**Supplementary Files - "Combination of cord blood-derived human hepatic progenitors and hepatogenic factors strongly improves recovery after acute liver injury in mice through modulation of the Wnt/β-catenin signaling"**

## Supplementary Methods

### Purification of CB-derived CD133+/OV6+ cells

According to the guidelines of the Ethical Committee of the FBF S. Peter Hospital, full-term delivery umbilical cord blood (UCB) specimens obtained with informed consent (committee's reference number n. 64/2012/C.B) were diluted 1:4 with Phosphate Buffered Saline (PBS) supplemented with 2mM EDTA and 1% BSA (PEB). MNC cells were isolated from diluted UCB by Ficoll-Paque separation: briefly, diluted UCB was layered in 1:2 ratio on top of Ficoll-Paque solution, centrifuged at 800g 10 min RT. Interface MNC layer was collected, washed twice with PEB and pelleted at 800g 10 min RT. Cells were suspended in PEB (108cells/300µl); aliquots were taken for antigen immunophenotyping and viability/counting using trypan blue assay (Crema A, Lisi A, Sanchez M, & et al, 2011). Cells were isolated by immunomagnetic CD133+ positive selection from MNC using mini-MACS selection kit (Miltenyi Biotec, Germany) following manufacturer's instructions.

### *In vitro* expansion

CD133+/OV6+ cells were cultured in expansion medium, consisting of ISCOVE medium supplemented with 25% fetal bovine serum (FBS) and 20 ng/ml stem cell factor (SCF) and grown at 37°C in an atmosphere of 5% CO2.On day 14 of culture, proliferating non-adherent cells were induced to hepatic commitment.

### Antigen immunophenotyping

Stem cell surface marker analysis was performed before and after CD133+ purification and expansion, during hepatocyte differentiation. Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against CD2, CD7, CD10, CD33, CD38, CD43, CD44, CD45, CD90 and CD117 (Immunotools) , as well as Allophycocyanine (APC)-conjugated CD34 and phycohoerythrin (PE)-conjugated CD133, (Miltenyi Biotec), were tested. Cells were incubated 30 min 4°C in the dark with selected mAbs, in presence of FcR blocking reagent (Miltenyi Biotec); then washed twice with PBS. Cells suspended in 1 x PBS were analyzed by flow cytometry using the FACSAria (Becton Dickinson, San Jose, CA) equipped with three air-cooled and solid state lasers (488-nm, 633-nm and 407-nm). Cells labelled with appropriated fluorochrome-conjugated isotype antibodies (BD) were used to gate

non-specific fluorescence signals, while dead cells were excluded on the basis of propidium iodide (5 μg/mL, Sigma) fluorescence intensity. Antibodies used are listed in Table S1.

**Flow cytometry of NP-enriched cells from explanted livers**

Briefly**,** $1x10^6$ RBC depleted liver NP cells were suspended in PBS. Following Fc blocking, combinations of the following human-specific antibodies were added: CD45 APC, CD133 PE, OV6 FITC, and c-Kit FITC incubated at 4°C for 30 min. Cells were washed twice with PBS prior to analysis using a FACSCalibur (BD). Analysis was done using the Flo-Jo program (Tree Star, Ashland, www.flojo.com). Positive and negative gates were determined using IgG stained and unstained controls. Antibodies used are listed in Table S1.

**Immunofluorescence**

Cells were washed twice with PBS and fixed 20 min RT with 4% paraformaldehyde (SIGMA-Aldrich). Cells were permeabilized with 0.2% Triton X-100 (SIGMA-Aldrich), 10 min RT and incubated with mAbs in wet chamber, 30 min in the dark at +4°C. When necessary a secondary antibody was used. Each step was followed by 3 fold washing with PBS + 1% BSA. Antibodies used are listed in Table S1.

**Analysis of the molecular pathways controlling proliferation and differentiation during *in vitro* hepatic commitment**

***Cellular fractionation protocols***

Cellular fractionation was performed as described by Li et al. (Li et al., 2011). Before induction (Day 0) and at day 2 and 7 of *in vitro* hepatic differentiation, the cells were harvested, washed twice with phosphate-buffered saline (137 mM NaC1, 2.7 mM KC1, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.4) and incubated on ice for 20 min with hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.2 mM PMSF, and 0.5 mM DTT). Cells were then homogenized with 20 strokes on Dounce homogenizer, to ensure that >95% of the cells were lysed. The cells were centrifuged at 3300×g, 4°C for 15 min, and the supernatant saved as the cytosolic fraction, instead the nuclear pellet was treated with nuclei lysis buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 0.2 mM EDTA, 0.2% NP-40, 10% Glycerin, and protease cocktail) and then centrifuged at 13,200×g for 20 min. Finally, the supernatant obtained was saved as nuclear fraction. Equal amount of proteins from these two fractions, cytosolic and nuclear, were used for western blotting analysis.

***Western Blotting analysis***

Equal amounts of proteins from the different samples were loaded for each lane. Electrophoresis was carried out on 8 % SDS polyacrylamide gel at 60V for 2 hours. Transfer on nitrocellulose membranes (Biorad) was subsequently performed at 300 mA for 2 hours. After a 5% fat-free-milk block for 1 hour RT, membranes were incubated with the following monoclonal antibodies: anti- β-Catenin (1:1000), anti- Wnt5a (1:100), anti β-Actin (1:10000) and revealed by chemiluminescence (ECL) system (Amersham). Protein expression levels were determined semiquantitatively by a densitometry analysis with Quantity One software- 4.4.0 (BioRad) and the control β-Actin protein was used to calculate the relative density.

**Tissue Immunohistochemistry and Immunofluorescence**

***Immunohistochemistry (IHC)***

Formalin fixed paraffin embedded (FFPE) tissues were cut into 4-µm sections, deparaffinized with xylene and rehydrated with decreasing alcohol series. For antigen retrieval, the sections were microwave treated in 1 mM EDTA at pH 8 for 10 min and allowed to cool for 20 min. The sections were then added with PBS containing 0.5 g/L Triton x100 and incubated at RT for 5 min. Endogenous peroxidase activity was blocked with 3% H2O2 for 3 minutes at RT. Endogenous biotin was saturated using a biotin blocking kit (Vector Laboratories; Burlingame, CA, USA). For detection of human cells in mouse parenchyma different human-specific antibodies were used: mouse mono-clonal anti-human hepatocyte (HepPar1), mouse monoclonal anti-human oval cell marker OV6 (OV6), goat polyclonal anti-human albumin (ALB). Antibodies used are listed in Table S1.

Unstained slides were heated in a 60°C oven for 30', cooled, deparaffinized, and rehydrated through xylenes and graded ethanol solutions to water. For heat-induced antigen retrieval, slides were taken 20 min in high pH Target Retrieval Solution (Dako). Endogenous peroxidases were quenched by incubation for 5 minutes in 0.3% hydrogen peroxide and immunohistochemistry was performed using LSAB/HRP kit (cod. K 0679, Dako). For antigen detection with mouse primary antibody, blocking of endogenous Mouse IgG was performed by pre-incubation with mouse IgG blocking reagent (Vector Labs). Horseradish peroxidase-coupled (HRP) goat anti-mouse IgG (Amersham, Les Ulis, France) was incubated for 30 min and visualized with 3,3'-diaminobenzidine (DAB). Finally, slides were counterstained in Mayer hematoxylin, dehydrated through graded ethanol solutions, cleared with xylene and cover-slipped. The results were evaluated by light microscopy (Microscope Labophot-2, Nikon).

***Immunofluorescence (IF)***

Fresh frozen liver tissues were subjected to 4-8 µm frozen sectioning. The sections were placed at RT for 30 min, fixed at 4°C in cold acetone for 10 min and stored in liquid nitrogen. For IF staining, fresh frozen slides were thawed at RT for 5 min, washed 3 fold with PBS and incubated for 30 min in 10% horse serum in PBS to suppress non-specific binding of IgG. The sections were then incubated with the antibodies listed in Table S1and kept at 4°C overnight. After washing, a second blocking was performed, then the labelled secondary antibodies were added: FITC-conjugated or Texas Red (TR)-conjugated goat anti-mouse IgG and incubated at RT in dark for 45 min. The sections were mounted with buffering glycerol 8:1 in PBS and observed under fluorescence microscopy (Olympus IX51, RT Slider SPOT - Diagnostic instruments) equipped with 20x, 40x and 60x objectives and with a cooled CCD camera (Spot RT Slider; Diagnostic Instruments). For negative control, PBS replaced the primary antibody.

**Liver gene expression analysis**

***Quantitative RT-PCR (qRT-PCR) on liver tissues***

One microgram of total RNA was used to synthesize first-strand cDNA with random primers using 100 U of ImProm-II TM RT-PCR kit (Promega) according to the manufacturer. The quantification of all gene transcripts was carried out by qRT-PCR. Experiments were conducted to contrast relative levels of each transcript and endogenous control GAPDH in every sample. The data were analyzed using the equation described by (Livak & Schmittgen, 2001) as follows:

Amount of target- 2-ΔCt.

ΔCt = (average target Ct - average GAPDH Ct)

Before using ΔCt method for quantification, we performed a validation experiment to demonstrate that efficiency of target genes and reference GAPDH were equal. qRT-PCR was conducted using Sybr Green I Mastermix (Applied Biosystems) using an ABI PRISMTM 7000 Sequence Detection System. Each reaction was run in triplicate and contained 0,5 µl of cDNA template along with 250 nM primers in a final reaction volume of 25 µl. Cycling parameters were 50°C for 2 min, then 95°C for 10 min to activate DNA polymerase, then 40 cycles of 95°C for 15 second and finally 60°C for 1 min. Melting curves were performed using Dissociation Curves software (Applied Biosystems) to ensure only a single product was amplified. As negative controls, we used tubes where RNA or reverse transcriptase was omitted during the RT reaction. Primers used are listed in Table S2

## References

Crema A, Lisi A, Sanchez M, & et al. (2011). Umbilical Cord Blood derived CD133+ cells can be differentiated in vitro into hepatocytes. *J Hepatol, 48*, S177.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods, 25*, 402-408.

## Supplementary Tables

**Table S1 -** List of antibodies

| Antibody | Manufacturer | Application | Dilution |
|---|---|---|---|
| Mouse monoclonal anti-human CD45 APC, | Miltenyi Biotec | FCM | 1:100 |
| Monoclonal anti-human CD133/2 (293C3)-PE | Miltenyi Biotec | FCM | 1:100 |
| Mouse monoclonal anti-oval cell marker OV6 | Santa Cruz Biotechnology | FCM/IHC/IF | 1:100 |
| Mouse monoclonal anti-human αFP clone C3 | Santa Cruz Biotechnology | IF | 1:100 |
| Goat Polyclonal anti-Human Albumin Gene ID 213 | BETHYL Laboratories Inc. | IHC/IF | 1:100 |
| Rabbit Monoclonal anti-human Thy1 | ORIGENE | IF | 1:100 |
| Mouse monoclonal anti-mouse Thy1-1.2 | NOVUS Biologicals | IF | 1:100 |
| Mouse monoclonal anti-human c-Kit | Immunotools | IF | 1:100 |
| Mouse monoclonal anti-human CD29 | Immunotools | IF | 1:100 |
| Rabbit anti-human CK18 | Santa Cruz Biotechnology | IF | 1:200 |
| Goat anti-human CK19 | Santa Cruz Biotechnology | IF | 1:100 |
| Monoclonal mouse anti-human HepPar1(OCH1E5) | Dako | IHC/IF | 1:100 |
| Goat polyclonal anti- Wnt-1 (A-20) | Santa Cruz Biotechnology | IF | 1:100 |
| Mouse monoclonal anti-Wnt-5a (A-5) | Santa Cruz Biotechnology | IF | 1:100 |
| | | WB | 1:100 |
| Mouse monoclonal anti-human β-Catenin | Santa Cruz Biotechnology | IF | 1:100 |
| | | WB | 1:1000 |
| Mouse monoclonal anti- β Actin | Sigma | WB | 1:10000 |
| Mouse monoclonal anti-Ki67 | Dako | IHC | 1:150 |
| Mouse monoclonal anti-EpCAM | antibodies-online | IF | 1:50 |
| Mouse monoclonal anti-CD56 (NCAM) | ImmunoTools | IF | 1:100 |

**Table S2-** List of primers

| Target | Sequence | Annealing T |
|---|---|---|
| **THY1** | 5'-GCTCTCAGTCTTGCAGGTG-3'<br>5'-GATGGAGTTATCCTTGGTGTT-3' | 60°C |
| **CK19** | 5'-TCGGATTGAGGAGCTGAAC-3'<br>5'-CACGCTCTGGATCTGTGAC-3' | 60°C |
| **C-KIT** | 5'-CGTCTCCTGGCGTTCATAAT-3'<br>5'-AAGATGAACCCTCAGCCTCA-3' | 60°C |
| **GAPDH** | 5'-CACCACCAACTGCTTAGCC-3'<br>5'-GGATGCAGGGATGATGTTCT-3' | 60°C |
| **EpCAM** | 5'-GACCGGAAGTGGCAGAAGAG-3'<br>5'-GTCTTCATCTTCCCCAGGTT-3' | 60°C |
| **Sox9** | 5'- GAAGCTGGCAGACCAGTACC-3'<br>5'- GGTCTCTTCTCGCTCTCGTT -3' | 60°C |

**Table S3 -** AST† and ALT‡ serum levels of transplanted animals and Sham group

| Group | AST§ | St. Dev | *p* value | | ALT§ | St. Dev | *p* value | |
|---|---|---|---|---|---|---|---|---|
| Sham | 233.6 | 126.0 | | | 62.6 | 61.7 | | |
| iBHPs-Saline | 129.6 | 103.2 | 0.078 | | 42.9 | 28.8 | 0.418 | |
| iBHPs-HM | 143.2 | 81.9 | 0.073 | 0.759 | 21.1 | 6.9 | 0.062 | 0.043 |

† alanine aminotransferase ;‡ aspartate aminotransferase ;§ units/liter

Data from AST and ALT serum levels were analyzed by unpaired, two-tailed t-test. *p* value under 0.05 were considered statistically significant.

## Supplementary Figures

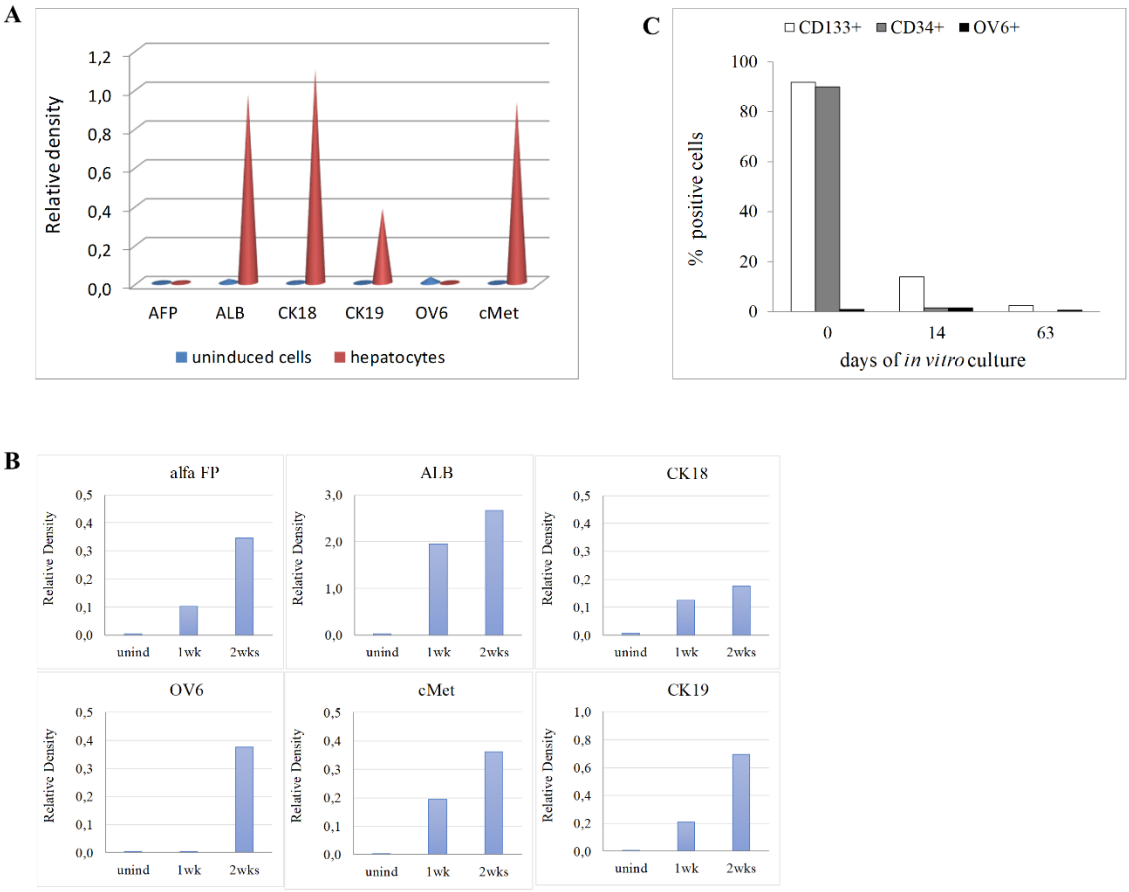


**Figure S1.** Expression of stemness and hepatic markers in cultured cord blood-derived $CD133^+CD34^+OV6^+$ precursor cells. **A**. Uninduced cells (before *in vitro* hepatic induction) were analyzed for the expression of hepatic markers and compared to human hepatocytes. **B**. Cells were analyzed for the expression of hepatic markers before (unind) and at the onset of *in vitro* hepatic induction (1-2 weeks culture with hepatogenic factors) by western blot. **C**. $CD133^+CD34^+OV6^+$ precursor cells were checked for the maintenance of stemness markers in long term culture with basal medium. Data in panels A and B were normalized *vs.* beta-actin. Unind: uninduced cells cultured without hepatogenic factors; AFP: alphafetoprotein; ALB albumin; CK18: cytokeratin 18; CK19: cytokeratin 19; OV6: oval cell antigen 6; cMet: HGFreceptor

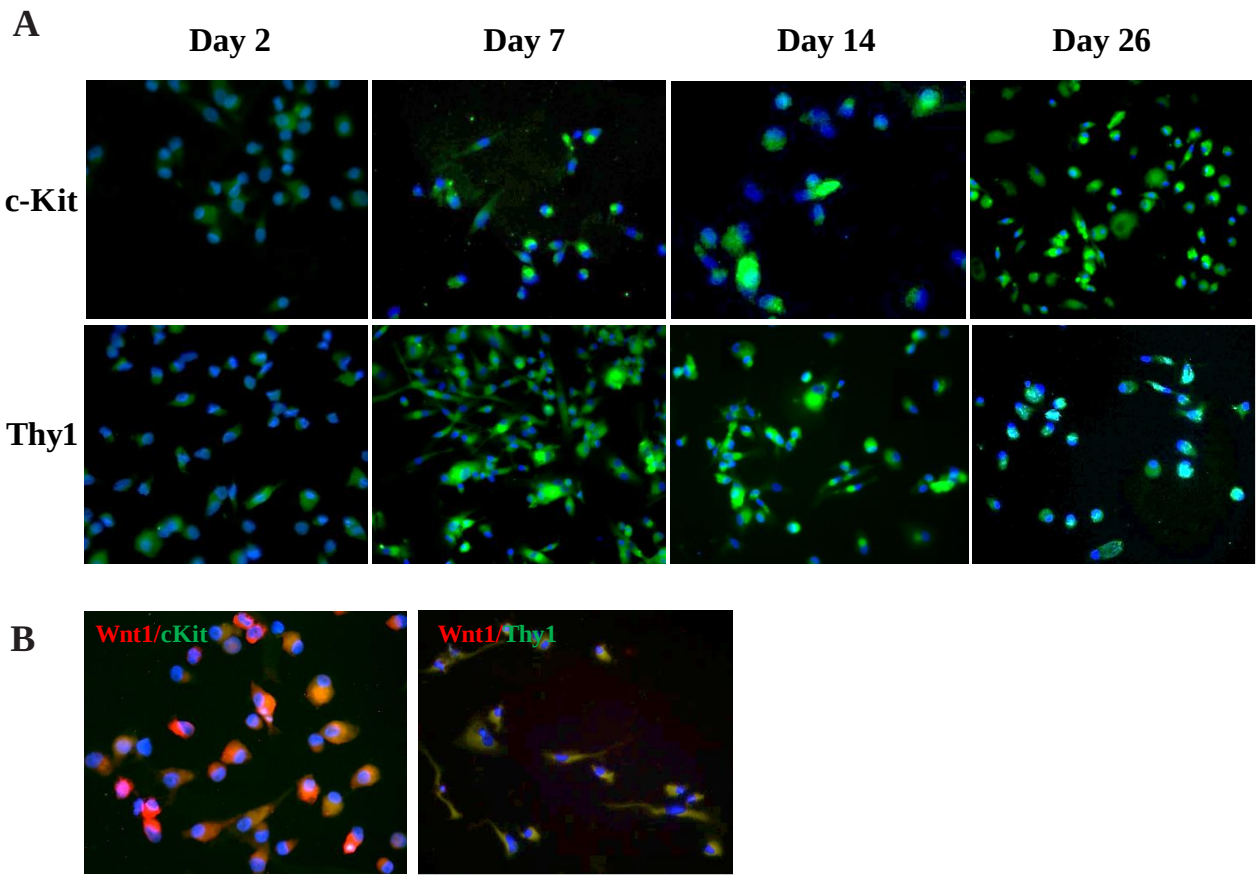


**Figure S2**. Immunofluorescence analyses. **A** Expression of the hepato-mesenchymal markers SCF receptor cKit (upper panels) and Thy1 (lower panels) throughout *in vitro* differentiation. **B** colocalization of Wnt1 (red)/cKit (green, left panel) and Wnt1 (red)/Thy1 (green, right panel) at early times post induction. Nuclei were counterstained with Hoechst (*blue*). Original magnification: 10 X.

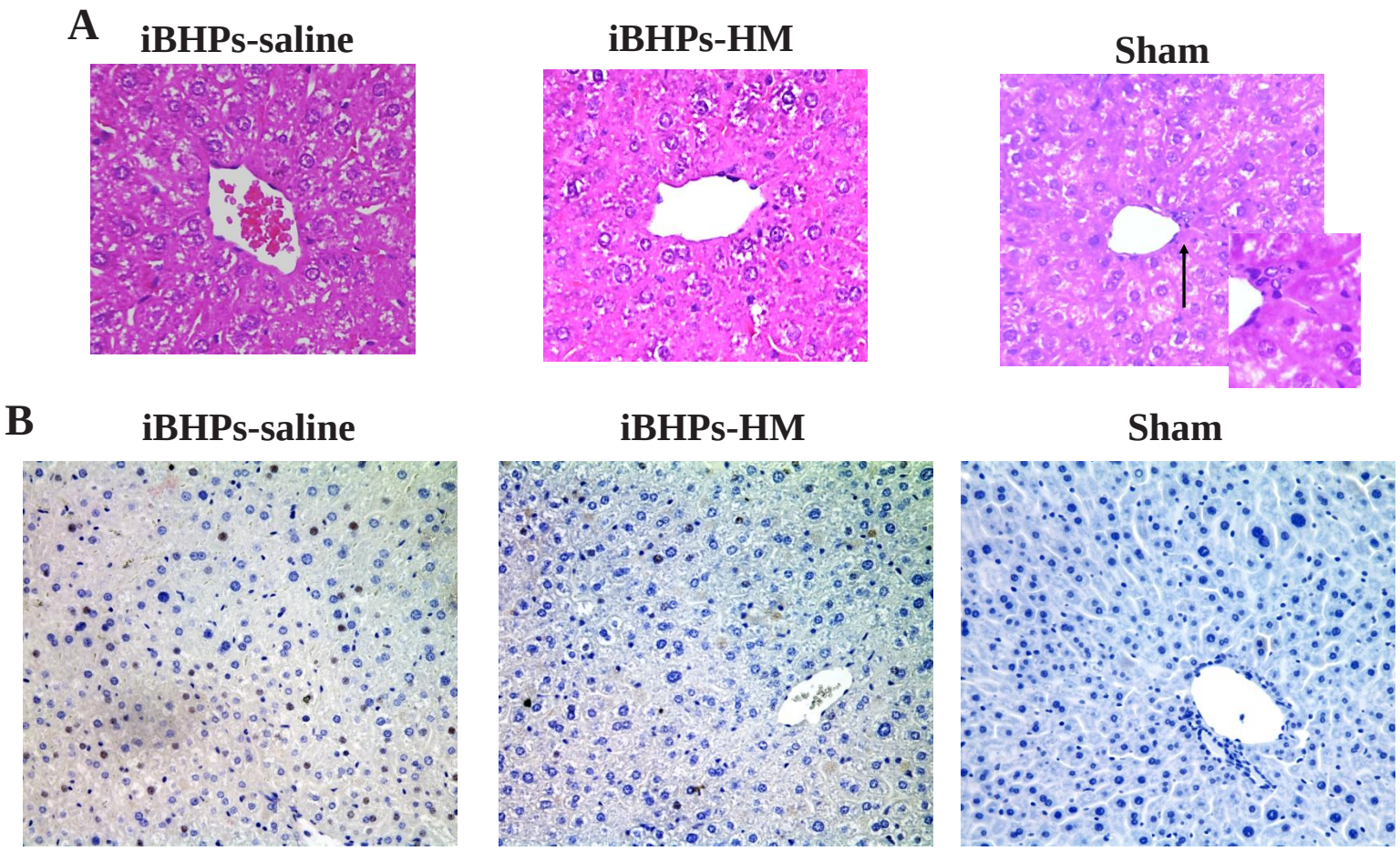


**Figure S3**.

**A**. Hematoxylin-Eosin centrilobular staining of liver sections of transplanted iBHPs-Saline (left), iBHPs-HM (centre) and sham (right) animals. A small group of necrotic cells was detectable only in sham control (arrow). **B** Immunohistochemical detection of proliferation markers Ki67. Widespread Ki67 positivity was present in both groups of transplanted animals. Any Ki67 positivity was detectable in sham group.

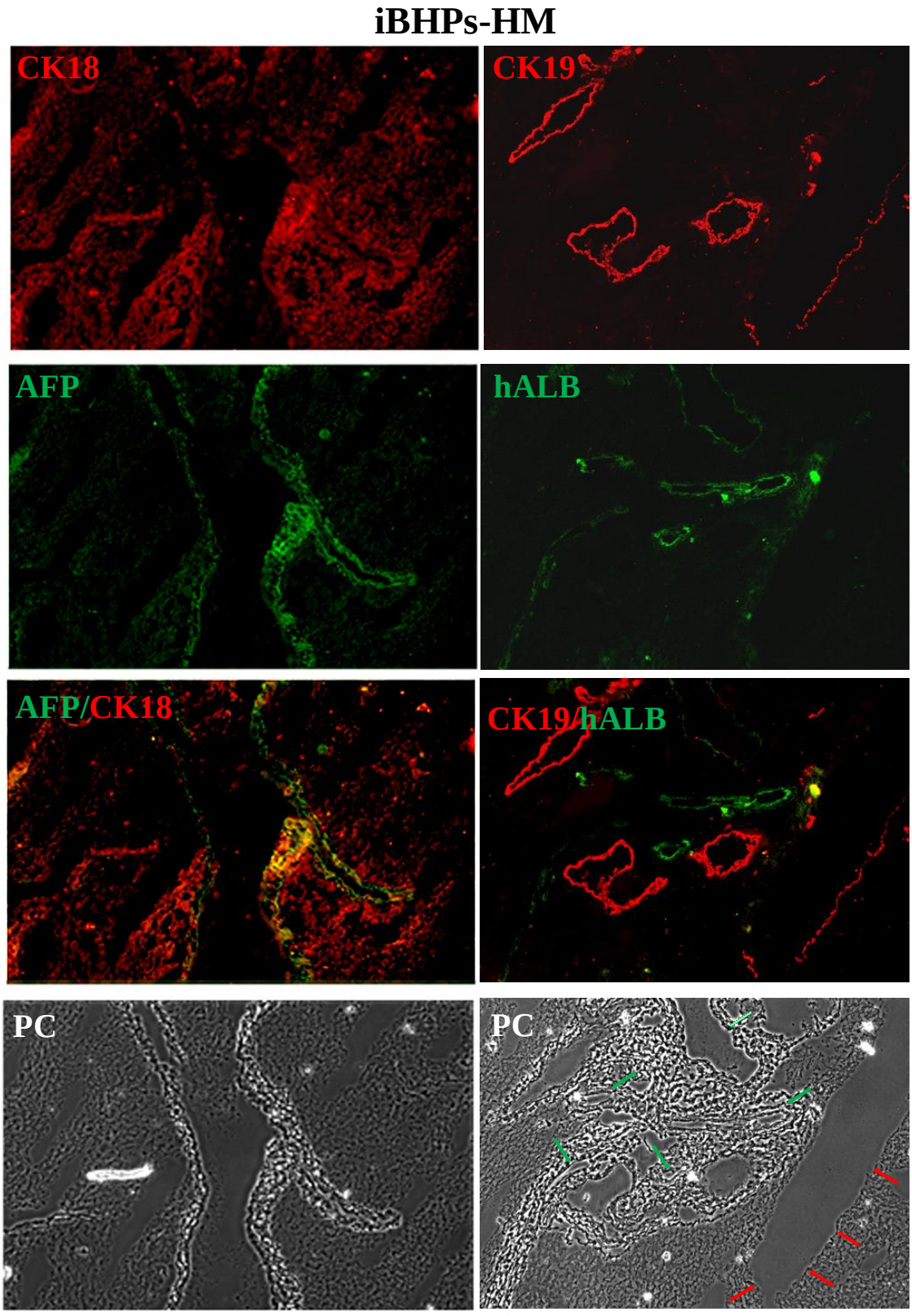


**Figure S4.** Expression and colocalization of early alpha-fetoprotein (AFP) and late ALB hepatic functional markers. Double immunofluorescence CK18 (red)/AFP (green) and CK19 (red)/ALB (green) Original magnification: 10 X.

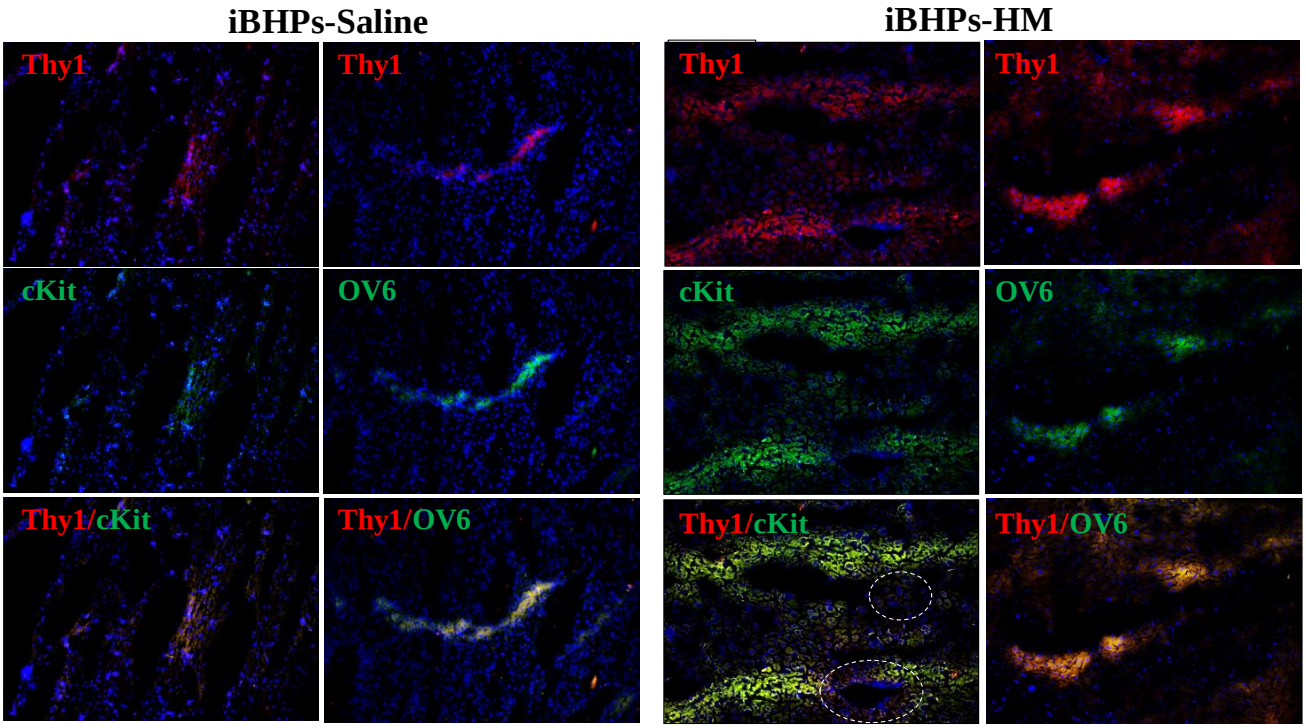


**Figure S5.** Colocalization of Thy1(red) with cKit (green) and with OV6 (green) in livers of recipient animals**.** Dotted lines: cell populations Thy1+cKit-. Original magnification: 10 X.

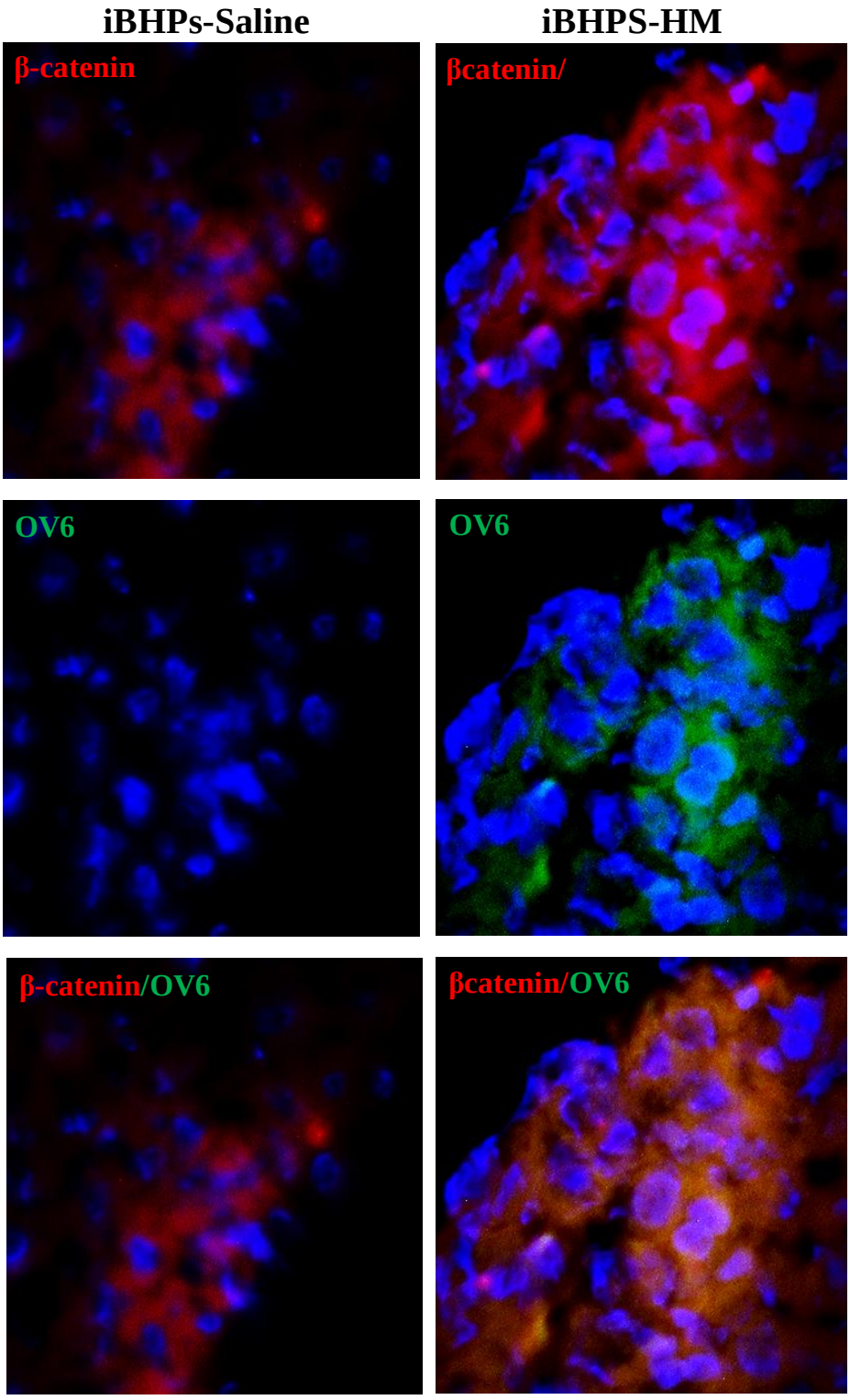


**Figure S6.** Effects of combination of human cells and hepatogenic factors on oval cell activation. Colocalization of OV6 (green) and β-catenin (red) in livers of recipient animals. Original magnification: 40 X.

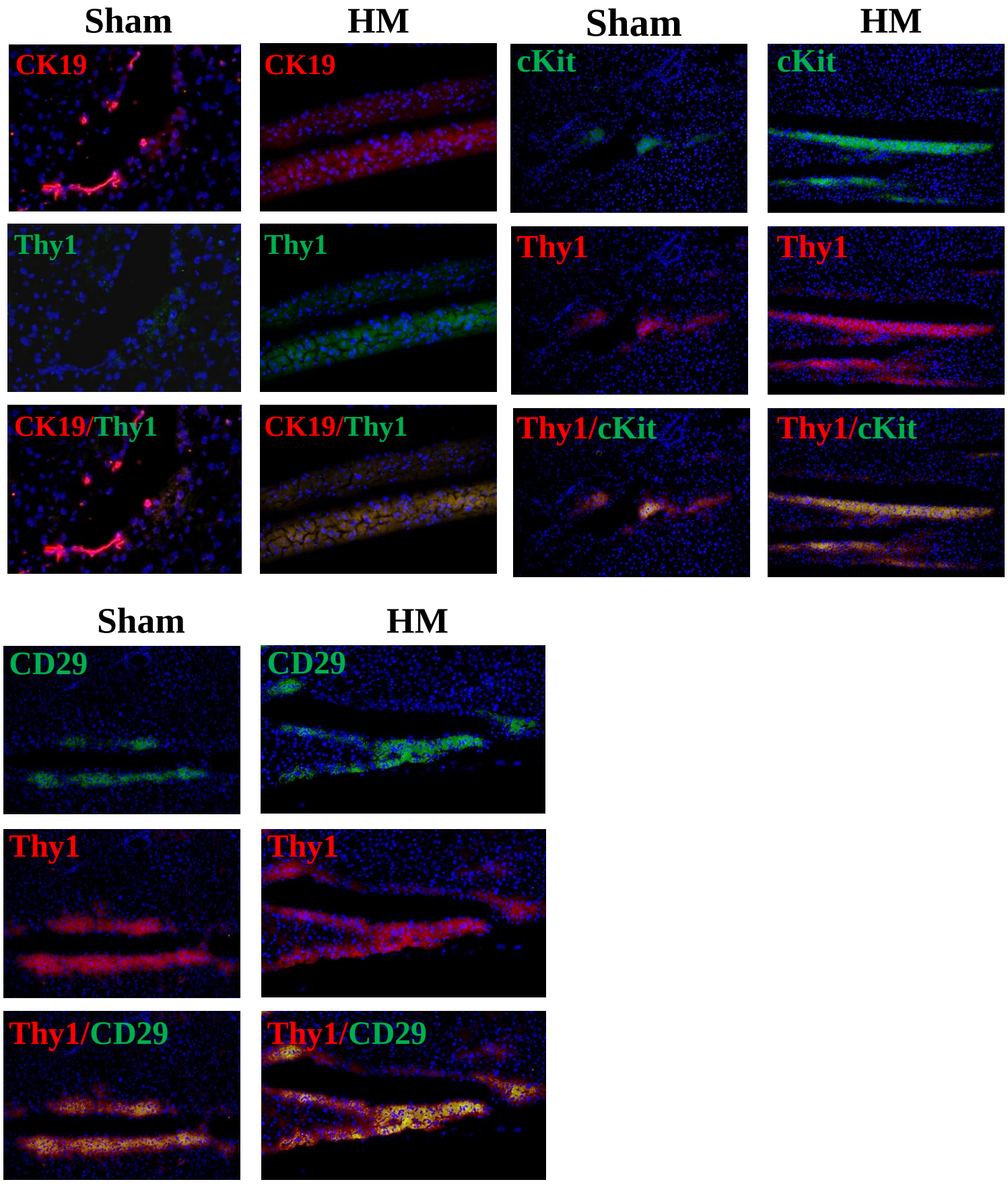


**Figure S7. Treatment with hepatogenic factors triggers EMT program in injured livers**. Co-expression of Thy1 with CK19, CD29 and cKit in HM injected animals and in sham group. Original magnification: 20 X.

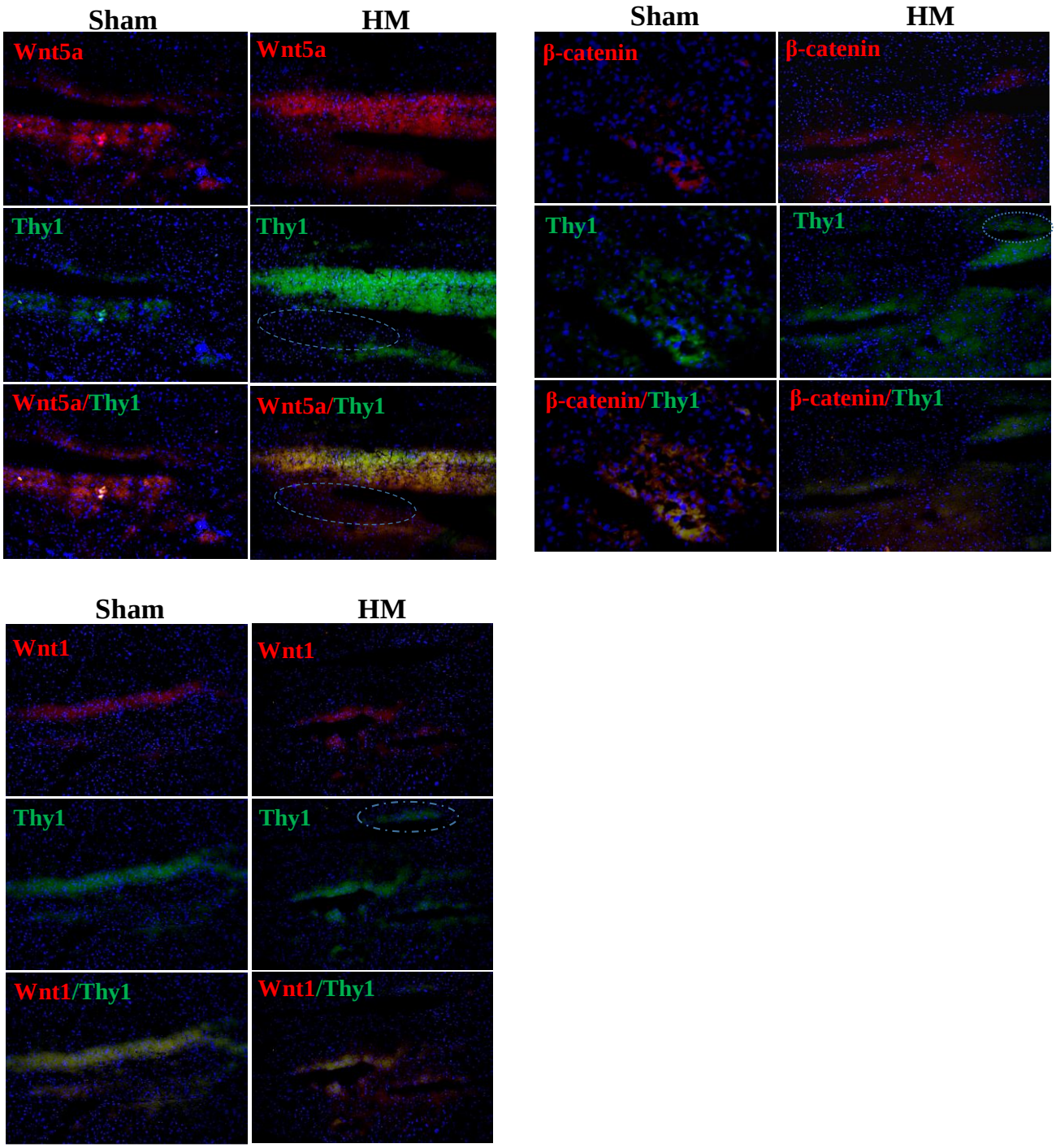


**Figure S8**. Relative expression of Wnt1, Wnt5a and Thy1 and βcatenin in HM injected animals. In HM group Thy1 mostly co-localized with Wnt1, Wnt5a and with β-catenin, but small populations Thy1-Wnt5a+, Thy1+Wnt1- and Thy1+ βcatenin- (dotted circles) were also present. In controls, Thy1 100% co-localized with all tested markers.

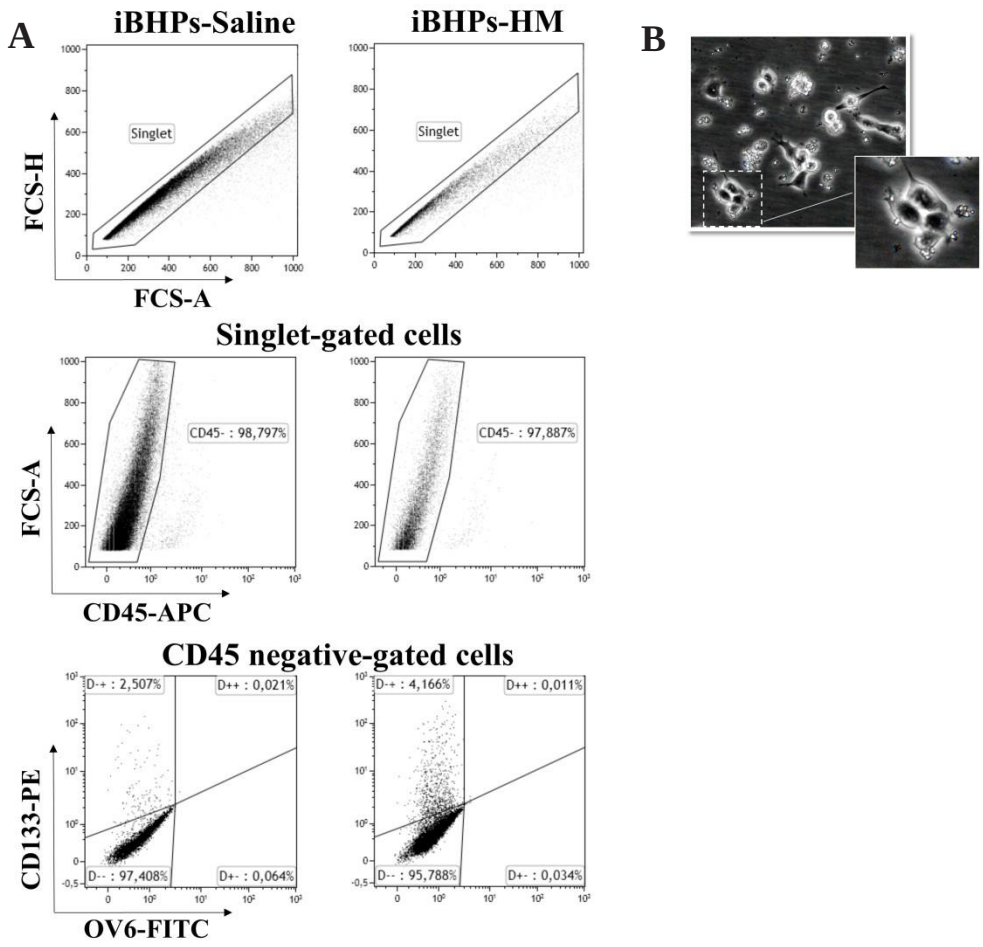


**Figure S9. Human-derived cells proliferate and differentiate within mouse NP-enriched cell culture. A** Cytofluorimetric analysis for detection of human cells within NP-enriched mouse liver cells after 3 weeks of in vitro culture. Left panels: iBHPs-Saline group (n = 3): Right panels: iBHPs-HM group (n = 3). In the dot plots on the top single cells were distinguished from aggregates on the basis of FSC height vs FCS area pulse. In the central dot plots are identified CD45 negative cells present in singlet-gated cells and in the dot plots on the bottom the CD45 negative-gated cells were analysed for CD133 and OVO6 phenotypic expression. **B** Cultured cells from iBHPs-HM group with mesenchymal-like structures. Original magnification: 20 X.