Stem Cell Reports

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

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Article
Keywords:	Liver, 3D Organoid, Citrullinemia, EpCAM
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Abstract:	Organoid technologies become a powerful emerging tool to model liver diseases, for drug screening, and for the design of personalized treatments. These applications are, however, limited to generate functional hepatocytes in a reproducible and efficient manner. Here, we generated and characterized the hepatic organoid (eHEPO) culture system using human iPSC-derived EpCAM-positive endodermal cells as an intermediate. eHEPOs can be produced within two weeks and expanded long-term without any loss of differentiation capacity to mature hepatocyte (>16 months). Then, starting from patient-specific iPSCs, we modeled Citrullinemia type 1, a urea cycle disorder caused by mutations in the argininosuccinate synthetase (ASS1) enzyme. The disease-related ammonia accumulation phenotype in eHEPOs could be reversed by the overexpression of the wild type ASS1 gene which also indicated that this model is amenable to genetic manipulation. Thus, eHEPOs are excellent unlimited cell sources to generate functional hepatic organoids in a fast and efficient manner.

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Dear Prof. Mummery,

We would like to submit our manuscript entitled "Robust, long-term culture of endoderm derived hepatic organoids (eHEPOs) for disease modeling" by Akbari et al. for consideration in *Stem Cell Reports* as an *Research Article*.

In this work, we were inspired by the work of Hans Clevers (Hubrecht Institute) in generating liver organoids from adult liver biopsies and worked together with his former postdoctoral fellow Onur Basak (UMC Utrecht) to establish a rapid and efficient method to generate liver organoids from patient-specific induced pluripotent stem cells (iPSCs). A critical step in our method that differs from previously published organoid protocols is the induction of endoderm lineage from iPSCs by the combined action of Activin A and Wnt3a/R-spondin1 and isolation of EpCAM-positive endodermal precursors cells for subsequent steps. Endoderm derived hepatic organoids (eHEPOs) could be obtained within 8-10 days and stably cultured long-term. eHEPOs are resilient to freeze/thaw cycles for biobanking and allow the generation of large amounts of hepatocytes in a rapid manner. Notably, our culture system produced functional organoids including biliary ductal structures and well-organized hepatocytes. We have carried out extensive *in vitro* and *in vivo* characterization of the resulting cells as well as RNA-sequencing analyses of each step of differentiation from iPSCs to functional hepatocytes. We also used our method to generate an organoid model of a metabolic disease of the liver, Citrullinemia. Importantly, disease-specific eHEPOs exhibited the pathophysiological phenotype of the disease in the form of ammonia accumulation. Furthermore, we were able to rescue this disease-relevant phenotype in patient-specific organoids via overexpression of the wild type *ASS1* gene mutated in Citrullinemia patients which demonstrated that eHEPOs are amenable to genetic manipulation.

Genetic and chronic liver diseases represent an important health care problem and there is a growing unmet clinical need for novel therapeutic strategies for such diseases. Cellular therapies may offer an alternative approach if adequate amounts of metabolically active and functional hepatocytes can be produced. Patient-specific liver organoids have great potential in this regard. Our work establishes a highly reproducible, rapid and efficient iPSC-derived liver organoid method which mimics liver development. We have extensively characterized this method based on its molecular signature, functionality and ability to accurately model a metabolic liver disease. In light of the journal's broad readership and demonstrated interest in the field of organoid biology, we believe that the enclosed manuscript is well-suited for publication in *STEM CELL REPORTS*. We hope that you will share our enthusiasm and look forward to your editorial feedback.

With best regards,

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We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. *Also, all authors have approved the manuscript and agree with its submission to STEM CELL REPORTS*.

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

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Highlights

- Liver organoid was generated from human iPSCs derived EpCAM+ endodermal cells
- We developed a robust culture protocol of endoderm derived hepatic organoid (eHEPO)
- The eHEPOs exhibit liver characteristics
- The eHEPOs were utilized for modeling of urea cycle disorder, Citrullinemia

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

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Running Title: "Endoderm derived hepatic organoids"

Summary

Organoid technologies become a powerful emerging tool to model liver diseases, for drug screening, and for the design of personalized treatments. These applications are, however, limited to generate functional hepatocytes in a reproducible and efficient manner. Here, we generated and characterized the hepatic organoid (eHEPO) culture system using human iPSC-derived EpCAM-positive endodermal cells as an intermediate. eHEPOs can be produced within two weeks and expanded long-term without any loss of differentiation capacity to mature hepatocyte (>16 months). Then, starting from patient-specific iPSCs, we modeled Citrullinemia type 1, a urea cycle disorder caused by mutations in the argininosuccinate synthetase (ASS1) enzyme. The disease-related ammonia accumulation phenotype in eHEPOs could be reversed by the overexpression of the wild type *ASS1* gene which also indicated that this model is amenable to genetic manipulation. Thus, eHEPOs are excellent unlimited cell sources to generate functional hepatic organoids in a fast and efficient manner.

Key words: Liver, 3D organoid, Citrullinemia, EpCAM

Introduction

Cellular therapies provide promising treatment options for liver pathologies ranging from cancer to genetic metabolic diseases. One of the main difficulties associated with cellular therapy approaches is the generation and expansion of mature autologous hepatocytes. While the ability to derive patient-specific induced pluripotent stem cells have solved the problem of generating autologous cells, differentiation and expansion of mature patient-specific hepatocytes remain a challenge.

Hepatocyte-like cells can be derived from human induced pluripotent stem cells (iPSC) via their step-wise differentiation. Initial works focused on developing

differentiation protocols to achieve hepatocyte-like cells were in standard two-dimensional (2D) conditions (Chen et al., 2012; Liu et al., 2011; Si-Tayeb et al., 2010). Such step-wise protocols utilize cocktails of growth factors/ cytokines to recapitulate embryonic liver development *in vitro*, from definitive endoderm to hepatic progenitors and then to functional hepatocytes. However, the cell populations produced by these different protocols vary considerably in their maturation status and, in most cases, represent immature hepatocytes. This is in part due to the absence of tissue-specific architecture, mechanical and biochemical cues and cell-cell communication under 2D conditions (Luo et al., 2018; Pampaloni et al., 2007). To overcome these limitations, an alternative approach has been utilized to induce human iPSCs into hepatic endodermal cells and then mix them in a three dimensional (3D) co-culture system with stromal cells to generate liver bud-like aggregates (Takebe et al., 2013; Takebe et al., 2017). This liver bud-like structure is able to vascularize and perform hepatic functions after transplantation into mice. However, the long-term stability and expansion of such structures is currently unknown.

A 3D organoid culture system of adult hepatic stem/progenitor cells has been recently developed. These cultures are derived from Lgr5-positive cells sorted from injured murine liver and epithelial cell adhesion molecule (EpCAM) positive ductal cells from intact human liver (Broutier et al., 2016; Huch and Dolle, 2016; Huch et al., 2013; Huch et al., 2015). During liver development and homeostasis, EpCAM has a dynamic expression pattern and its expression in immature cells is gradually lost upon their maturation into hepatocytes (Schmelzer et al., 2007). EpCAM is expressed in mouse liver bud at E9.5 and EpCAM+DLK1+ hepatoblasts with high proliferation potential have capacity to differentiate into bile duct and hepatocytes (Tanaka et al., 2009). Additionally, only EpCAM+ endodermal cells in zebrafish are able to license hepatic development by sequestering Kremen1 on the cell membrane and allowing the formation of Lrp6 signalosomes to activate Wnt2bb target genes (Lu

et al., 2013). Whether human EpCAM+ endodermal cells that arise during development can form liver organoids remain unknown.

Here, we report a human iPSC-derived hepatic organoid culture system for producing functional hepatocytes using EpCAM-positive endodermal cells as an intermediate. These endoderm derived hepatic organoids (eHEPOs) could be produced within 14 days and expanded more than one year without any loss in culture efficiency. Using eHEPOs, we modeled the urea cycle disorder Citrullinemia, demonstrating the power of this system for disease modeling. Finally, we performed ectopic expression of wild type allele of the disease-causing gene, *ASS1* in eHEPOs which rescued the phenotype of the disease and showed that eHEPOs are amenable to genetic manipulation.

Results

Endodermal Progenitors Generated from hiPSCs

To test whether hepatic organoids can be generated from iPSCs, we first derived transgene-free iPSCs from a healthy donor via non-integrative episomal plasmid-based reprogramming of dermal fibroblasts (Fidan et al., 2015). Teratoma formation in immunocompromised mice confirmed the pluripotency of the iPSCs (Figure S1). iPSCs expanded in feeder-free culture were incubated with modified endodermal induction medium containing Activin A, Wnt3a and R-spo1 (Chen et al., 2012; Si-Tayeb et al., 2010). After 5 days, cells no longer exhibited typical embryonic stem cell (ESC) morphology and adopted a polygonal shape (Figure 1A). Immunostaining revealed that almost all of the cells were positive for the endoderm markers SOX17, FOXA2 and EpCAM at day 5 indicating that the human iPSCs efficiently differentiated into definitive endoderm during the 2D monolayer endodermal induction period (Figure 1B). Additionally, flow cytometry analyses showed that majority of cell populations expressed both FOXA2 and SOX17 at day 5 (Figure S2). Notably, modification of the endoderm induction medium by adding R-spondin1, a

well-known Wnt signal potentiator in the stem cell growth (Carmon et al., 2011; de Lau et al., 2011; Planas-Paz et al., 2016) caused a significant increase in the number of EpCAM+ cells (Figure 1C). Thus, our modified protocol allows more efficient generation of a pure endodermal progenitor population.

Generation of organoids from Endodermal EpCAM+ cells

We inoculated Fluorescence-activated cell sorting (FACS) purified EPCAM+ or EPCAM- endodermal progenitors in the liver organoid expansion medium as defined in adult liver organoid cultures. As early as 1 week, EpCAM+ endodermal cells generated 3D hollow structures similar to the adult stem cell derived liver organoids, while EpCAM- cells lacked this ability. When culturing EpCAM+ derived organoids, structures with round shaped organoid morphology, approximately 100 nm in diameter with distinct edges were observed. Notably, organoid cultures could be passaged weekly at 1:5 ratio and could be expanded for >16 months without any loss of phenotypic characteristics and differentiation capacity at passage 30 (Figure 1D). The organoids comprised of CK19+ cells surrounding tightly self-organized ductal-like structures, indicating that cells with hepatoblast and/or bile duct progenitor characteristics persist in the structure (Figure 1E). Besides, some EpCAM+ cells were still present in the organoids showing the continuity of precursor cells of liver. Notably, HNF4A staining showed that organoids have already committed toward hepatic lineage (Figure 1E). Further immunohistochemical analysis of organoids showed that CK18+ and AFP+ cells form pseudostratified epithelial structures as observed in epithelium development (Figure 1F). We then performed RNA sequencing of iPSCs, endodermal progenitors and organoids cultured in expansion medium (EM) conditions to perform an unbiased characterization of their identity at whole transcriptome level (see experimental procedure). Gene set enrichment analysis (GSEA) indicated that gene sets of gastrulation, endoderm formation and endoderm development were highly enriched upon endoderm induction from iPSCs. Conversely, pluripotency associated genes were beginning to be down regulated at

the same stage (Figure 1G). Taken together, these data indicate that this protocol faithfully simulates the stepwise developmental process of hepatic differentiation.

In Vitro Differentiation of Organoids into Functional Hepatocytes and Transplantation

To further the maturation of the emerging hepatic cells, we cultured organoids in differentiation medium (DM) for 14 days (Huch et al., 2015) and analyzed the expression of liver specific genes and structural organization by immunostaining. Differentiated organoids comprised ALB+ and CK18+ hepatocytes with typical polar structure and ZO-1 expression indicating the existence of tight junctions separating apical and basolateral domains (Figure 2A). Further immunohistochemical analysis revealed that organoids have both ALB+ and CK19+ cells indicating mature hepatocyte and cholangiocyte in ductal-like structure, respectively. Also, E-CAD+ cells represented polygonal epitheloid structures reflecting hepatocyte-like phenotype (Figure 2B). Ultrastructural analysis of organoids demostrated the presence of a layer of live cells with apical and basolateral polarity and a luminal area containing the residual of apoptotic and multivesicular bodies. Of note, the junctional complex between cells defined us the characteristic of epithelial cells surrounding lumen (Figure 2C and Figure S3).

To further characterize the maturation of the iPSC-endoderm derived hepatic organoids (eHEPOs), we developed an Albumin-green fluorescent protein (GFP) reporter system. An albumin enhancer/promoter driving GFP expression was cloned into a lentiviral backbone between two flanking insulator elements and integrated into human iPSCs which enabled us to monitor real time differentiation of iPSCs to mature hepatocytes in organoids. Organoids were established from reporter bearing iPSCs which, after 5 days in DM culture conditions became GFP-positive (Figure 2D). Consistent with reporter activity, differentiated organoids secreted significant amounts of Albumin to the medium as an indication of hepatocyte functionality (Figure 2E). Upon differentiation, organoids also gained mature hepatocyte functions

such as CYP3A4 activity, LDL-uptake and glycogen storage (Figure 2F, 2G and 2H). For detailed assessment of the differentiation status of organoids, we generated global expression profiles after DM induction. GSEA analysis showed that liver specific genes were highly upregulated upon culture in DM conditions (NES 1.81, FDR q-value = 0). This finding supports our hypothesis that liver specific genes are further upregulated in DM conditions compared to EM condition (Figure 2I). Most of the key enzymes and receptors involved in different aspects of liver function including glucose homeostasis (*DCXR*, *IGFBP4*, *PGM1*), lipid metabolism (*RXRA*, *GHR*, *SOD1*, *APOC3*, *APOB*, *APOA1*, *LPIN1*) and gluconeogenesis (*PPP1R3B*, *GBE1*) were induced upon DM culture (Figure 2J). qPCR validation of RNA-sequencing data confirmed the upregulation of mature hepatocyte markers such as *ALB*, *A1AT*, *CYP3A7*, *CYP3A4* and downregulation of the endoderm stage marker *EpCAM* (Figure 2K). To test the ability of the ALB-GFP reporter organoids to engraft as functional hepatocytes in vivo, we treated NSG mice with Dimethylnitrosamine (DMN) for 14 days to induce acute liver damage. GFP+ cells were detected 2 days after transplantation into mouse liver (Figure 2L). These results indicate that the mature, functional hepatocytes in our organoid cultures have the ability to engraft in the mouse liver.

Disease specific iPSC- endoderm derived hepatic organoids (eHEPOs)

We next investigated whether the eHEPO system can be utilized for disease modeling. To this end, we generated iPSC lines from two patients who presented with neonatal hyperammonemia and clinically diagnosed with classical Citrullinemia type 1 (CTLN). Citrullinemia type 1 is an autosomal recessive urea cycle disorder caused by defects in the argininosuccinate synthetase (ASS) enzyme due to mutations in the *ASS1* gene (Quinonez and Thoene, 2016). An impairment of ASS1 function can lead to a wide spectrum of phenotypes, from life-threatening neonatal hyperammonemia to a later onset with mild symptoms. Patient-specific iPSCs were grown under feeder-free conditions and displayed typical pluripotent stem cell

morphology (Figure 3A). PCR amplification and sequencing of all coding exons of *ASS1* indicated that both the patients' fibroblasts and iPSCs harbored homozygous G390R mutations in exon 15, which is one of the most common mutations in classical Citrullinemia (Figure S4, Figure 3B) (Kose et al., 2017; Quinonez and Thoene, 2016). Established iPSC lines were devoid of episomal vector sequences as shown by genomic DNA PCR (Figure 3C). One iPSC clone from each patient was further analyzed by chromosomal G-banding and was confirmed to have a normal karyotype (Figure 3D). CTLN-iPSCs were positive for pluripotency markers OCT4, NANOG and SSEA-4 by immunofluorescence (Figure 3E). RT-PCR analyses indicated a high expression of *OCT4*, *SOX2*, *NANOG* and *LIN28* mRNA in patient-derived iPSCs, but not in the original dermal fibroblasts (Figure 3F). As expected, the ASS1 protein expression was detected in the healthy donor derived iPSCs but was undetectable in patient-specific iPSCs (Figure 3G). Finally, both of the CTLN-iPSC lines formed well-differentiated teratomas containing cells derived from all three germ layers (Figure 3H). Taken together, these data confirm the pluripotency of Citrullinemia patient-derived iPSCs.

Following the protocol described above, we successfully generated CTLN-organoids that could be passaged for more than 6 months in culture (Figure 4A). Immunofluorescence staining of HNF4A, ZO-1, ALB, CK18 and CK19 showed a similar pattern/structure with the healthy donor derived organoids (Figure 4B). RNA-sequencing of patient derived cultures and k-means clustering of Pearson correlation of whole transcriptomes showed clear separation of organoids from less differentiated cell types (Figure 4C). While iPSCs and endodermal cells were similar in molecular identity, they still clustered separately, indicating proper discrimination of cell types (Figure 4C). Most importantly, patient derived cells were almost indistinguishable from their healthy counterparts. DM cultures of healthy and CTLN-patient derived organoids were almost identical (2 genes with FDR<0.01) indicating that CTLN disease specific mutation does not affect the differentiation capacity of

patient-derived cells.

We then compared the healthy donor and patient derived eHEPOs in terms of their ability to eliminate ammonia. Similar to disease pathology, patient organoids were significantly impaired in their ability to eliminate ammonia when compared to healthy donor organoids (Figure 4D). To investigate whether the re-expression of *ASS1* can rescue this disease-related phenotype in hepatic organoids, full length *ASS1* cDNA was cloned into a lentiviral vector and transduced to iPSCs. CTLN-derived hepatic organoids ability to detoxify ammonia was restored upon ASS1 expression (Figure 4D). Taken together these data indicate that hepatic organoids can faithfully recapitulate the disease phenotype, and restoration of gene function can be carried out in the eHEPO model.

Discussion

Hepatocyte-like cells differentiated from iPSCs have been used to model several liver-related diseases, including a1-antitrypsin deficiency, citrin-deficiency (CD) and citrullinemia type 1 (CTLN1), highlighting the pathophysiology to pursue new medical strategies (Kim et al., 2016; Yoshitoshi-Uebayashi et al., 2017; Yusa et al., 2011) . Although these models represent the phenotype of disease in dish, the major limitation is the lack of protocols that produce fully functional mature hepatocytes (Baxter et al., 2015; Gieseck III et al., 2014; Godoy et al., 2015). Additionally, long-term expansion/maintenance of these cells without losing function is very limited and freeze/thawing of these cells in 2D culture is one other big challenge in this era, to our knowledge.

The 3D cell culture models have recently become important for hepatic functional studies because they often induce levels of cell differentiation and tissue organization with proper cell-cell contact not observed in conventional 2D culture systems (Duval et al., 2017; Pampaloni et al., 2007). Studies have shown that the gene expression profiles (Godoy et al., 2015) as well as the responses to drug toxicity (Takayama et

al., 2013) in the multicellular spheroid 3D models resemble more closely to in vivo situation.

The recent availability of stem cell- derived organoid systems to provide 3D self-organized tissue models provides a compelling new class of biological model to serve as both tissue and organ proxies (Lancaster and Knoblich, 2014). Organoids recapitulate a large number of biological parameters including the spatial organization of heterogeneous tissue-specific cells, cell-cell interactions, cell-matrix interactions, and certain physiological functions generated by tissue-specific cells within the organoid. Researchers are toiling to refine organoid culture systems to make them more complex, more mature and more reproducible.

Here, we developed a novel human iPSC-derived 3D organoid culture system for producing functional hepatocytes using EpCAM+ endodermal cells as an intermediate. EpCAM has been identified as a surface marker on human hepatic stem/progenitor cells (Schmelzer et al., 2006; Yoon et al., 2011), (Dollé et al., 2014) and freshly isolated EpCAM+ cells from either fetal or postnatal livers are shown to be expandable in culture and have the ability to form human liver tissue after transplantation into livers of NOD/SCID mice (Schmelzer et al., 2007). Moreover, endoderm specific EpCAM is a key regulator of licensing hepatic commitment in zebrafish embryos (Lu et al., 2013). EpCAM+ liver stem cells isolated from adult liver can be expanded in long-term organoid culture in a genome stable manner. Based on these findings, we hypothesized that freshly isolated EpCAM+ progenitor cells from iPSC-derived endoderm may be used as cell sources to generate hepatic organoids a more simple and reproducible manner yielding more mature cell types. To test this, we sorted EpCAM+ cells after endoderm induction from iPSCs and used them to make 3D organoids. At the initial step of our protocol, we added R-spo1 as a secreted Wnt pathway agonist to the endoderm induction culture consisting of Activin A and Wnt3a to get increased numbers of EpCAM+ cells. We showed that only freshly isolated EpCAM+ endodermal cells can readily make 3D organoids within 8-

10 days in EM culture conditions while their EpCAM- counterparts cannot do so. Of note, these endoderm derived hepatic organoids (eHEPOs) have been expanded for long-term in culture (passage number 30) and still produced albumin after differentiation by Huch's DM medium. Notably, we showed that organoids originated from endoderm could be produced in Huch's medium which was originally defined only for organoids derived from adult stem cells. We also transplanted eHEPOs into NSG mice and showed human specific Albumin+ hepatocytes embedding mouse liver. Finally, we used this technology to model liver metabolic disease, citrullinemia and showed pathophysiological phenotype of disease as evidenced by a decrease in ammonia detoxification capacity. The rescue of disease phenotype via overexpression of wild type of the ASS1 gene also demonstrates us the potential of eHEPOs in the treatment of disease via genetic manipulation.

Recently, Guan et al. developed a model system where iPSC lines differentiate into 3D human hepatic organoids (HOs) and introduced certain known mutations to model genetic diseases such as Alagille syndrome (ALGS) and Tetralogy of Fallot (TOF) (Guan et al., 2017). In this model, iPSCs were first differentiated to Hepatoblast (HB) by a modified Hannan 2D hepatocyte differentiation protocol for 9 days. Cells were then cultured in suspension for additional 12 days until large and complex structures with limited oxygen and nutrient availability in their centers formed. Since these Hepatic Organoids (HO1) structures were not suitable for further differentiation, single cell dissociation was obligatory in this protocol. The dissociated cells could be grown further and differentiated toward hepatic organoids (HO2) for additional 30 days while maintaining their ability to form organoids with luminal structures over a 4-month period. In our protocol, eHEPOs expressed CK18 (hepatocyte-like cell marker) and CK19 (bile duct-like cell) as well as bipotent markers such as AFP and HNF4a at an early time point starting at day 10. However, in the Guan's protocol, only parenchymal type HO1 which is minor type expressed

mature hepatocyte markers such as A1AT and Albumin after day 50. eHEPOs offer the possibility to get more clonal parenchymal types from hepatic organoids in an efficient way. In addition, the long duration of organoid formation from earlier 3D models may render them inconvenient in comparison to our robust eHEPO model to get functional hepatocyte from hiPSCs. Furthermore, our extensive transcriptomic analyses further validate the fidelity of differentiation from eHEPOs at various steps. We anticipate that generation of eHEPOs from diverse set of genetic metabolic and chronic diseases will significantly accelerate the development of new therapeutic strategies against such diseases.

Experimental Procedures

Derivation of human fibroblasts and iPSC generation

After informed consents were obtained from Citrullinemia patients and healthy donors, skin punch biopsies were taken according to a protocol approved by the relevant Institutional Review Boards of Dokuz Eylul University and Koc University. Human primary fibroblast cultures and iPSCs were generated as described in supplementary information.

Teratoma formation and characterization

iPSCs were expanded on Matrigel (Corning) in mTesR1 medium (Stem Cell Technologies) and harvested with Dispase (Stem Cell Technologies), resuspended in a 1:1 mixture of DMEM, and hESC-qualified Matrigel. Cell suspensions were injected intramuscularly into SCID mice (100 µl suspension per leg). Teratoma formation was observed in 6–8 weeks. Teratomas were fixed, paraffin-embedded, sectioned and H&E stained (Koc University Histopathology Core, School of Medicine).

Mutation analysis of iPSCs

The Citrullinemia type I (CTLN 1) patients whose fibroblasts were reprogrammed carry homozygous ASS-p~G390R (c.1168G>A) mutations in the *ASS1* gene.

Genomic DNAs were amplified by PCR using the following primers: Exon15-Fwd: CAGTCCTCCCTTCAAGCAGA, Exon15-Rev: GCAGTCAAGGTCGCATCAAA. Sanger sequencing was performed which confirmed the presence of homozygous single nucleotide mutation that leads to ASS-p~G390R (c.1168G>A).

In vitro differentiation of human iPSCs into definitive endoderm

When iPSCs had attained a confluence of 70%, the MEF-conditioned medium was replaced with Roswell Park Memorial Institute/B27 with 100 ng/mL Activin A (PeproTech), 50 ng/mL Wnt3a (R&D Systems) and 5 ng/ml R-spo1 (R&D Systems) for 5 days.

Organoid culture of EpCAM+ hepatic progenitors

After 4 days of 2D monolayer induction of definitive endoderm, cells were harvested and trypsinized by TrypLE (Gibco) for 3-4 min at 37^0C. The dissociated cells were washed with PBS and stained with anti-human CD326 (EpCAM) (Milteny) antibody and sorted on BD FACSARIA III sorter. EpCAM+ cells were mixed with Matrigel (BD Biosciences) and seeded into 24-well/plate at ratio of 3000, 5000 or 10000 cells per well. Non-attachment plates were used in all procedure (Sarsted). After Matrigel had solidified, culture medium was added. Culture media that is called as expansion medium (EM) comprised AdDMEM/F12 (Invitrogen) supplemented with 1% B27 (GIBCO), 1.25 mM N- Acetylcysteine (Sigma), 10 nM Gastrin (Sigma), and the growth factors: 50 ng/ml EGF (Peprotech), 10% R-spo1 conditioned media (homemade), 100 ng/ml FGF10 (Peprotech), 25 ng/ml HGF (Peprotech), 10 mM Nicotinamide (Sigma), 5 µM A83.01 (Tocris), and 10 uM FSK (Tocris). Only for the first 3 days, the medium was supplemented with %5 Noggin and 30% Wnt conditined medium (both prepared as described (Barker et al., 2010), and 10 µM Y27632 (Sigma Aldrich). After 10–11 days, organoids were removed from the Matrigel and mechanically dissociated into small fragments before transferring to fresh Matrigel containing dishes. In each passage, split ratio was 1:4–1:8 which was performed in a once every 7–10 days for 9-12 months.

Hepatocyte differentiation and in vitro functional studies

To induce differentiation, organoids were cultured 3-5 days with EM media in fresh matrigel as described above supplemented with BMP7 (25 ng/ml). Medium was then replaced to differentiation media (DM) which comprised AdDMEM/F12 medium supplemented with 1% B27 and containing EGF (50 ng/ml), Gastrin (10 nM, Sigma), HGF (25 ng/ml), FGF19 (100 ng/ml), A8301 (500 nM), DAPT (10 μM), BMP7 (25 ng/ml), and Dexamethasone (30 μM). This media was refreshed every 3 days for 10 days.

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 in IBG-Vivarium Mouse Facility.

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, xylazine 8mg/kg), and 0.2×10^6 ALB-GFP iPSC derived organoid cells in 30 μl cell suspension were intrahepatically transplanted to the DMN-treated mice. Two days after transplantation, mice were sacrificed and liver samples were obtained.

Messenger RNA-sequencing

Messenger RNA-sequencing was performed using the CEL-Seq2 method (Basak et al., 2017; Hashimshony et al., 2016). In brief, total RNA was extracted with RNeasy mini kit (Qiagen). 10 ng of total RNA per sample was precipitated with 2 μg GlycoBlue (Ambion) overnight at −80°C. The pellet was dissolved in 4-UMI barcode containing primers and incubated at 70 °C for 2 minutes. dNTPs (Promega) were added and the mRNA was reverse transcribed using the Superscript II reverse transcription kit (ThermoFischer Scientific). The second strand was synthesized

using the DNA ligase I (ThermoFischer Scientific). Samples were pooled for *in vitro* transcription through the T7 binding sequence (Ambion Megascript kit). Amplified RNA was converted into cDNA using the Superscript II reverse transcription kit (ThermoFischer Scientific). DNA libraries were generated using Phusion Polymerase (NEB) and the Illumina Truseq small adapter primers (Hashimshony et al., 2016). Ampure XP beads (Beckman Coulter) were used for nucleic acid clean-up of double stranded DNA, amplified RNA and the final DNA library. Libraries were sequenced on an Illumina Hiseq4000 using 150-bp paired-end sequencing at the Genomescan. All the samples were sequenced in a single run to an average depth of ~10 million reads per sample. Reads were mapped to the latest human coding transcriptome and normalized as described elsewhere (Basak et al., 2017). The data was processed using the DESeq2 package (Love et al., 2014). For heat maps, the values of the variance stabilizing transformation of the data are displayed (see DESeq2 manual). For the cluster heatmap, k-means clustering of the Pearson correlation of the whole transcriptome of the samples is displayed.

Data analysis

Statistical analysis was carried out using GraphPad Prism (GraphPad Software, Inc, California, USA). Differences between groups were considered significant at $*P < 0.05$, $**P < 0.001$, and $***P < 0.0001$.

Data availability

The datasets generated in the current study are available in the GEO repository, accession number GSE120145.

For additional and detailed experimental about in vitro functional assays (Periodic Acid-Schiff (PAS) staining, LDL uptake, measurement of CYP3A4 activity, RNA isolation and real-time PCR, ammonia elimination, determination of albumin secretion, immunostaining, Transmission Electron Microscopy and Flow Cytometry analysis) please refer to Supplementary Information.

Author Contributions

E.E., T.T.O. and S.A. conceived of the presented idea, designed and analyzed experiments. G.G.S performed the experiments for the production and characterization of iPSCs. B.S. and B.O. assisted in iPSC experiments. S.A. designed and performed the all experiments related 3D liver organoid culture and disease modeling including FACS, immunostaining, confocal imaging, in vitro assays. A.B. and N.E. performed organoid transplantation in the DMN induced-animal model and the all in vivo assays. S.A. and K.K. performed in vitro functional assays and analyzed the data. E.E. prepared the ALB promoter GFP reporter plasmid. E.O. prepared the ASS1 lentiviral expression vector. S.A., T.T.O, E.E. together with O.B. designed RNA-sequencing experiments. O.B. supervised next-generation sequencing, S.A. prepared RNAs. O.B. and K.S. performed bioinformatics analyses. N.A. provided human donor biopsies and patient material. S.A. cultured biopsy material and performed primary fibroblast culture. S.A. and K.K. performed patient phenotype rescue assays. G.G.S, S.A and T.T.O prepared all figures. E.E, T.T.O, S.A, G.G.S and O.B wrote the paper. All authors commented on the manuscript.

Financial Support: This research was supported by TUBİTAK (The Scientific and Technological Research Council of Turkey) via projects SBAG-115S465 and SBAG-213S182 and by an EMBO Installation Grant (T.O.). K.S. was supported by a TUBITAK BIDEB Scholarship. The authors gratefully acknowledge use of the services and facilities of the Koç University Research Center for Translational Medicine (KUTTAM), funded by the Republic of Turkey Ministry of Development. The content is solely the responsibility of the authors and does not necessarily represent the official views of the Ministry of Development.

Acknowledgements

We would like to thank to Prof. Dr. Hans Clevers (Hubrecht Institute/KNAW, Utrecht-Netherlands) for hosting Soheil Akbari in the Hubrecht Institute (Utrecht, Netherlands) and for the kind gift of L-Wnt3a and Noggin cell lines. We are also immensely grateful to the members of Clevers Lab for sharing their experience about organoid culture.

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Figure Legends

Figure 1. Establishment of endoderm derived hepatic organoids (eHEPOs). A) Schematic representation and timeline of organoid culture establishment. B) Expression of OCT3/4 (pluripotency marker), FOXA2, SOX17 and EpCAM (endoderm markers), in the different stages by immunostaining (20X magnification). C) Flow cytometric analysis demonstrating the increase in EpCAM level with addition of R-spo1. D) Organoid formation potency of sorted EpCAM+ and EpCAM- cells. E) Confocal images for EpCAM, CK19 and HNF4A. Nuclei were co-stained with DAPI. F) Immunohistochemical staining of CK18 and AFP in organoids. G) GSEA plots for

differentially expressed genes during iPSC to endoderm induction and endoderm to organoid differentiation. Normalized enrichment scores (NES) and FDR q-values are listed for each gene set analyzed.

Figure 2. Characterization of functional hepatocyte differentiation in eHEPOs. A) Confocal images of organoids cultured in DM conditions stained for CK18, E-Cadherin, A1AT, ZO1 and ALB. Nuclei were stained with DAPI. B) Immunohistochemical staining of organoids for ALB, CK19 and E-cadherin. C) Transmission electron microscopy image of organoid. D) Lentiviral Albumin promoter-GFP reporter to monitor Albumin expression in organoids. Representative bright-field and fluorescence microscopy images of pALB-GFP reporter bearing iPSCs at the indicated stages of differentiation. E) Albumin secretion from organoids cultured in EM and DM conditions as measured by ELISA. F) CYP3A4 activity in organoids cultured in EM and DM conditions expressed as RLU per ml per million cells. G) Uptake of low-density lipoprotein detected on day 14 by immunofluorescence staining in DM organoids. H) Glycogen storage function of EM and DM organoids as determined by Periodic-Acid Schiff (PAS) staining (magenta). I) GSEA analysis of differentially regulated genes in DM versus EM conditions. Normalized enrichment scores (NES) and FDR q-value is listed for the liver specific gene set analyzed. J) Heatmap showing the expression of EM and DM related genes. K) qPCR based mRNA expression analysis of indicated genes in EM and DM organoids (n=4, * $p<0.01$) L) Immunohistochemistry with anti-GFP antibody in DMN-treated NSG mice liver sections transplanted with organoids. The presence of GFP+ cells demonstrates engraftment of hepatocytes into mice liver.

Figure 3. Derivation and characterization of Citrullinemia-specific iPSCs. A) Morphology of wt iPSC, CTLN iPSC-1 and CTLN iPSC-2. B) Sequence analysis *ASS1* exon15 mutations in healthy and patient-derived fibroblasts and the iPSCs. C) PCR-based integration analysis of the episomal reprogramming vectors. D) Karyotype analysis of WT iPSC, CTLN iPSC-1 and CTLN iPSC-2. E)

Immunofluorescence images iPSCs stained for NANOG, OCT4 and SSEA-4. Cell nuclei were counterstained with Hoechst (Scale bars, 100 μm). F) mRNA expression levels of pluripotency related genes. G) Western Blot for ASS1 using two independent antibodies in wt iPSCs, CTLN1 iPSC overexpressing GFP or ASS1 cDNA (OE). H) Teratoma formation of CTLN iPSC-1 and CTLN iPSC-2 in SCID mice (Scale bars, 100 μm).

Figure 4. eHEPOs for modelling Citrullinemia. A) Bright Field image of Citrullinemia patient-derived hepatic organoid cultures. B) Confocal images of CTLN-organoids stained for HNF4A, CK18, ZO-1, CK19 and ALB. Nuclei were stained with DAPI. C) Cluster Heatmap of WT and CTLN-iPSCs, endoderm and organoids. D) Ammonia detoxification capacity of healthy donor (WT means organoids with wild type ASS1 gene) and patient derived organoids expressing either control (GFP) or ASS1 vector as determined by ammonia elimination assay. Data are expressed as nM/h/million cells (n=3, **p<0.0001).

Figure 1.

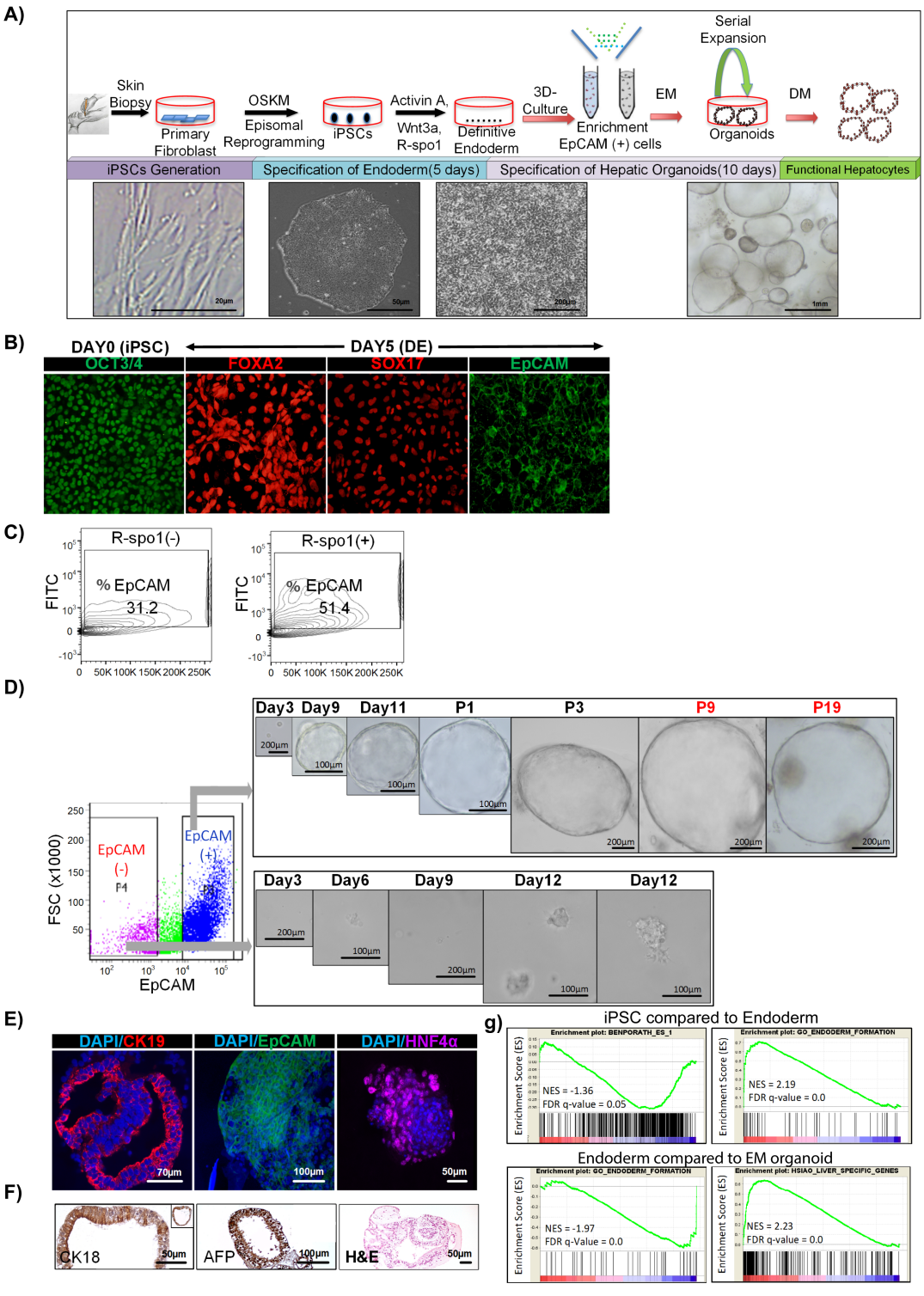

A)
Skin Biopsy
Primary Fibroblast
OSKM Episomal Reprogramming
iPSCs
Activin A, Wnt3a, R-spo1
Definitive Endoderm
3D-Culture
Enrichment EpCAM (+) cells
EM
Serial Expansion
Organoids
DM
iPSCs Generation
Specification of Endoderm(5 days)
Specification of Hepatic Organoids(10 days)
Functional Hepatocytes
B)
DAY0 (iPSC)
DAY5 (DE)
OCT3/4
FOXA2
SOX17
EpCAM
C)
R-spo1(-)
% EpCAM 31.2
R-spo1(+)
% EpCAM 51.4
FITC
0 50K 100K 150K 200K 250K
D)
Day3 Day9 Day11 P1 P3 P9 P19
EpCAM (-)
EpCAM (+)
FSC (x1000)
EpCAM
Day3 Day6 Day9 Day12 Day12
E)
DAPI/CK19
DAPI/EpCAM
DAPI/HNF4α
F)
CK18
AFP
H&E
g)
iPSC compared to Endoderm
NES = -1.36
FDR q-value = 0.05
NES = 2.19
FDR q-value = 0.0
Endoderm compared to EM organoid
NES = -1.97
FDR q-Value = 0.0
NES = 2.23
FDR q-value = 0.0
Enrichment Score (ES)

Figure 2.

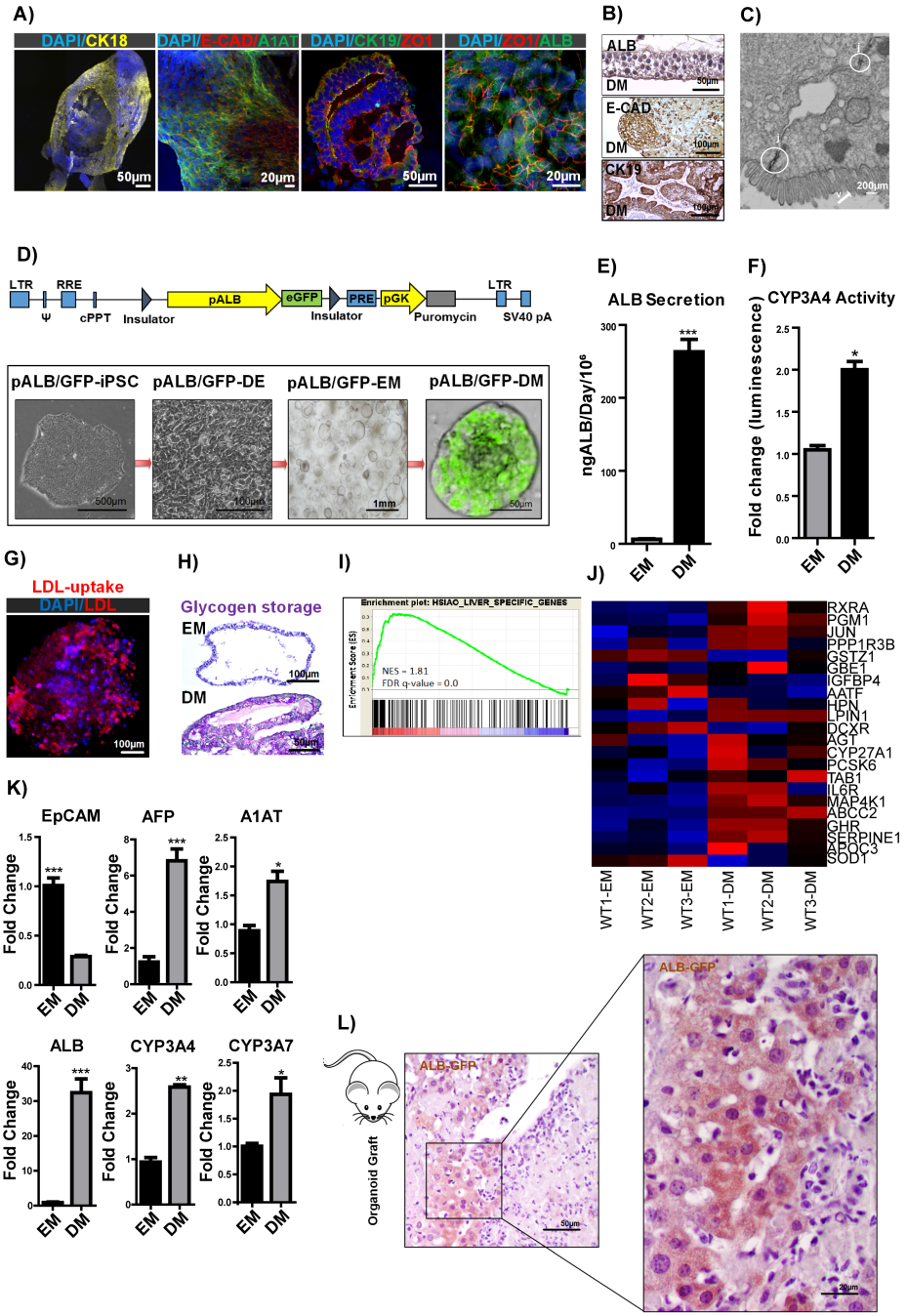

A)
DAPI/CK18
DAPI/E-CAD/A1AT
DAPI/CK19/ZO1
DAPI/ZO1/ALB
50μm
20μm
50μm
20μm
B)
ALB
DM
E-CAD
DM
CK19
DM
C)
200μm
D)
LTR
RRE
Ψ
cPPT
Insulator
pALB
eGFP
Insulator
PRE
pGK
Puromycin
LTR
SV40 pA
pALB/GFP-iPSC
pALB/GFP-DE
pALB/GFP-EM
pALB/GFP-DM
500μm
100μm
1mm
50μm
E)
ALB Secretion
ngALB/Day/10^6
***
EM
DM
F)
CYP3A4 Activity
Fold change (luminescence)
*
EM
DM
G)
LDL-uptake
DAPI/LDL
100μm
H)
Glycogen storage
EM
DM
100μm
100μm
I)
Enrichment plot: HSIAO_LIVER_SPECIFIC_GENES
Enrichment Score (ES)
NES = 1.81
FDR q-value = 0.0
J)
RXRA
PGM1
JUN
PPP1R3B
GSTZ1
GBE1
IGFBP4
AATF
HPN
LPIN1
DCXR
AGT
CYP27A1
PCSK6
TAB1
IL6R
MAP4K1
ABCC2
GHR
SERPINE1
APOC3
SOD1
WT1-EM
WT2-EM
WT3-EM
WT1-DM
WT2-DM
WT3-DM
K)
EpCAM
AFP
A1AT
ALB
CYP3A4
CYP3A7
Fold Change
EM
DM
L)
Organoid Graft
ALB-GFP
ALB-GFP
50μm
20μm

Figure 3.

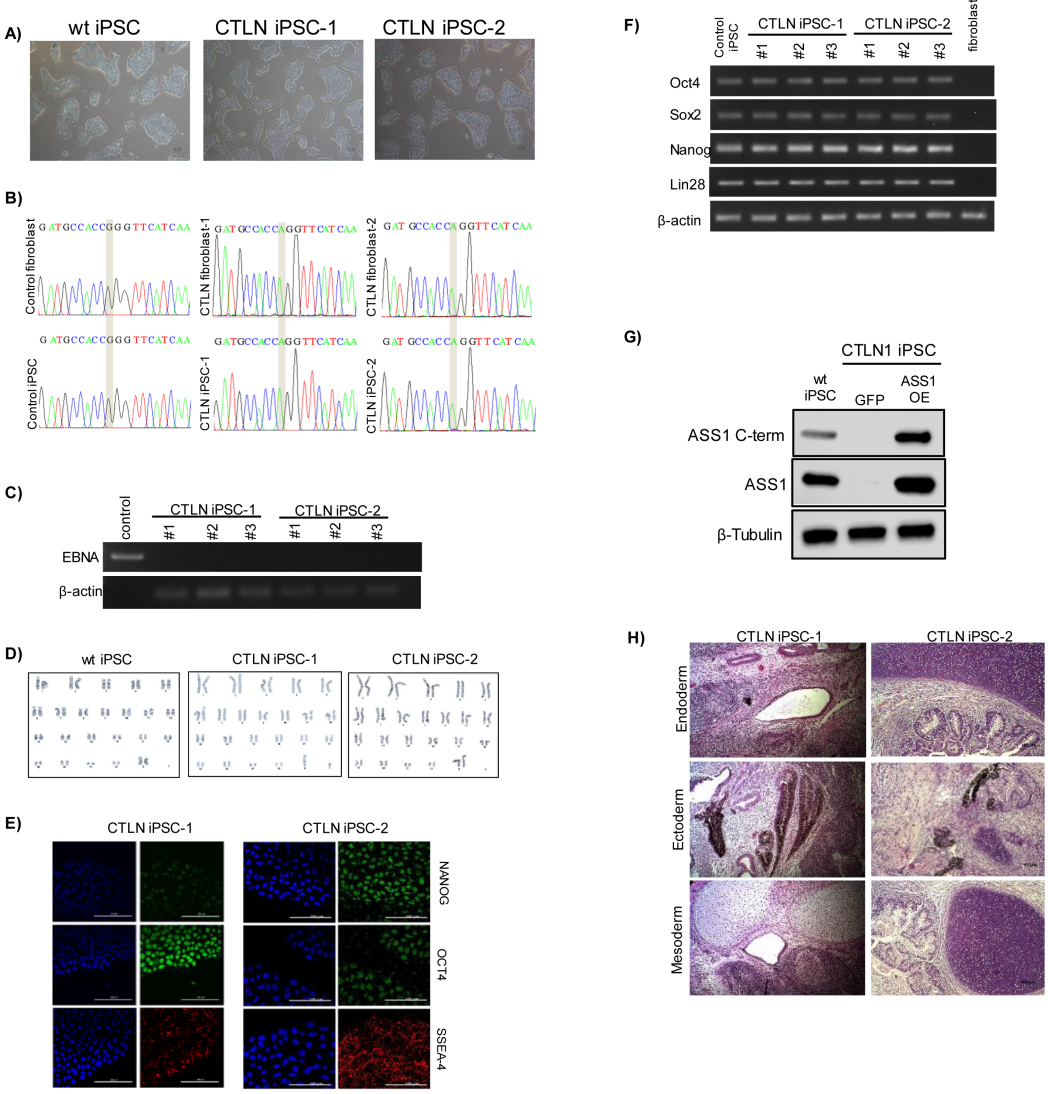
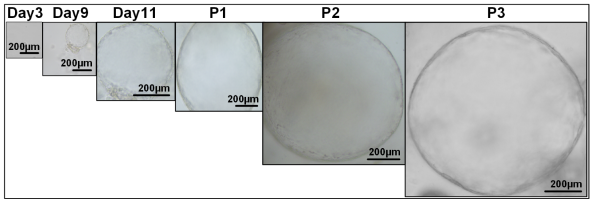
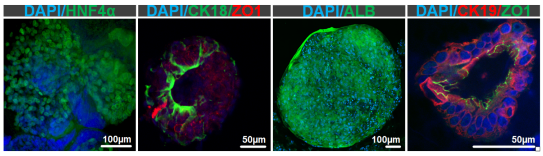

A)
wt iPSC
CTLN iPSC-1
CTLN iPSC-2
B)
Control fibroblast
CTLN fibroblast-1
CTLN fibroblast-2
Control iPSC
CTLN iPSC-1
CTLN iPSC-2
C)
control
CTLN iPSC-1
CTLN iPSC-2
#1
#2
#3
EBNA
β-actin
D)
wt iPSC
CTLN iPSC-1
CTLN iPSC-2
E)
CTLN iPSC-1
CTLN iPSC-2
NANOG
OCT4
SSEA-4
F)
Control iPSC
CTLN iPSC-1
CTLN iPSC-2
fibroblast
Oct4
Sox2
Nanog
Lin28
β-actin
G)
CTLN1 iPSC
wt iPSC
GFP
ASS1 OE
ASS1 C-term
ASS1
β-Tubulin
H)
CTLN iPSC-1
CTLN iPSC-2
Endoderm
Ectoderm
Mesoderm

Figure 4.

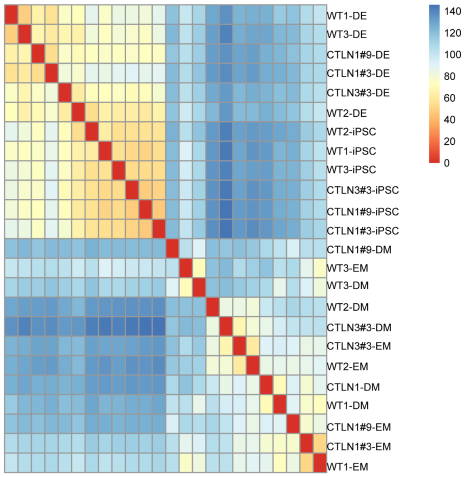
A)



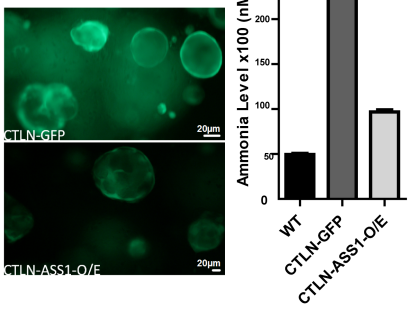
B)



C)



D)



Supplemental Information

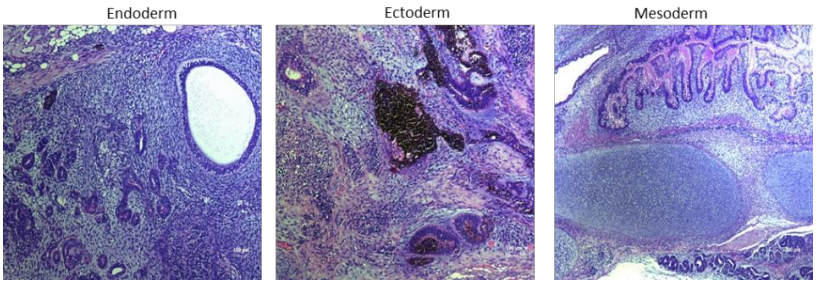


Figure S1. Teratoma formation of CTLN iPSC-1 and CTLN iPSC-2 in SCID mice.

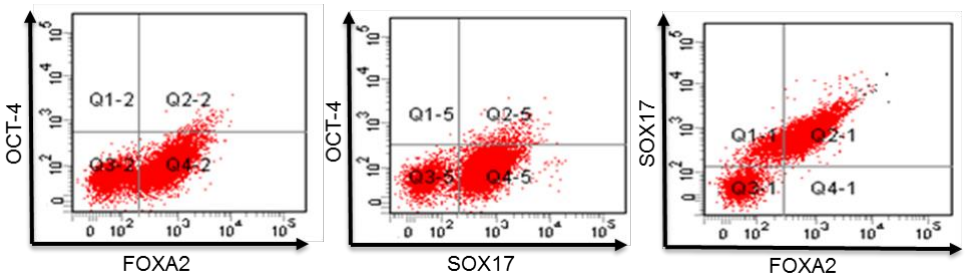


Figure S2. Flow cytometric analysis of Oct3/4, FOXA2 and SOX17 at day5.

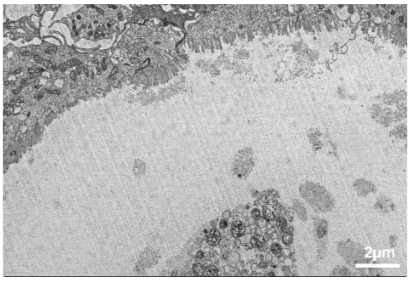


Figure S3. Ultrastructural image of eHEPO via TEM

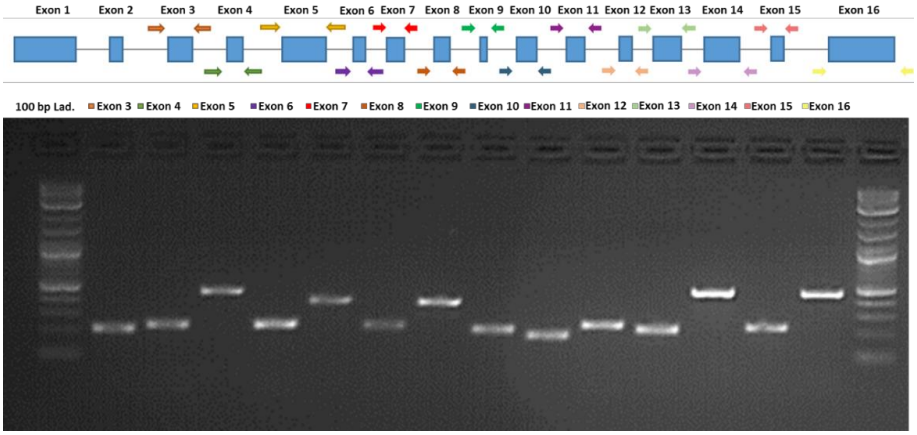


Figure S4. PCR amplification of ASS1 exons for Sanger sequencing

Supplemental Table

Table S1. Primers used in this study

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
ASS1-RT primer1_forward	
ASS1-RT primer1_reverse	

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.

Ammonia elimination

One day before the last differentiation step, 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 24h incubation, medium was removed and 1.5 mM NH4Cl (Santa Cruz) in Hanks buffer (Biological Industries) was added to organoids. After 2 hr incubation, ammonia concentration was determined by Ammonia Assay kit (Sigma).

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.

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 Tra-1-81/A488 (BD), SSEA-4/A647 (BD), Nanog (Abcam), OCT4 (Abcam) SOX17 (Abcam), FOXA2 (Abcam) and Epcam (Milteny). Donkey anti-rabbit IgG/A555 (Molecular Probes), Goat anti-Rabbit (Invitrogen) and Goat anti-Mouse (Invitrogen) 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), E-Cadherin (Cell Signaling), A1AT (Abcam) EpCAM (Milteny), CK19 (Santa Cruz), ALB (Abcam) and ZO1 (Invitrogen) were diluted in blocking buffer, samples were incubated for 24 h at 4°C. Goat anti-Rabbit (Invitrogen) and Goat anti-Mouse (Invitrogen) 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), CK19 (Santa Cruz), CK18 (Santa Cruz), ALB (Abcam) and

E-CAD (Cell Signaling) 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 for 1 hour at 4^{o}C (BD). 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 0.2×10^{6} ALB-GFP iPSC derived organoid cells in 30 µl cell suspension were intrahepatically transplanted to the DMN-treated mice. Two 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 antibody GFP (Cell Signaling, 1:100) incubated at 4 °C for overnight. Secondary antibody (Goat Anti-Rabbit, Thermo, 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.