *t*-test. (N=4 donor livers).



**Supplemental Figure S5. hBEC transplanted in the IRI immunocompromised model** (related to Figure 5).

**(A)** H&E staining of lung and spleen of hBEC transplanted mice (intrasplenic injection) show no abnormalities. SB=250  $\mu$ m. Below, STEM121 immunostaining shows no presence of hBEC in lung or spleen upon transplantation. SB=120  $\mu$ m. (N=6).

**(B)** Immunohistochemistry for GFP-positive hBEC (green) adopting biliary. Scale bars = 60  $\mu$ m.

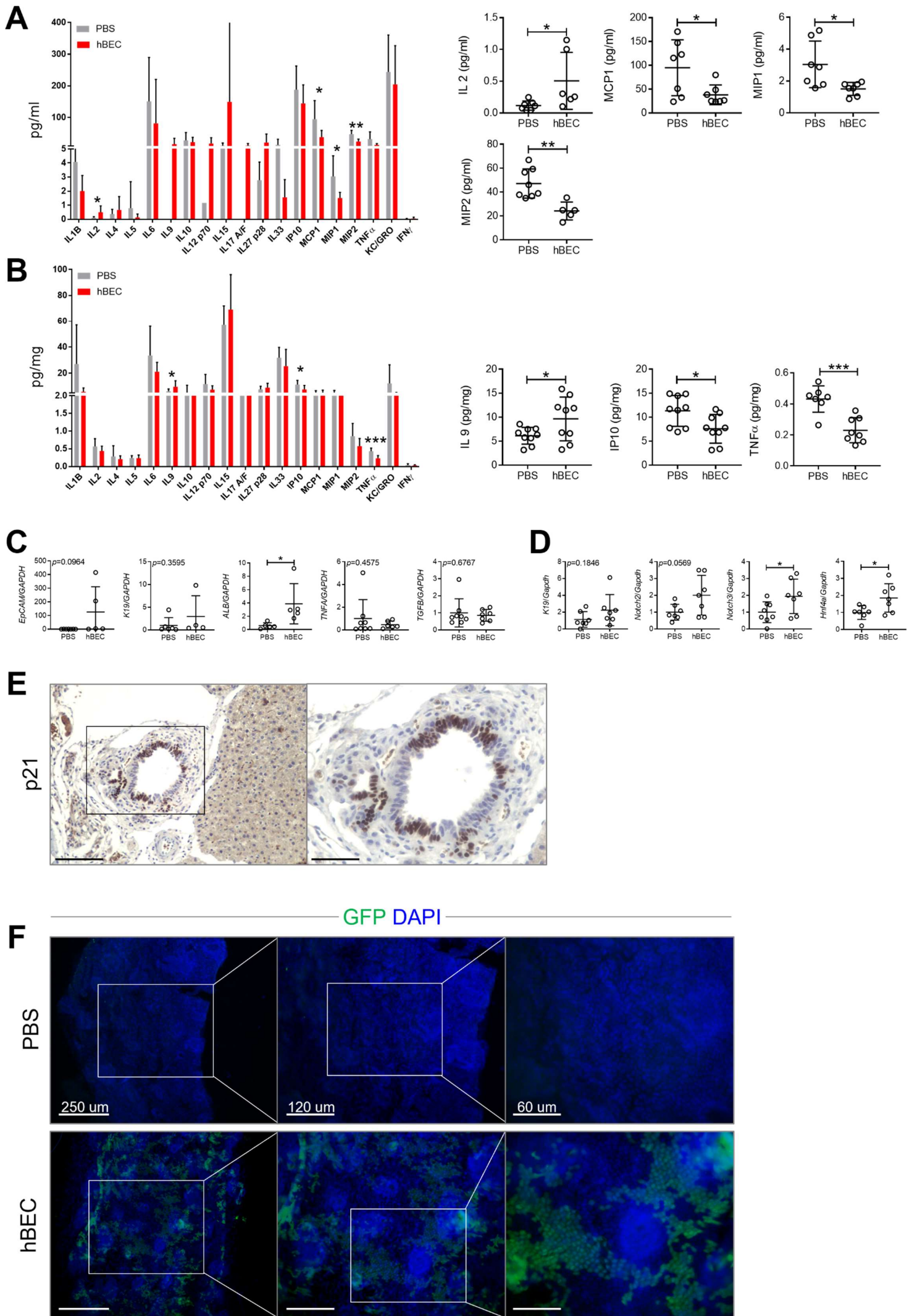
**(C)** Immunofluorescence for Keratin 19 (grey) and GFP-hBEC (green) showing homing of hBEC towards the native biliary tracts. White arrows indicate some sections of the host murine biliary tracts. Scale bars = 60  $\mu$ m.

**(D)** Immunofluorescence for marker of proliferation KI67 (red) and GFP-hBEC. White arrows indicate presence of proliferating hBEC. Scale bars = 60  $\mu$ m.

**(E)** Immunofluorescence for marker of proliferation PCNA (red) and GFP-hBEC. White arrows indicate presence of proliferating hBEC. Scale bars = 60  $\mu$ m.

**(F)** hBEC present markers of cholangiocytes/hepatocytes depending on the engraftment area in the IRI model. Immunofluorescence for GFP-positive hBEC and CYP2D6 in distal parenchymal areas in the IRI model. Scale bars = 60  $\mu$ m.

**(G)** Immunofluorescence for GFP-positive hBEC and HNF4 $\alpha$  near the host biliary tract in the IRI model. Scale bars = 60  $\mu$ m.



**Supplemental Figure S6. Cytokine analysis in blood and liver, mRNA expression and extrahepatic findings upon hBEC transplantation** (related to Figure 5).

**(A)** Cytokine analysis in blood in PBS control (grey) and hBEC transplanted mice (red). Results expressed as pg per ml of blood. Right, differentially expressed statistically significant cytokines.

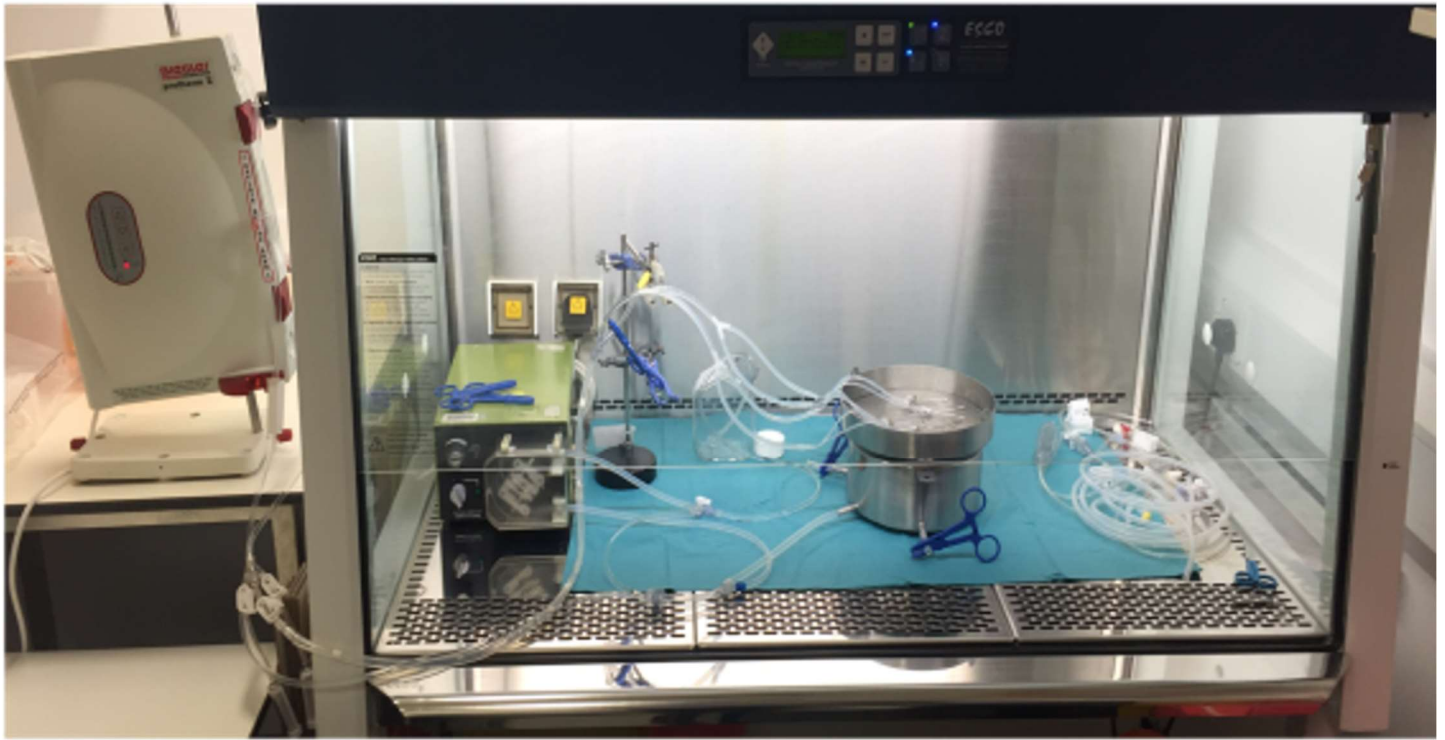
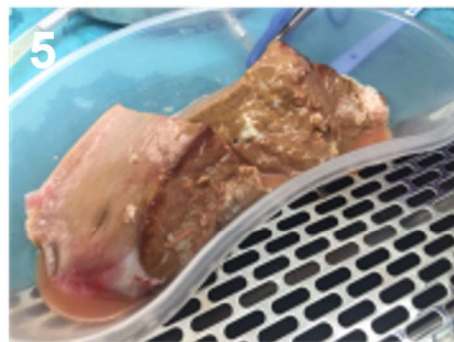
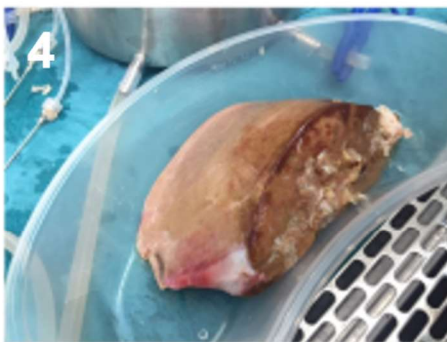
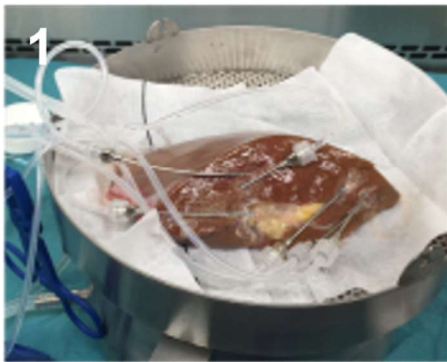
**(B)** Cytokine analysis in liver in PBS control (grey) and hBEC transplanted mice (red). Results expressed as pg per mg of tissue.

**(C)** mRNA expression of human genes of interest in whole liver of PBS control and hBEC-transplanted mice. \* denotes  $p < 0.05$ , (Mean $\pm$ SEM), Student t-test ( $N \geq 6$ ).

**(D)** mRNA expression of murine genes of interest in whole liver of PBS control and hBEC-transplanted mice. \* denotes  $p < 0.05$ , \*\* denotes  $p < 0.005$ , \*\*\* denotes  $p < 0.001$  (Mean $\pm$ SEM), Student t-test, ( $N \geq 6$ ).

**(E)** p21 immunohistochemistry in the *Krt19Cre<sup>ER</sup>Mdm2<sup>fl/fl</sup>Rag2<sup>-/-</sup>Il2rg<sup>-/-</sup>* (plus DDC diet) murine model of biliary disease indicate presence of senescence markers in the extrahepatic areas. Scale bars, left, 120  $\mu\text{m}$ ; right 60  $\mu\text{m}$ .

**(F)** GFP-positive hBEC engraft in the common bile duct upon transplantation in the model. Scale bars are indicated in the figure.

**A****B**

**Supplemental Figure S7. Isolation procedure for large sections of liver** (related to Figure 6).

**(A)** Equipment setup in a class 2 biosafety hood for hBEC isolation from fresh liver in sterile conditions, from left to right: fluid warmer, peristaltic pump, cannulas and sieve.

**(B)** Perfusion and processing of the liver:

1 represents early stages of perfusion while 2 is an advance stage after 2 hours of perfusion.

3, 4 cannulas detached and liver transferred to a disposable kidney dish.

5-8 disaggregation of the tissue.

9, transference to gentleMACS C-tubes (Miltenyi Biotec).