Stem Cell Reports, Volume *13*

Supplemental Information

Robust, Long-Term Culture of Endoderm-Derived Hepatic Organoids for Disease Modeling

Soheil Akbari, Gülben Gürhan Sevinç, Nevin Ersoy, Onur Basak, Kubra Kaplan, Kenan Sevinç, Erkin Ozel, Berke Sengun, Eray Enustun, Burcu Ozcimen, Alper Bagriyanik, Nur Arslan, Tamer Tevfik Önder, and Esra Erdal

Supplemental Information

Robust, long-term culture of endoderm derived hepatic organoids for disease modeling

Soheil Akbari, Gulben Gurhan Sevinc, Nevin Ersoy, Onur Basak, Kubra Kaplan, Kenan Sevinc, Erkin Ozel, Berke Sengun, Eray Enustun, Burcu Ozcimen, Alper Bagriyanik, Nur Arslan, Tamer Onder, Esra Erdal

Supplemental Figure S1. Akbari et al.

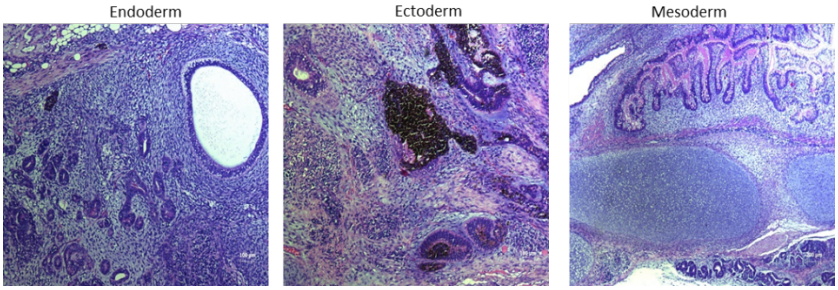

Figure S1. Teratoma formation of iPSC from a healthy donor in SCID mice, Related to Fig 1. Hematoxylin and eosin stained sections of teratomas derived from a healthy donor IPSC line. Panels show glandular epithelium (endoderm), pigmented neural tissue (ectoderm) and cartilage tissue (mesoderm).

Supplemental Figure S2. Akbari et al.

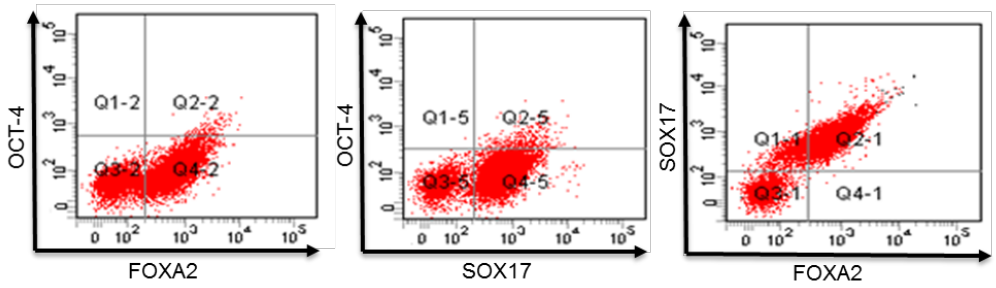

Figure S2. Flow cytometric analysis to assess the efficiency of endoderm generation at day 5, Related to Fig 1B. (OCT3/4 as pluripotency markers; FOXA2 and SOX17, as endoderm markers).

Supplemental Figure S3. Akbari et al.

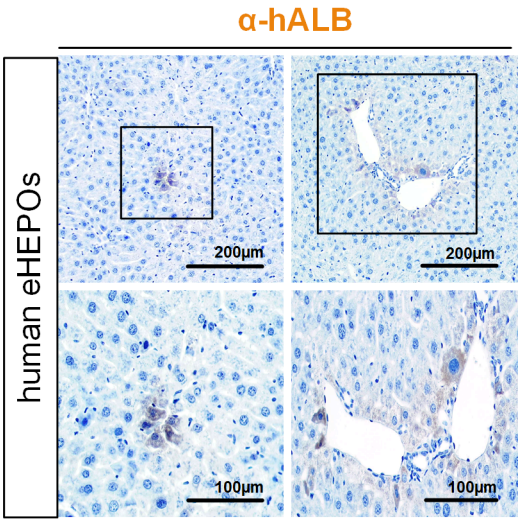

Figure S3. Engraftment of eHEPOs to mouse liver, Related to Fig 4E. Immunostaining of mouse liver after 32 days transplantation with anti-GFP and human specific anti-ALB antibodies

Supplemental Figure S4. Akbari et al.

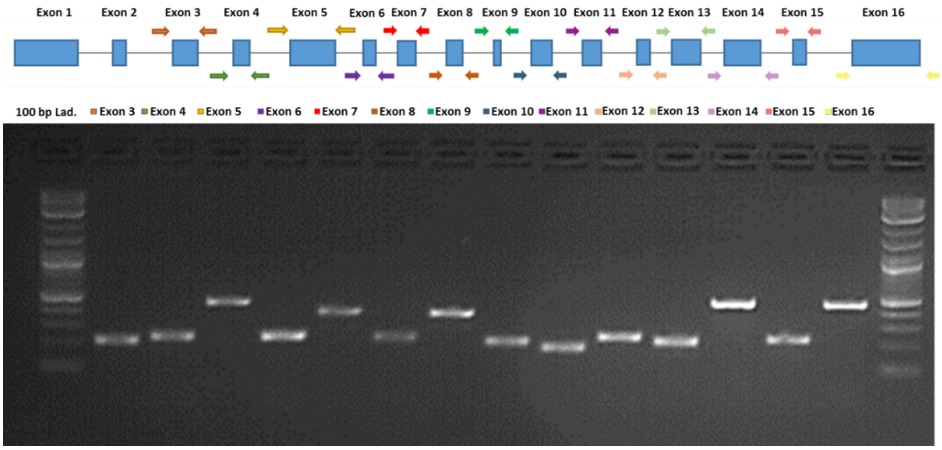

Figure S4. PCR amplification of *ASS1* exons for Sanger sequencing, Related to Fig 5.

Supplemental Figure S5. Akbari et al.

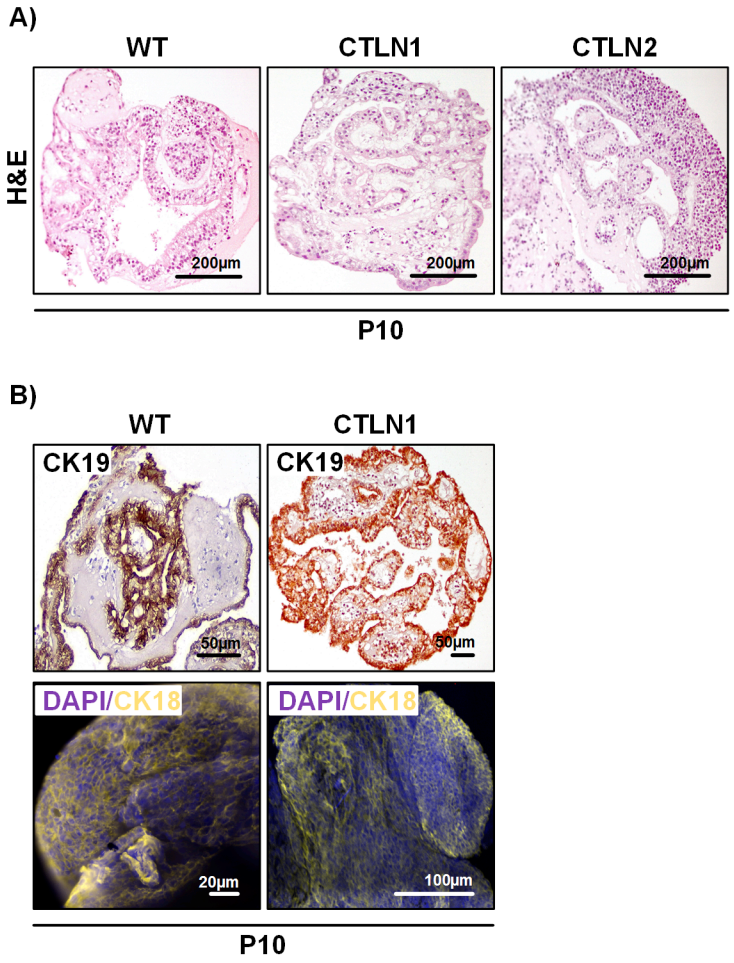

Figure S5. Comparison of structural organizations of organoids, Related to Fig 6. A) H&E stainings of WT and CTLN organoids. B) Immunostainings with CK18 and CK19 for WT and CTLN organoids.

Supplemental Figure S6. Akbari et al.

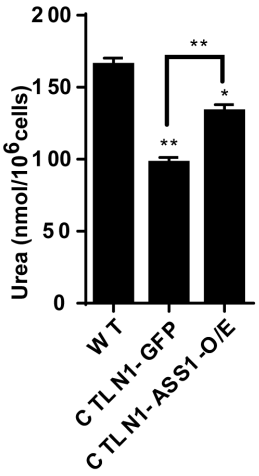

Figure S6. Ureagenesis of WT, CTLN1-GFP and CTLN1-ASS1-O/E organoids, Related to Fig 6. Error bars are ±SD of three independent experiments.

Supplemental Table S1. Akbari et al.

Table S1. Primers used in this study, Related to Fig 5B and Fig S4

ASS1-Ex-Primer3_forward	CAGCTTGCCCAGGAGACAA
ASS1-Ex-Primer3_reverse	CGAGAGAAGAGGCCAGGC
ASS1-Ex-Primer4_forward	CATGCGGATGGTGTGAACTC
ASS1-Ex-Primer4_reverse	CACACTGGAAGGGATGGGAT
ASS1-Ex-Primer5_forward	GAAGAAGCACCAGGTACAGC
ASS1-Ex-Primer5_reverse	TGGGGTCTAAACAAAGCCATG
ASS1-Ex-Primer6_forward	GTCCTTGTCCTCACGTCCTC
ASS1-Ex-Primer6_reverse	GATTCTGTGCCTGTCCTGTG
ASS1-Ex-Primer7_forward	TGTCCTTCCCCTCCTTCATG
ASS1-Ex-Primer7_reverse	GCTGGGGTGGAAGTTCAATC
ASS1-Ex-Primer8_forward	ACCCCTTGTCCTATGTCCAG
ASS1-Ex-Primer8_reverse	CAGCTGGGGTGGAAGTTCA
ASS1-Ex-Primer9_forward	AGAATGTTTCAGGCAGGTTGG
ASS1-Ex-Primer9_reverse	GGGAGGTCTTCAAATGCAGC
ASS1-Ex-Primer10_forward	GAGGTGGGCTGTAGGGTG
ASS1-Ex-Primer10_reverse	GAGGGGAAAGGAGCCAGG
ASS1-Ex-Primer11_forward	CTGCCCTCTCTTCCCACC
ASS1-Ex-Primer11_reverse	GCCACAACCATTAGCTGCAA
ASS1-Ex-Primer12_forward	AATGCTGCCTAATGTGTGGC
ASS1-Ex-Primer12_reverse	ACAGGGTCTCAGGGATCTCT
ASS1-Ex-Primer13_forward	TGCTGACAGTTTGGGTTTCA
ASS1-Ex-Primer13_reverse	GCAGCCATAGAGTCTTACGC
ASS1-Ex-Primer14_forward	ATGGTCCTCAACTCAGCCAC
ASS1-Ex-Primer14_reverse	CGGGAGCATAGTGGTGTCTA
ASS1-Ex-Primer15_forward	CAGTCCTCCCTTCAAGCAGA
ASS1-Ex-Primer15_reverse	GCAGTCAAGGTCGCATCAAA
ASS1-Ex-Primer16_forward	CCCCAGCTCTGCCTGAATTA
ASS1-Ex-Primer16_reverse	ACTTAGGTCCGAAAACACAAAGG

Supplemental Experimental Procedures

Derivation of Human Fibroblasts and iPSC Generation

Skin punch biopsies were obtained from Citrullinemia patients and healthy donors according to a protocol approved by the relevant Institutional Review Boards of Dokuz Eylul University and Koc University. Human fibroblasts were generated from the 3 mm forearm dermal biopsies and were transfected with 1 µg of the four plasmids encoding the reprogramming factors (pCXLE-hOCT3/4- shp53-F, Addgene plasmid 27077; pCXLE-hUL, Addgene plasmid 27080; pCXLE-hSK, Addgene plasmid 27078; pCXWB-EBNA, Addgene plasmid 37624) using the Lonza-Amaxa 4D-Nucleofector System as described (1). Transfected fibroblasts were cultured in Dulbecco's Modified Eagle Medium (Gibco Invitrogen) supplemented with 10% FBS (Sigma Aldrich) and Pen/Strep (Gibco Invitrogen) at 37°C and 5% CO_2 for seven days. On day 7, patient fibroblasts were passaged onto Vitronectin (VTN-N) coated plates (Lifetech) and cultured in serum free Essential 8 medium (Thermo Fisher). Emerging iPSC colonies were manually picked and expanded on hESC-qualified Matrigel matrix basement membrane (Corning) with mTeSR1 medium (Stem Cell Technologies). To check for genome integration of episomal vectors, genomic DNA was isolated from iPSCs using a commercial genomic DNA isolation kit (Macherey-Nagel NucleoSpin Tissue). PCR was carried with primers pairs designed to detect the EBNA sequences encoded by all four vectors used. The primer sequences for *EBNA* and *GAPDH* control can be found in Supplementary Table 1. Karyotyping was performed at the Clinical Genetics Laboratory of Koc University Hospital, Istanbul Turkey.

Periodic Acid-Schiff (PAS) Staining

Paraffin sections were prepared as described below. Slides were washed with 1x PBS and then either treated or not treated with diastase (Sigma-Aldrich) for 1 h at 37^0C. The organoids were oxidized in 1% periodic acid (Abcam) for 5 min, washed three times in de-ionized H2O, treated with Schiff's reagent (Abcam) for 20 min in dark, and further stained with hematoxylin for 1 min. Finally, they were mounted and visualized under light microscope (Olympus).

LDL Uptake

The organoids were incubated for 4 h with acetylated LDL conjugated with fluorescent dye Dil (Invitrogen) at 37^0C. The experiment was carried out according to the manufacturer's instructions and was analyzed with fluorescent microscope (Olympus).

Measurement of CYP3A4 Activity

Organoids were removed from Matrigel and seeded onto suspension plates. The organoids were cultured with Hepatozyme medium including %10 FBS (Gibco) and 50µM luciferin-PFBE substrate for 8 h incubation at 37°C. After incubation 50 µl of medium was added to 50 µL Luciferin Detection Reagent and Cyp3A4 activity was measured P450-Glo Assays kit (Promega) according the manufacturer's instructions.

RNA Isolation and Real-time PCR

Total RNA was extracted with RNeasy mini kit (Qiagen). RNA was converted to cDNA with Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to manufactuer's instructions. Quantitative real-time RT-PCR (q-PCR) was performed on a quantitative PCR system (Applied Biosystems) using Power SYBR Green Master mix (Applied Biosystems). Relative gene expression was normalized to *GAPDH, RPL41, RPL13* and calculated by using the $2^{-\Delta\Delta Ct}$ method.

Lentiviral Expression Vectors

To generate the pALB-GFP-PURO reporter, Albumin promoter sequences were amplified from pALB-GFP plasmid (Addgene, #55759) with primers: 5'-AGCAGGTACCCACCGCGGTGGCGGCCGCTC-3'(forward), 5'-AGCATTAATTAAGGGCCCCCCCTCGAGGTCGAC -3' (reverse) that would allow for KpnI and PacI cut sites and cloned into pENTR1A-UBE2C-eGFP plasmid backbone (Gift of Nathan Lack, Koc University). Gateway Cloning with Gateway LR Clonase II Enzyme mix (Thermo Fisher) was used to move the reporter casette into pLENTI X1 Puro DEST (694-6) (Addgene, #17297) backbone. To generate ASS1 lentiviral overexpression vector, ASS1 cDNA and an IRES-GFP cassette was amplified by PCR cloned into pENTR1A noccDB plasmid (Addgene plasmid #17389). Gateway Cloning was used to transfer this cassette to pLenti PGK PURO DEST (Addgene #19068). All vectors were confirmed by Sanger Sequencing.

Production of Viral Particles

2.5 x 10^6 of HEK293T cells were seeded to 10 cm tissue culture plates. Next day, cells were transfected with 250 ng envelope protein pCMV-VSV-G (Addgene, #8454), 2250 ng 8.2DeltaVPR (Addgene, #8455) or PSPAX2 (Addgene, #12260), and 2500 ng viral vector of interest using 20 µl FuGENE 6 Transfection Reagent (Promega) in a total volume of 400 µl DMEM. Viral supernatants were collected at 48 hours and 72 hours after the transfection. Supernatants were centrifuged at 1500 rpm for 5 minutes and filtered through a 45 µm low protein binding syringe filter. To concentrate the viral particles, 50% PEG-8000 (Sigma) solution was prepared in PBS as 5X and added to collected viral supernatants. The mixture was incubated for two days at 4°C and then centrifuged at 2500 rpm for 20 minutes at 4°C. The supernatant was discarded, and pellet was resuspended in cold PBS.

Determination of Albumin Secretion

Secreted Albumin was analyzed from the media collected after 24 hours incubation by Human Albumin Elisa kit (Bethyl) according to manufacturer's instructions. HepG2 and Hek293T cells were cultured in same medium and were used as positive and negative control respectively. Concentration of albumin was calculated as ngAlb/day/10^6 cell.

Ammonia and Urea Measurement

Organoids were grown in DM medium for 14 days, then medium was refreshed and supplemented with 5 mg/mL insulin (Sigma), 50 mM hydrocortisone hemisuccinate (Sigma), 2mM glutamine (Gibco), 50 mg/mL penicilline/streptomycine mix (Gibco), 2.27 mM D-galactose (Sigma), 2 mM L-lactate (Sigma) and 2 mM ornithine hydrochloride (Sigma). After 24 h, medium was replaced with 1.5 mM NH4Cl (Santa Cruz) containing phenol red free medium (Gibco) for another 24 hours and supernatants were collected. Ammonia and urea concentrations were determined by Ammonia Assay kit (Sigma) and Urea Assay kit (Sigma). For both assays, colorimetric measurements were taken by Varioskan Flash (Thermo) and values were calculated based on manufacturers' instructions and normalized by cell number.

Immunostaining

Characterization of integration free iPSC lines was performed by immunofluorescence staining as described previously. Briefly, iPSC colonies and definitive endoderm cells were fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature, washed three times with PBS and incubated overnight at 4°C with primary antibody. The antibodies used for staining were SSEA-4/A647 (BD, #560218), NANOG (Abcam, #ab21624), OCT4 (Abcam, #ab19857) SOX17

(Abcam, #ab84990), FOXA2 (Abcam, #ab60721) and EpCAM (Milteny, #130-080-301), Donkey anti-rabbit IgG/A555 (Molecular Probes, #A-31572), Goat anti-Rabbit (Invitrogen, #A11034, A11037) and Goat anti-Mouse (Invitrogen, #A11001, A11005) were used as secondary antibodies. Nuclear staining was performed with Hoechst (Invitrogen) and DAPI (Sigma). All antibodies were diluted in 3% goat serum, 3% BSA Fraction VII and 0.01% Triton X-100 and 0.3M Glycine in PBS. Imaging was performed using a confocal microscope Nicon 90i and Zeiss LSM 880.

For organoid immunofluorescence staining, matrigel was removed by washing with cold PBS for four times in a 15-ml tube. Free organoids were then fixed with %4 PFA for 30 minutes on ice and washed with 1x PBS for three times. After fixation, organoid staining was performed either by whole-mount [10] or frozen sectioning. To prepare frozen sections, organoids were first embedded into mold containing OCT (Tissue-Tek) at -20°C and serial cryo-sections were made at 7µ thickness. In both stainings for whole-mount and frozen section, blocking was done by 0.1%–1% Triton X-100 (depending on the protein localization), 1% DMSO, 1% BSA and 1% serum in 1x PBS for 3h in RT. After antibodies CK18 (Santa Cruz, #SC51582), E-Cadherin, Santa Cruz, #8426), A1AT (Abcam, #166610) EpCAM (Milteny, #130-080-301), CK19 (Santa Cruz, #SC6278), ALB (Abcam, #ab10241), HNF4α (Abcam, #ab55223) and ZO1 (Invitrogen, #402-2000) were diluted in blocking buffer, samples were incubated for 24 h at 4°C. Goat anti-Rabbit (Invitrogen, #A11034, A11037) and Goat anti-Mouse (Invitrogen, #A11001, A11005) used as secondary antibodies. Nuclear staining was done by DAPI and imaging was performed by using confocal microscopy Zeiss LSM 880.

For immunohistochemistry staining, matrigel was removed and organoids were fixed as described above. Organoids were embedded in paraffin after serial dehydration steps by applying different concentrations of ethanol. 5µ sections were first blocked as described above. Then antibodies for AFP (Abcam, #ab3980), CK19 (Santa Cruz, #SC6278), CK18 (Santa Cruz, #SC51582), ALB (Abcam, #ab10241), and E-CAD (Santa Cruz, #8426) were used. To analyze morphological organization in the organoid sections, H&E staining was performed.

Flow Cytometry Analysis

iPSCs were fixed with %4 PFA for 15 minutes on ice, washed three times with 1x PBS. The cells were permeabilized with %0.5 Triton X-100 in PBS for 30 minutes on ice and then blocked with %10 FBS and incubated with conjugated primary antibodies OCT4 (BD, #563524), SOX17 (BD, #562594) and FOXA2 (BD, #561586) for 1 hour at 4^{o}C. Flow cytometry analysis was carried out with BD canto II. Data were analyzed with FlowJo software.

Transmission Electron Microscopy

The organoids were fixed in 2,5% gluteraldehit in 0.1 M sodium cacodylate for 48 h at 4°C and then washed with Sorenson's Buffer thoroughly and post-fixed in 2% aqueous osmium tetroxide for 1 h. After dehydration step in serial dilutions of aceton and propylene oxide, the organoids were embedded in mixture of Embed 812, dodecenyl succinic anhydride(DDSA), Araldite 502 and BDMA (Electron Microscopy Sciences, Hatfield, PA) and incubated at 63°C for 48 h for complete polymerization. Ultrathin sections (50nm thick) were cut with an ultramicrotome (Leica EM UC7). The sections were viewed using Zeiss Sigma 500 electron microscopy.

Transplantation

Immunodeficient NSG mice (NOD.Cg-*Prkdcscid Il2rgtm1Wjl*/SzJ) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Animals were housed and maintained under specific

pathogen-free conditions in accordance with institutional guidelines under approved protocols. Six to 8 week-old NSG mice received DMN (7,5 mg/kg, Sigma, 1.0% dissolved in saline, intraperitoneally) 3 consecutive days per week for 2 weeks. After a 2-week treatment, one day after the final DMN injection, mice were anaesthetized (ketamine 100mg/kg, xylazin 8mg/kg), and 2×10^6 ALB-GFP iPSC derived organoid cells in 30 µl cell suspension were intrasplenically transplanted to the DMN-treated mice. 32 days after transplantation, mice were sacrificed and liver samples were obtained. For histopathological analysis, liver samples were fixed in a 10% neutral buffered formaldehyde solution, embedded in paraffin and serial sectioned. Immunohistochemistry was performed on 5 µm tissue sections fixed on slide glass, deparaffinized with xylene and rehydrated with ethanol. Antigen epitope retrieval was performed by heating in 0.1 M citrate buffer (pH 6.0) in microwave for 15 to 20 min and cooling at room temperature. Non-specific binding was blocked with 5% normal goat serum for 1 h. The slides were washed with 1×TBS containing 0.1% Tween-20 and bound with the following primary antibodies anti-GFP (Cell Signaling, #CS12/2016 1:100) and anti-hALB (Bethyl, #A80-229P) incubated at 4 °C for overnight. Secondary antibody (Goat Anti-Rabbit, Thermo, #31460, 1:100) was incubated for 45 minutes at room temperature, signal was visualized with 3,3'-diaminobenzidine (DAB) (Roche) and sections were counterstained with hematoxylin.

Supplemental References

1. Fidan K, Ebrahimi A, Çağlayan ÖH, Özçimen B, Önder TT: Transgene-free disease-specific iPSC generation from fibroblasts and peripheral blood mononuclear cells. In: Patient-Specific Induced Pluripotent Stem Cell Models: Springer, 2015; 215-231.