Biofabrication of synthetic human liver tissue with advanced programmable functions

Rodrigo M. Florentino, Kazutoyo Morita, Nils Haep, ..., D. Lansing Taylor, Ira J. Fox, Alejandro Soto-Gutierrez
als208@pitt.edu

Highlights
Edited human iPSCs gain can control transcriptional activation
CYP3A4 and UGT1A1 activity in human iHep gain are similar to human hepatocytes
Human iHep gain generate synthetic liver tissue with advanced programmable functions

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Biofabrication of synthetic human liver tissue with advanced programmable functions

Rodrigo M. Florentino,1,2,9 Kazutoyo Morita,1,9 Nils Haep,1,9 Takashi Motomura,1 Ricardo Diaz-Aragon,1 Lanuza A.P. Faccioli,1 Alexandra Collin de l’Hortet,1 Zeliha Cetin,1 Carla Frau,1 Lawrence Vernetti,2,3,4 Anna-Klara Amler,5 Alexander Thomas,3 Tobias Lam,3 Lutz Kloke,5 Kazuki Takeishi,1,6 D. Lansing Taylor,2,3,4 Ira J. Fox,4,7,8 and Alejandro Soto-Gutierrez1,2,3,8,10,*

SUMMARY
Advances in cellular engineering, as well as gene, and cell therapy, may be used to produce human tissues with programmable genetically enhanced functions designed to model and/or treat specific diseases. Fabrication of synthetic human liver tissue with these programmable functions has not been described. By generating human iPSCs with target gene expression controlled by a guide RNA-directed CRISPR-Cas9 synergistic-activation-mediator, we produced synthetic human liver tissues with programmable functions. Such iPSCs were guide-RNA-treated to enhance expression of the clinically relevant CYP3A4 and UGT1A1 genes, and after hepatocyte-directed differentiation, cells demonstrated enhanced functions compared to those found in primary human hepatocytes. We then generated human liver tissue with these synthetic human iPSC-derived hepatocytes (iHeps) and other non-parenchymal cells demonstrating advanced programmable functions. Fabrication of synthetic human liver tissue with modifiable functional genetic programs may be a useful tool for drug discovery, investigating biology, and potentially creating bioengineered organs with specialized functions.

INTRODUCTION
Liver failure causes more than 30,000 deaths per year in the US alone. Aggressive clinical management can extend life, but the only definitive therapy for end-stage liver disease is allogeneic orthotopic liver transplantation.1,2 Widespread use of liver transplantation is limited by a shortage of donor organs,1 primary graft dysfunction,1 the need for long-term immune suppression, and high cost.3 Many strategies are being explored to increase the availability of donor livers, including resuscitation of marginal-quality livers by machine perfusion4 and transplantation of livers from non-human donors, such as the pig,5 all of which currently have various technical and practical difficulties. As the liver can regenerate even after severe injury, temporary hepatic support is beneficial in some circumstances.5 The ability to engineer livers from autologous cells such as patient-derived induced pluripotent stem cells might ultimately provide transplantable organs and eliminate the need for life-long immune suppression.7 However, liver tissue engineered in this way has produced limited clinically relevant functions.

Use of programmable DNA-binding proteins, such as CRISPR-Cas9, has revolutionized genome modulation and has been used as a platform for modulating endogenous gene expression.10–13 The catalytically deficient, Cas9 synergistic activation mediator (dCas9-SAM) system10 is an engineered protein complex used for transcriptional activation of endogenous genes, where it has been shown to be highly efficient at activating gene expression and function when compared to other systems.14 We have previously generated protocols for differentiating human-induced pluripotent stem cells (hiPSCs) into hepatocyte and other liver non-parenchymal cells,9,15 and have now created hiPSCs with programmable expression of specific genes based on use of the dCas9-SAM system. Following differentiation of these cells into human iPS-derived hepatocytes (iHeps), we examined the extent of hepatic gene activation in a guide RNA (gRNA)-dependent manner. We demonstrated stable programmable expression derived from the dCas9-SAM system in human iPSCs (Human iPSCs-Cas9gain) at any stage of hepatocyte-directed...
differentiation. As proof of principle, we selected two genes, CYP3A4 and UGT1A1, normally expressed only by mature human hepatocytes, but poorly expressed in iPSC-derived hepatocytes to test the synergistic activation mediator system. By delivering gRNAs for these two genes into human iHepsgain, we found that mRNA and protein expression of CYP3A4 and UGT1A1 were specifically upregulated and functional activity to the level found in freshly isolated human hepatocytes in both 2D and 3D liver tissue configurations. Therefore, we believe that biofabrication of synthetic human liver tissue with advanced programmable functions could represent a viable approach to interrogate maturation mechanisms and functions.

RESULTS

Establishment of human fibroblasts with inducible dCas9 and a gene expression activator system

CRISPR-Cas9 is a simple and efficient tool for genome editing and has enabled genome-wide CRISPR screens in mice and cell lines.16–19 We, therefore, sought to generate human iPSCs with inducible dCas9 expression. We first transduced human primary fibroblasts (HF) with Tet-on-3G doxycycline (Dox)-inducible lentiviral vectors containing inducible dCas9 gain (dCas9-VP64 and the synergistic activation mediator (SAM) system) (neomycin resistant) and inducible SAM containing GFP (puromycin resistant) (Figure 1A). After neomycin and puromycin selection, the transduced fibroblasts (HF-dCas9 gain) exhibited a Dox-dependent 28-fold Tet-On-3G induction of Cas9 (*p = 0.0168 in mRNA) and a 62.5-fold induction of SAM (*p = 0.0394) as assessed by mRNA and GFP expression (Figure 1A).

Generation of human iPSCs from edited fibroblasts carry programmable Cas9-gain-of-function

The stably transduced human fibroblast (HF-dCas9 gain) was then programmed into iPSCs as described.20 The resulting human iPSC colonies showed, by mRNA and protein production, high expression of inducible dCas9 gain and SAM-GFP after Dox exposure. The resulting human iPSCs were cultured for >10 passages in the presence of neomycin and puromycin for enrichment and stability. We named these cells “human iPSCs-dCas9 gain”. The human iPSCs-Cas9 gain showed a 15-fold induction of Cas9 (p < 0.0001 in mRNA) and 322.5-fold (p = 0.0087 in mRNA) induction of SAM-GFP after Dox exposure, as assessed by mRNA and protein expression for either dCas9 or GFP (Figure 1B).

Human iPSCs-dCas9 gain showed normal pluripotent morphology, consisting of compact colonies with distinct borders similar to that seen in human embryonic stem cells (hESCs),21 expressed NANOG, OCT4, TRA-1-60, and SSEA4 (Figure 1C) and displayed uniform mRNA expression of pluripotency markers (C-Myc, Lin28, SOX2, Nanog, and Oct3/4) comparable to that seen in human iPSCs (WTC-11) (Figures 1C and S1). The cells also had a normal karyotype (Figure 1D). Embryoid bodies derived from human iPSCs-Cas9 gain spontaneously expressed ectodermal (SOX1 and Otx-2), mesodermal (Brachyury and HAND1), and endodermal (GATA-4 and SOX17) markers (Figure 1E). Thus, engineering of human iPSCs-Cas9 gain did not affect pluripotency or produce chromosomal alterations. Finally, the human iPSCs-dCas9 gain were tested for the presence or absence of three single nucleotide polymorphisms that could affect hepatic differentiation and/or function22: PNPLA3 (rs738409C>G) MBOAT7/TMC4 (rs641738C>T) and GCKR (rs78094C>T). Human iPSCs-Cas9 gain were “heterozygous” for the PNPLA3 C allele, had a “normal” C/C MBOAT7 allele, and were “heterozygous” for the GCKR C allele (Figure 1F).

Hepatocyte-directed differentiation of gene programmable human iPSCs

We then differentiated the human iPSCs-dCas9 gain toward hepatocytes using our previously published protocol9,15,23,24 with modifications. Cells were first cultured in monolayers with a combination of activin A, bone morphogenetic protein 4 (BMP4), and fibroblast growth factor (FGF)-2 to induce definitive endoderm (Stage 1 & 2) (Figure 2A). Greater than 80% of cells expressed the definitive endoderm markers SOX17 (Figure 2B) and CXC chemokine receptor 4 (CXCR4; Figure 2C) at day 4. Then, cells were cultured for 14 days in the presence of dimethyl sulfoxide (DMSO) and human hepatocyte growth factor (hHGF) to induce hepatocyte specificity (Stage 3). Finally, cells were cultured for 4 additional days in DMSO and HGF with low glucose in the presence of endodermal growth factor, dexamethasone, hydrocortisone, free fatty acids, bile acids, and cholesterol (Figure 2A)(Stage 4) to induce hepatic maturation.
Figure 1. Generation of custom-engineered hiPSCs-dCas9 gain

(A) Schematic diagram of the lentiviral vectors: pcLVi(3G)-SAM containing a TetOn system comprising of a tetracycline response element (pTRE-3g), a reverse tetracycline-controlled reverse transactivator (rtTA-3G), green fluorescent protein (GFP), a puromycin antibiotic selection cassette gene (PuroR), and the synergistic activation mediator (SAM). pcLVi(3G)-dCas9-VP64 containing a tetracycline response element (pTRE-3g), a neomycin antibiotic selection cassette gene (NeoR), and the deactivated CRISPR/Cas9 transcription activator VP64 (dCas9-VP64). Quantitative gene expression analysis of dCas9 and SAM normalized to ACTB in human fibroblasts transduced with lentiviral vector for dCas9-VP64 (HFs-dCas9 gain) and non-transduced HF as control in the presence or absence of DOX (*p = 0.0168, Welch’s t-test). Quantitative gene expression analysis of SAM normalized to ACTB transduced with lentiviral vector for SAM in HFs or HFs-dCas9 gain (*p = 0.0394, Welch’s t-test). In-live GFP-fluorescence of synergistic activation mediator in HFFs or HFFs-dCas9 gain with and without DOX treatment. Data are represented as mean ± SD

(B) Quantitative gene expression analysis of dCas9 and SAM normalized to ACTB in human iPSCs derived from fibroblasts transduced with lentiviral vector for dCas9-VP64 (Human iPSCs-dCas9 gain) and non-transduced human iPSCs as control in the presence or absence of DOX (*p < 0.0001, Welch’s t-test). Quantitative gene expression analysis of SAM normalized to ACTB transduced with lentiviral vector for SAM in Human iPSCs or Human iPSCs-dCas9 gain (*p < 0.05, Welch’s t-test). Immunofluorescence micrographs of dCas9 and GFP in Human iPSCs or Human iPSCs-dCas9 gain with and without DOX treatment; HEK293-dCas9 was used as control. Western blot analysis of dCas9 and β-actin in human iPSCs and human iPSCs-Cas9 gain in the presence or absence of DOX; HEK239-dCas9 was used as control. Also shown is the Quantification of GFP and Cas9 in human iPSCs, human iPSCs-Cas9 gain, and HEK293-Cas9 in the presence or absence of DOX. Data are represented as mean ± SD

(C) Immunofluorescence micrographs of pluripotency markers Nanog, Oct4, TRA-1-60, and SSEA-4 in human iPSCs-Cas9 gain. Quantitative gene expression analysis of pluripotency markers c-myc, Lin28, Sox2, Nanog, and Oct3/4 normalized to ACTB of human iPSCs and human iPSCs-Cas9 gain. Human iPSCs-Cas9 gain carries normal male karyotype in G-banding analysis.

(D) Bright-field micrographs of human iPSCs-dCas9 gain forming embryonic bodies after 20 days in culture. Immunofluorescence micrographs of the three germ layer markers: Ectoderm (Otx-2, SOX1), Mesoderm (HAND1, Brachyury), and Endoderm (SOX17, GATA-4). Genotyping results of human iPSCs-Cas9 gain: PNPLA3 (rs738409) CT-heterozygous, MBOAT7 (rs641738) CC-major homozygous, and GCKR (rs780094) CT-homozygous.

(E) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(F) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(G) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(H) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(I) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(J) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(K) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(L) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(M) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(N) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(O) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(P) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(Q) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(R) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(S) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(T) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(U) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(V) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(W) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(X) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(Y) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

(Z) Human iPSCs-dCas9 gain transduced with lentiviral vector containing Cas9 and SAM in the presence or absence of DOX. Data are represented as mean ± SD

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Programmable activation of hepatocyte-specific genes in Hepsgain using guide RNAs

Since expression levels of CYP3A4 and UGT1A1 were not detected following the final maturation step of hepatocyte-directed differentiation, these genes were selected to test the efficiency of our gene programmable system in human iHeps\textsuperscript{gain}. To induce these clinically relevant metabolic genes, iHeps\textsuperscript{gain} were transduced with lentiviral vectors coding gRNAs for CYP3A4 and UGT1A1, with >90% transduction efficiency (Figure 5A). We designed two guide RNAs for transcriptional activation of CYP3A4 to bind in the promoter region of CYP3A4 on Chromosome 7 and three guide RNAs for the transcriptional activation of UGT1A1 were also designed to bind in the promoter region of UGT1A1 on Chromosome 2. Only specific guide RNAs with no predicated off-target effects were screened and selected. gRNAs were packed in a lentiviral vector for transduction.

Once gRNAs were transduced into human iHeps\textsuperscript{gain}, they were differentiated in a defined medium containing Activin A, BMP4, FGFR2 (stage 1); Activin A (stage 2); dimethyl sulfoxide (DMSO), and hepatocyte growth factor (HGF) (stage 3); epidermal growth factor (EGF), dexamethasone (DEX), hydrocortisone (Hydro), free fatty acids (FFA), cholesterol (Chol), and bile acids (stage 4). Exposure of iHeps\textsuperscript{gain} resulted in expression of the adult isoform of CYP3A4 comparable to primary human hepatocytes (>80% respectively), expression of albumin comparable to primary human hepatocytes (>80% respectively), and no expression of α-fetoprotein (AFP), freshly isolated human fetal and adult hepatocytes were used as controls. Graphs showing quantification of cells expressing HNF4α, albumin, and α-fetoprotein positive cells in Hepsgain, human fetal hepatocytes, and human adult hepatocytes.

Quantitative gene expression analysis of undifferentiated hiPSCs and human iPSCs-Cas9\textsuperscript{gain} for the expression of the adult isoform of CYP3A4 and UGT1A1 were 44% and 61%, respectively, at the end of stage 3 and 4 of hepatic differentiation (D14). Stage 3 of the hepatic differentiation (D18) qPCR is shown for genes encoding octamer-binding transcription factors 3/4 (OCT3/4), C-X-C motif chemokine receptor 4 (CXCR4), SRY-box 17 (Sox17), hepatocyte nuclear factor-4-alpha (HNF4α), hepatocyte nuclear factor-1-alpha (HNF1α), forkhead box A1 (FOXA1), forkhead box A2 (FOXA2), CCAAT enhancer-binding protein alpha (CEBPa), constitutive androstane receptor (CAR), liver X receptor (LXR), retinoid X receptor (RXR), peroxisome proliferator-activated receptor alpha (PPARα), Met, ATP binding cassette subfamily B member 11 (BSEP), ATP binding cassette subfamily C member 2 (MRP2), alpha-fetoprotein (AFP), UDP glucuronosyltransferase family 1 member A1 (UGT1A1) and cytochrome P450 family 3 subfamily A member 4 (CYP3A4). Human adult hepatocytes and human fetal hepatocytes were included as controls. Data are represented as mean ± SD (Figure 2A) Immunofluorescence micrographs of hiPSCs-Cas9\textsuperscript{gain} at stage 3 of hepatic differentiation (D14) stained for CYP3A4 and UGT1A1 compared to primary human adult and fetal hepatocytes.

Quantitative gene expression analysis of dCas9\textsuperscript{gain} for transcriptional activation of CYP3A4 was used in iHeps\textsuperscript{gain}. (Figure 2B). On day 14 of differentiation (stage 3), more than 80% of human iPSCs-dCas9\textsuperscript{gain}-derived hepatocytes (human iHeps\textsuperscript{gain}) expressed the adult isoform of HNF4α and albumin, and human iHeps\textsuperscript{gain} did not express the hepatocyte immature marker α-fetoprotein (AFP) (Figures 2B and S2). Analysis of mRNA expression at the end of stage 3 and 4 of differentiation showed upregulation of liver-enriched transcription factors such as HNF4α, hepatocyte nuclear factor 1 (HNF1α), forkhead box protein A1 (FOXA1), forkhead box protein A2 (FOXA2), CCAAT/enhancer-binding protein 1 alpha (CEBPa), constitutive androstane receptor (CAR), liver X receptor (LXR), retinoid X receptor (RXR), and peroxisome proliferator-activated receptor (PPARα) to levels approximating those for human primary hepatocytes (Figure 2C). However, we did not detect expression of Cytochrome P450 3A4 (CYP3A4) or glucuronosyltransferase 1 family, polypeptide A1 (UGT1A1), as assessed by mRNA and protein expression (Figures 2C and 2D). To test if hepatocyte-directed differentiation could be enhanced by programmable expression of dCas9 and the gene activator (SAM), protein and mRNA expression of these two genes were assessed at the different stages of differentiation. Cells at the definitive endoderm stage and iHeps derived from human iPSCs-dCas9\textsuperscript{gain} showed highly inducible dCas9 and SAM-GFP expression and function after Dox exposure (Figures 2E, 2F, and S3).
Biofabrication of synthetic human liver tissue with programmable gene activation

After generating hepatocytes from human iPSCs-dCas9<sup>gain</sup>, we determined that generating human liver tissue would require reproducing other elements of the liver microenvironment.<sup>7,27,28</sup> Therefore, to biofabricate synthetic human liver tissue, human iPSCs-dCas9<sup>gain</sup>, human vascular endothelial cells, human fibroblasts, and human mesenchymal stem cells were populated onto 3D-printed constructs (Figures 4A and 4B). Although hepatocytes constitute most of the parenchymal space, supportive non-parenchymal cells (mesenchymal stromal cells, endothelial cells, and fibroblasts) enhance structural polarity and cellular stability.<sup>27,29–31</sup> iPSCs-dCas9<sup>gain</sup> (representing 85% of the cellular mass), human vascular endothelial cells (representing 10% of the cellular mass), human mesenchymal stem cells (hMSCs, representing 2.5% of the cellular mass), and human fibroblasts (representing 2.5% of the cellular mass) were seeded onto a 3D-printed construct based on the cellular content of a liver lobe.<sup>32</sup> Expression of CD31 for human endothelial cells, CD44 for human mesenchymal stem cells, and zSMA for human fibroblast was confirmed in the synthetic human liver<sup>gain</sup> tissue (Figure 4B). iHep<sup>gain</sup> showed inducible SAM-GFP expression under Dox exposure while both iPSCs-dCas9<sup>gain</sup> (Dox and +Dox) showed similar expression of RFP/gRNAs transduction (>90%) in the synthetic human liver<sup>gain</sup> tissues well (Figures 4C and S4A). Moreover, synthetic human liver<sup>gain</sup> expressed dCas9, CYP3A4, and UGT1A1 protein diffusely (Figures 4D and 4E). By applying a different format of 3D-multicellular approach, we corroborated CYP3A4 activity under Dox exposure and critical markers of cell-cell and cell-extracellular matrix contacts such as ZO-1 and ITGB1 were significantly improved when compared to 2D cell culture format (Figures 4D, S9, and S10), indicating that the biofabricated 3D synthetic human liver<sup>gain</sup> tissue was functionally programmable and expressed critical cellular communication molecules to a high degree.
DISCUSSION

Generating human liver tissue that incorporates elements from synthetic biology that allows augmentation of the cell’s genetic programs to change the tissue functional capabilities and behavior would not only provide an alternative strategy for the treatment of patients with terminal liver failure but also represent a powerful method to study genomic scale gain-of-function approaches to uncover biological mechanisms, as well as to enhance drug discovery/development. In this study, we demonstrated that human liver tissue
generated from human iPSCs genetically modified to express an inducible dCas9-based gene activation system is responsive and programmable to express mature and clinically relevant genes CYP3A4 and UGT1A1 under control of guide RNA delivery, thus, inducing post-translational activation and functions comparable to freshly isolated human primary hepatocytes.

By engineering human-induced pluripotent cells carrying an inducible dCas9-based activation system or CRISPRa, programmed guide gene expression exhibited a high degree of consistency, with nearly all resulting cells effectively targeted by guide RNAs. This is an important consideration when consistent and adult-level gene expression is desirable across a large population of cells and tissues. The guide RNA delivery method can be selected depending on the desired gene, timeline, and specificity of the study. In the present study and as a proof-of-principle, we use a lentiviral vector to guarantee high transduction efficiency in human cells. However, other delivery methods could be used efficiently such as lipid nanoparticles, adeno associate viruses, or fusion proteins.

Moreover, human-induced pluripotent cells are generated by forced expression of specific pluripotency transcription factors and they have the ability to proliferate infinitely and differentiate into cells of all three germ layers, and although a great deal of progress has been made investigating not only hepatocyte differentiation capacity of iPSCs but other cell types of the liver, cells that replicate the ability of human primary adult hepatocytes to metabolize drugs in vitro, regenerate livers, or to have advanced functions on demand has not been achieved. First, this human iPSC-CRISPRa-based system provides the advantage of upregulating endogenous genes that can be studied in both healthy and diseased genetic backgrounds, which could be a very valuable tool for drug discovery and preclinical programs. Second, the system goes beyond drug discovery or basic biology and could represent a reliable source for future liver bioengineering approaches that can be adapted to other organ systems. Clinical application of bioengineered liver tissue or grafts will require the generation of high numbers of iPS-Heps with functionality equal to that of primary human hepatocytes. As a proof-of-principle, we programmed the expression and function of two mature, clinically relevant liver-specific enzymes CYP3A4 and UGT1A1 which were not fully expressed using our conventional hepatocyte-directed differentiation protocol (Figure 2). Using this inducible dCas9-based activation system strategy, we were able to maintain the expression of hepatocyte-enriched transcription factors important for hepatocyte function, to induce expression of the clinically relevant enzymes CYP3A4 and UGT1A1, and most importantly to demonstrate metabolic activity at equal levels or higher than those observed in freshly isolated primary human adult hepatocytes. These human iPSCs-dCas9gain could be used to generate any cell type or tissue and programmed expression of one or many genes. Thus, the current approach represents a powerful tool that can be used to investigate signaling pathways and to generate human disease models as well. Furthermore, applications of human iPSCs-dCas9gain in positive and negative selection screens will enable the analysis of many kinds of genetic components, varying from protein-coding genes or non-coding RNA elements to even epigenetic modifiers in diverse biological processes.

Future programming of engineered human iPSCs carrying the dCas9 complex can further expand the dCas9 toolbox. Additional developments of the dCas9-based gene activation system may include improvement of features such as customizability of the gRNA scaffold sequence composition and stability or improvements in the potency of a new generation of gene activation systems such as the catalytic domain of the histone acetyltransferase CBP or SPH which combines components of SAM and other activators. An important concern for any dCas9-based technology is the specificity of the desired effect. It has been demonstrated that dCas9 binds promiscuously throughout the genome, causing potential off-target effects which is a critical concern for the preclinical and clinical application of the dCas9-based gene activation system and the engineered cell therapies. However, several encouraging strategies have been developed to mitigate any potential off-target responses, such as the sgRNA design optimization using computational methods, transcriptome analysis, and functional screening after dCas9 treatment. A recent CRISPR/Cas9 clinical application has demonstrated the safety of the system with no evidence of off-target editing in at least two patients.

Importantly, using custom-engineered human iPSCs and multicellular systems, human synthetic liver tissue was biofabricated that was able to be genetically programmed. While entire genetic programs that control the more than 500 hepatocyte functions represent a complex system and many genes and susceptibilities play important roles, this study was limited to the modification of only two clinically relevant genes. Future
human synthetic liver tissue prototypes should incorporate a greater number of programmable gene induction simultaneously by utilizing multiple gRNAs against many genes. Moreover, while the biofabricated human synthetic liver tissue was able to demonstrate cytochrome activity at the level of freshly isolated human hepatocytes, we were not able to document comparable inducibility after exposure to rifampicin (a prototypical CYP3A4 inducer). The lack of expression and function of other gene programs related to the capacity of cytochrome inducibility might be the major reason responsible for this failure. However, systems such as human iPSCs-Cas9<sup>gain</sup> can be used to interrogate metabolic inducibility and maturation by screening activators or co-activators of functional enzymes to design improved hepatic differentiation protocols so that hepatocytes express CYP3A4 and UGT1A1 naturally. In the current 3D cell culture formats, we observed increase in cell-cell and cell-matrix proteins, when compared to 2D cultures. However, the 3D spheroid multicellular system did not show additive effect on the gene functions targeted by the Dox-mediated gene activation (dCas9-SAM system) such CYP3A4 activity. Future experiments will explore the incorporation of other organotypic cell culture formats and the use of animal models or liver repopulation and regeneration to decipher critical gene circuits and programs.

Synthetic biology is poised to improve gene- and engineered-cell-based treatments for many diseases by providing precise control over the intensity, timing, and context of therapeutic intervention. The proof of principle experiments presented here used human iPSCs with controllable expression of the CRISPR-Cas9 guide RNA-directed synergistic activation mediator (SAM) which is capable of transcriptionally activating target endogenous genes to generate human liver tissue with advanced programmable functions. This strategy could be adapted to interrogate other pathways, molecules, cell types, and diseases. Future studies will optimize these tools for disease modeling within structured microphysiology systems (MPS) and organoid-MPS, including critical diseases impacting the liver. These studies outline a strategy for custom-engineering liver tissue for future biological, drug discovery/development, and potential clinical applications.

Limitations of the study

The present study demonstrated that synthetic human liver tissue with modifiable functional genetic programs can be generated. Importantly, using human iPSCs with target gene expression controlled by a guide RNA-directed CRISPR-Cas9 synergistic activation mediator (SAM), the fabricated synthetic human liver tissues is capable of transcriptionally activating target endogenous genes such as CYP3A4 and UGT1A1. While we demonstrated activity of both CYP3A4 and UGT1A1 proteins, this study was limited to the modification of only these two genes. Future human liver tissue prototypes should incorporate a greater number of entire pathways by incorporating several genes and transcriptional factors as gRNA-mini libraries. Moreover, while the biofabricated human synthetic human liver tissue was used to activate targeted endogenous genes, this study did not test the knockdown or knockout of genes. Thus, future developments will incorporate the generation of human iPSCs with CRISPR-Cas9-based toolbox to perform any type of gene regulation. Our study also had limitations in the generation of only one liver cell type—hepatocyte—and in the full hepatic metabolic profiling due to the non-fully mature hepatocytes produced from human iPSCs.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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**QUANTIFICATION AND STATISTICAL ANALYSIS**

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105503.

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**AUTHOR CONTRIBUTIONS**

A.S.-G., conceived and designed the study; K.M., R.M.F., N.H., T.M., R.D.-A., L.A.P.F., A.C.-H., Z.C., and L.V., performed data acquisition; R.M.F., N.H., K.M., C.F., T.M., R.D.-A., L.A.P.F., A.C.-H., L.V., K.T., and A.S.-G., analyzed and interpreted data; R.M.F., N.H., K.M., C.F., A.C.-H., I.J.F., and A.S.-G., established the human iPS cell lines used in this report and performed hepatic differentiation; assembly and characterization; K.M., R.M.F., N.H., L.V., D.L.T., and A.S.-G., analyzed functional and metabolic data; A.K.A., A.T., T.L., and L.K., designed and 3D-printed liver scaffolds; R.M.F., N.H., and A.S.-G., wrote the manuscript; K.M., R.M.F., N.H., T.M., R.D.-A., L.A.P.F., A.C.-H., L.V., A.K.A., A.T., T.L., K.T., D.L.T., I.J.F., and A.S.-G., participated in critical revision of the manuscript for intellectual content; A.S.-G., obtained funding. All authors contributed to the preparation of the manuscript.

**DECLARATION OF INTERESTS**

A.C.-H., K.T., I.J.F., and A.S.-G. are inventors on a provisional international patent application that describes hepatic differentiation of human pluripotent stem cells and liver repopulation (PCT/US2018/018032) and a provisional international patent application that describes the use of human-induced pluripotent stem cells for highly genetic engineering (PCT/US2017/044719). A.S.-G., J.G.-L., A.C.-H., and I.J.F. are co-founders and have a financial interest in Von Baer Wolff, Inc. a company focused on biofabrication of autologous human hepatocytes from stem cells technology. I.J.F. and A.S.-G. are co-founders and have a financial interest in Pittsburgh ReLiver Inc, a company focused on programming liver failure and their interests are managed by the Conflict-of-Interest Office at the University of Pittsburgh in accordance with their policies.

**INCLUSION AND DIVERSITY**

One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse anti-Nanog    | Cell Signaling | Cat#4893; RRID:AB_10548762 |
| Rabbit anti-Octamer-binding transcription factor 3/4 | Santa Cruz Biotechnology | Cat#sc-9081; RRID:AB_2167703 |
| Mouse anti-Stage-specific embryonic antigen-4 | BD Pharmingen | Cat#560173; RRID:AB_1645379 |
| Mouse anti-TRA-1-60 | BD Pharmingen | Cat#560218; RRID:AB_1645389 |
| Mouse anti-Cas9     | Millipore Sigma | Cat#MAC133; RRID:AB_2744694 |
| Goat anti-SRY-Box (SOX)17 | Invitrogen | Cat#180003; RRID:AB_86566 |
| Mouse anti-Hepatocyte Nuclear Factor 4 α | Abcam | Cat#ab41898; RRID:AB_732976 |
| Goat anti-Human Albumin | Bethyl | Cat#A80-229A; RRID:AB_67018 |
| Rabbit anti-CD31    | Abcam | Cat#ab32457; RRID:AB_726369 |
| Rabbit anti-CYP3A4  | Abcam | Cat#ab124921 |
| Rabbit anti-UGT1A1  | Abcam | Cat#ab237810 |
| Rabbit anti-UGT1A1  | Abcam | Cat#ab170858 |
| Anti-Human Otx2 NL557-Conjugated | R&D Systems | Cat#SC022 |
| Anti-Human SOX1 NL493-Conjugated | R&D Systems | Cat#SC022 |
| Anti-Human Brachyury NL557-Conjugated | R&D Systems | Cat#SC022 |
| Anti-Human HAND1 NL637-Conjugated | R&D Systems | Cat#SC022 |
| Anti-Human GATA-4 NL493-Conjugated | R&D Systems | Cat#SC022 |
| Anti-Human SOX17 NL637-Conjugated | R&D Systems | Cat#SC022 |
| Mouse anti-GAPDH   | Proteintech | Cat# 60004-1; RRID:AB_2107436 |
| Rabbit anti-ACTB    | Cell Signaling | Cat#4970; RRID:AB_2223172 |
| Mouse anti-CD44     | Abcam | Cat#ab6124; RRID:AB_305297 |
| Mouse anti-sMA      | Abcam | Cat#ab7817; RRID:AB_262054 |
| Rabbit anti-Cleaved Caspase-3 | Cell Signaling | Cat#9641; RRID:AB_2341188 |
| Mouse anti-ZO-1     | Invitrogen | Cat#33-9100; RRID:AB_2533147 |
| Rabbit anti-Integrin beta 1 | Abcam | Cat#179471; RRID:AB_2773020 |
| **Biological samples** | | |
| Human adult Hepatocytes | This paper | This paper |
| Human fetal hepatocytes | This paper | This paper |
| Human fibroblast | This paper | This paper |
| **Chemicals, peptides, and recombinant proteins** | | |
| Accutase          | STEMCELL | 7920 |
| Activin A         | R&D Systems | 338-AC |
| 1 X B27 without insulin supplement | Thermo Fisher Scientific | A1895601 |
| BMP4              | R&D Systems | 314-BP |
| CELLMAXX BOVINE ALBUMIN STEM Cell Grade, Low Free Fatty Acid | Thermo Fisher Scientific | 219989980 |
| Cholesterol       | Sigma Aldrich | 12531-018 |
| Collagen I, High Concentration, Rat Tail | Thermo Fisher Scientific | 354249 |

(Continued on next page)
| REAGENT or RESOURCE                               | SOURCE                  | IDENTIFIER |
|-------------------------------------------------|-------------------------|------------|
| Collagenase                                      | Sigma Aldrich           | C7657      |
| CTS KnockOut SR XenoFree Medium                  | Thermo Fisher Scientific | 12618-012  |
| Defined Lipid concentrate                        | Thermo Fisher Scientific | 11905-031  |
| DMEM, low glucose, pyruvate, HEPES               | Gibco                   | 12-320-032 |
| DMEM, high glucose                               | Gibco                   | 11-965-092 |
| DMEM/F12, HEPES                                  | Gibco                   | 11-330-057 |
| Dispase                                          | STEMCELL                | 7923       |
| Dexamethasone                                    | Sigma Aldrich           | D2915      |
| EGM-2MV SingleQuots Kit                          | Lonza                   | CC-3202    |
| Epidermal Growth Factor                          | R&D Systems             | 236-EG-200 |
| FGF 2 Human                                      | BD Biosciences          | 354060     |
| GABA                                             | Sigma Aldrich           | A2129      |
| Gentamicin                                       | Thermo Fisher Scientific | 15750060   |
| Glutamax                                         | Thermo Fisher Scientific | 35050061   |
| Goat Serum                                       | Abcam                   | ab-7481    |
| HCM Bullet Kit                                   | Lonza                   | CC-3198    |
| HCM SingleQuot Kit                               | Lonza                   | CC-4182    |
| Hepatocyte Growth Factor                         | R&D Systems             | 294-HG-250 |
| Hoechst 33342                                    | Sigma Aldrich           | B2261      |
| Holo Transferin                                  | CalBiochem              | 616424     |
| HyClone Fetal Bovine Serum (U.S.)                 | Thermo Fisher Scientific | SH3007103  |
| iCell Endothelial Cells                          | Cellular Dynamics, Fujifilm | #R1112     |
| Medium Supplement                                |                         |            |
| Insulin                                          | Sigma Aldrich           | I9278      |
| Insulin                                          | Sigma Aldrich           | 91077C     |
| L-Glutamine                                      | Sigma Aldrich           | G8540      |
| Linoleic Acid                                    | Sigma Aldrich           | L1376-500MG|
| Lithium Chloride                                 | Sigma Aldrich           | L4408      |
| L-pipecolic acid                                 | Sigma Aldrich           | P2519      |
| Matrigel hESC-Qualified Matrix, LDEV-Free        | Thermo Fisher Scientific | 354277     |
| Matrigel Membrane Matrix-Growth Factor Reduced   | Thermo Fisher Scientific | CB-40230A  |
| MEM Non-Essential Amino Acids Solution (100x)     | Thermo Fisher Scientific | 11140-050  |
| MEM Non-Essential Amino Acids Solution (100x)     | Millipore               | TMS-001-C  |
| Normal Donkey Serum                              | Abcam                   | ab-7475    |
| Oleic Acid                                       | Sigma Aldrich           | O1008      |
| Palmitic Acid                                    | Sigma Aldrich           | P0500      |
| Penicillin-Streptomycin (10,000 U/ml)             | Thermo Fisher Scientific | 15140-122  |
| Penicillin-Streptomycin 100x                     | Millipore               | TMS-AB2-C  |
| Recombinant Human Activin A                      | Thermo Fisher Scientific | 88518      |
| Recombinant Human Basic Fibroblast Growth Factors (bFGF) | Thermo Fisher Scientific | 354060     |
| Recombinant human basic Fibroblast Growth Factor | Lifeline Cell Technologies | 61977      |

(Continued on next page)
Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| Recombinant Human Epidermal Growth Factor (rhEGF) | Lifeline Cell Technologies | LS-1046 |
| Rifampicin | Sigma Aldrich | R3501 |
| RPMI1640, HEPES | Gibco | 22-400-105 |
| RT2 SYBR Green ROX qPCR Mastermix | Qiagen | 330500 |
| TaqMan Fast Advanced Master Mix | Thermo Fisher Scientific | 4444557 |
| Trace Element B | Coming | 25-022-CI |
| Trace Element C | Coming | 25-023-CI |
| Transforming Growth Factor-β1 | Millipore | GF111 |
| Trypsin-EDTA (0.05%), phenol red | Sigma Aldrich | 25300054 |
| Urso deoxycolic acid | Sigma Aldrich | US127 |
| 7-hydroxywarfarin | Cayman | 9000672 |

Critical commercial assays

| RT² Profiler™ PCR Array Human Drug Metabolism (PAHS-002ZC-6) | Qiagen | Cat#PAHS-002Z |
| RT² Profiler™ PCR Array Human Drug Metabolism: Phase II Enzymes (PAHS-069Z-6) | Qiagen | Cat#PAHS-069Z |
| P450-GloTM CYP3A4 Assay (Luciferin-IPA) | Promega Corporation | Cat#V9001 |
| RNasy mini kit | Qiagen | Cat#74104 |
| SuperScript III First-Strand Synthesis System | Thermo Fisher Scientific | Cat#18080-051 |

Experimental models: Cell lines

| Adipose-Derived mesenchymal Stem Cells, Normal, Human | American Type Culture Collection (ATCC) | PCS-600-011 |
| Human neonatal microvascular endothelial cells | Lonza | CC-2543 |
| HEK293-Cas9 | American Type Culture Collection (ATCC) | CRL-1573Cas9 |

Oligonucleotides

| Octamer-binding transcription factor 4 | Life Technologies | Hs04260367_gH |
| Nanog homebox | Life Technologies | Hs02387400_g1 |
| c-Myc | Life Technologies | Hs00153408_m1 |
| Lin-28 homolog A | Life Technologies | Hs00702808_s1 |
| SRY-Box 2 | Life Technologies | Hs01053049_s1 |
| C-X-C chemokine receptor type 4 | Life Technologies | Hs00607978_s1 |
| Forkhead box protein A1 | Life Technologies | Hs04187555_m1 |
| Forkhead box protein A2 | Life Technologies | Hs00232764_m1 |
| Hepatocyte nuclear factor 1 alpha | Life Technologies | Hs0167041_m1 |
| Other Oligonucleotides | See Table S1 | N/A |

Recombinant DNA

| pCXLE-EGFP | (Okita et al., 2011) | Addgene, Cat#27082 |
| pCXLE-hOCT3/4-shp53-F | (Okita et al., 2011) | Addgene, Cat#27077 |
| pCXLE-hSK | (Okita et al., 2011) | Addgene, Cat#27078 |
| pCXLE-hUL | (Okita et al., 2011) | Addgene, Cat#27080 |
Continued

**REAGENT or RESOURCE** | **SOURCE** | **IDENTIFIER**
--- | --- | ---
Software and algorithms |  |  
Amaxa 4D-Nucleofector | Lonza | N/A
Image J areas quantification and Gel analysis tool | NIH | https://imagej.nih.gov/ij/
Image Lab | Bio-Rad | www.bio-rad.com/
NanoDrop | Thermo Scientific | assets.fishersci.com/
NIOS-Elements AR | Nikon | www.nikoninstruments.com/
Prism 6 | GraphPad | www.graphpad.com/
StepOnePlus system | Applied Biosystems | www.thermofisher.com/
TSQ Quantum Ultra Mass Spectrometer | Thermo Scientific | www.thermofisher.com/
Other |  |  
Haemobricks | Cellbricks | https://cellbricks.com/pages/haemobrick

**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Alejandro Soto-Gutierrez, MD, PhD (als208@pitt.edu).

**Material availability**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact.

**Data and code availability**

All data produced in this study are included in the published article and its supplementary information, or are available from the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Primary human fetal and adult hepatocytes**

De-identified tissues were obtained from Magee Women’s Hospital (Pittsburgh, PA) and the University of Washington Department of Pediatrics, Division of Genetic Medicine, Laboratory of Developmental Biology (Seattle, WA) after obtaining a written informed consent by a protocol approved by the Human Research Review Committee of the University of Pittsburgh (Honest broker approval number HB015 and HB000836). Human fetal hepatocytes were isolated using EMEM (Lonza, Walkersville, MD) containing 0.5 mg/mL of collagenase (Type XI, Sigma Aldrich, Saint-Louis MO, Cat. #C7657) on a lab shaker for 40 min and cultured with a DMEM medium (GIBCO, Life Technologies, Carlsbad, CA, USA) containing 1X Penicillin/Streptomycin (ThermoFisher Scientific, Waltham, MA), \(10^{-7}\) M of insulin (Sigma-Aldrich, Saint-Louis, MO), and 5% bovine serum albumin (GIBCO, Life Technologies, Carlsbad, CA, USA). The de-identified normal human liver cells were obtained through the Liver Tissue Cell Distribution System (Pittsburgh, PA) after obtaining written informed consent. The protocol was approved by the Human Research Review Committee at the University of Pittsburgh funded by NIH Contract # HSN276201200017C. Adult human hepatocytes were also obtained from the Human Synthetic Liver Biology Core from the Pittsburgh Liver Research Center, University of Pittsburgh. The Institutional Review Board of the University of Pittsburgh has determined that the human liver cell isolation protocol employed in this study is exempt from further review. Human hepatocytes were also procured from Ira J Fox Laboratory at Children’s Hospital of UPMC, after obtaining written informed consent by a protocol approved by the Human Research Review Committee and the Institutional Review Board (IRB#: PRO12090466) at the University of Pittsburgh. Specific information on age, gender, and cell viability of human liver tissue and hepatocytes used in this study is described in Table S2.
METHOD DETAILS

Production of high titer lentivirus

Lentiviral Tet-On system plcVi(3G) was produced by Sirion-Biotech. Briefly, lentiviral HIV-based, VSVG pseudo typed, self-inactivating high titer stocks were produced by co-transfection of 1x10^6 HEK293T cells with lentiviral packaging plasmids pRev, pMDL-GAG, and pMD2G and either the plcVi(3G)-SAM or plcVi(3G)-dCas9-VP64 lentiviral vectors. 48h after transfection, lentiviral particles in the culture medium were concentrated by precipitation with polyethylene glycol and dissolved in 400 μL DMEM + 10% FCS. The biological titration of each lentivirus was performed through Lenti X qRT-PCR Titration kit (Clontech). This assay measures the number of packaged lentiviral vector genomes of viral stocks. Each lentivirus yields >1 x 10^8 viral particles. Each stock was preserved at -80°C.

Lentiviral transduction of human fetal fibroblasts

Primary human fetal fibroblasts were isolated by digesting the tissue in EMEM (Lonza, Walkersville, MD) containing 0.5 mg/mL of collagenase (Type XI, SigmaAldrich, Saint-Louis MO, Cat. #C7657) on a lab shaker for 40 min. Viability was assessed by trypan blue exclusion test and was routinely >85%. Fetal fibroblasts were plated at a density of 1.3x10^5 cells/cm^2 on type I rat tail collagen-coated 12 well plates (Corning, Corning, NY). Cells were cultured and passaged 2 times to get a 100% pure population of human fetal fibroblasts, with a DMEM medium (GIBCO, Life Technologies, Carlsbad, CA, USA) containing 1X Penicillin/Streptomycin (ThermoFisher Scientific, Waltham, MA), 10^-7 M of insulin (Sigma-Aldrich, Saint-Louis, MO), and 5% bovine serum albumin (GIBCO, Life Technologies, Carlsbad, CA, USA). Human Fetal Fibroblasts were transduced with plcVi(3G)-dCas9-VP64 particles at an MOI of 15. The transduced cells were selected 72h after transduction with 200 μg/mL of neomycin for 21 days to generate a stable pool. The stable integration of cLVi(3G) lentiviral vectors in human fetal fibroblasts was confirmed by measuring the expression of the neomycin antibiotic cassette gene by qRT-PCR. The process was repeated using the plcVi(3G)-SAM and the transduced cells were selected with 0.5 μg/mL of puromycin. Transduction efficiency after the selection was measured by the expression of the puromycin antibiotic cassette gene by qRT-PCR. The cell pool generated was tested for the efficiency of the Tet-On-3G system by adding 1 μg/mL of doxycycline to the cells for 48h and checking GFP expression by live fluorescence. The dCas9-VP64 and the Synergistic Activation Mediator (SAM) expression were also measured by qRT-PCR, Western Blot, and immunostaining. Quality control tests included viability, sterility (with CASO-Bouillion, Heipha), and mycoplasma testing (Lonza, Walkersville, MD).

Generation and culture of human iPSC lines

The iPSC line used was generated from human fetal fibroblast transduced with both constructions as above mentioned. Reprogramming of fetal fibroblasts was performed using episomal plasmids vectors adapted from a previously described protocol. Briefly, for each nucleofection, 1 million cells were resuspended in 100 μL of the AmaxaTM NHDF Nucleofector kit (Lonza, Walkersville, MD), containing 3 μg of each of the four episomal plasmids vectors encoding OCT3/4 and p53 shRNA, SOX2 and KLF4, L-MYC and LIN28, and enhanced green fluorescent protein (eGFP) (Addgene, Boston, MA, USA). Cells were nucleofected using the Amaxa 4D-Nucleofector (Lonza, Walkersville, MD) and plated in mTeSR on human embryonic stem cell–qualified Matrigel (Corning, New York, NW)-coated plates. 2 hiPSC-dCas9^9iam^ colonies were isolated around 20-25 days after induction based on morphology. The presence of Tet-On-3G systems was tested in both clone cells by measuring the puromycin and neomycin antibiotic selection cassette gene expression by qPCR. Both clones were tested for their ability to induce the Tet-On-3G system by adding 1 μg/mL doxycycline and measuring either dCas9 expression by qRT-PCR and GFP expression by live fluorescence. One clone was selected, expanded for 10 passages, and tested to test the long-term efficiency of Tet-On-3G systems after multiple passages. The line was karyotyped, and pluripotency was validated by expression of NANOG, OCT4, and membrane markers SSEA and TRA160 at different passages and regularly tested negative for mycoplasma contamination.

Embryoid body formation

Embryoid bodies (EBs) cells were prepared by plating Accutase (StemCell Technologies, Vancouver, Canada) passaged iPSC cells at a density of 1 to 5x10^3 cells per cm^2 on low attachment Petri dishes for 48 hours in mTeSR. To induce differentiation, iPSC cell suspensions were subsequently incubated with mTeSR supplemented with 20% FBS. EBs started to form in suspension after one week of culture. On day 20, EBs were fixed in 4% paraformaldehyde for 12 h and 70% ethanol overnight at 4°C and then embedded in paraffin,
cut sections (5-7 micron) were mounted on glass slides, for immunofluorescence using Human Three Germ Layer 3-Color Immunocytochemistry Kit (R&D Systems, Minneapolis, MN).

**Cell culture**

HEK293-dCas9 cells were obtained from ATCC, maintained in DMEM medium (GIBCO, Life Technologies, Carlsbad, CA, USA) supplemented with 10% HyClone fetal bovine serum (ThermoFisher Scientific, Waltham, MA), 1% Penicillin/Streptomycin (ThermoFisher Scientific, Waltham, MA) and kept at 37°C in 5% CO₂. Human fibroblasts were cultured in DMEM/F12 medium (GIBCO, Life Technologies, Carlsbad, CA, USA) supplemented with 10% HyClone fetal bovine serum (ThermoFisher Scientific, Waltham, MA), 1% Penicillin/Streptomycin (ThermoFisher Scientific, Waltham, MA), bFGF (BD, Franklin Lakes, NJ), Insulin (Sigma-Aldrich, Saint Louis, MO) and EGF (BD, Franklin Lakes, NJ) and kept at 37°C in 5% CO₂. Human mesenchymal stem cells were used following the Mesenchymal Stem Cell Growth Kit for Adipose and Umbilical-derived MSCs - Low Serum instructions (ATCC). The human vascular endothelial cells were culture using EGM™-2 Endothelial Cell Growth Medium-2 BulletKit™ (Lonza, Walkersville, MD).

**Differentiation of human iPSCs into hepatocytes (iHepGain)**

Our hepatocyte differentiation protocol is summarized in Figure 2A. Human iPSCs were passaged with Accutase (StemCell Technologies, Vancouver, Canada) and re-plated at a density of 1 to 2x10⁷/cm² at growth factor reduced Matrigel (Corning Incorporated, Corning, NY) coated plates with mTeSR. The day after, cells were exposed to a defined differentiation medium containing RPMI (Invitrogen, Carlsbad, CA), 1X B-27 w/o insulin supplement (Invitrogen, Carlsbad, CA), 0.5% Penicillin/Streptomycin (Millipore, Billerica, MA), 0.5% of Non-Essential Amino Acids (Millipore, Billerica, MA), 100 ng/mL Activin A (R&D Systems, Minneapolis, MN), 10 ng/mL BMP4 (R&D Systems, Minneapolis, MN) and 20 ng/mL FGF2 (BD, Franklin Lakes, NJ) for two days and placed in a normal O₂ incubator (Stage 1, endoderm induction). Cells were subsequently maintained in a similar medium without FGF2 and BMP4 for two days in ambient O₂/5% CO₂ incubator (Stage 2, definitive endoderm). Cells were then grown for 10 days in a defined medium containing 45% DMEM low glucose 1g/l (ThermoFisher Scientific, Waltham, MA), 45% F-12 (ThermoFisher Scientific, Waltham, MA), 10% CTS KnockOut SR XenoFree Medium (ThermoFisher Scientific, Waltham, MA), 0.5% Non-Essential Amino Acids (ThermoFisher Scientific, Waltham, MA), 0.5% L-glutamine (ThermoFisher Scientific, Waltham, MA), 50 ng/mL HGF (R&D Systems, Minneapolis, MN) and 1% DMSO (Sigma-Aldrich, Saint Louis, MO), medium was changed every other day (Stage 3, hepatic specification). At the end of Stage 3, cells were detached and re-plated at a 30%-40% confluence in 3D sandwich culture for further maturation.

**dCas9Gain activation and gRNA delivery in the iHepGain**

At the end of Stage 3 and the re-plating, cells were grown in a defined medium containing 45% DMEM low glucose 1 g/l (ThermoFisher Scientific, Waltham, MA), 45% F-12 (ThermoFisher Scientific, Waltham, MA), 10% CTS KnockOut SR XenoFree Medium, 0.5% Non-Essential Amino Acids (ThermoFisher Scientific, Waltham, MA), 0.5% L-glutamine (ThermoFisher Scientific, Waltham, MA), 0.1% of Gentamicin/Amphotericin-B (ThermoFisher Scientific, Waltham, MA), 1% of Penicillin/Streptomycin (ThermoFisher Scientific, Waltham, MA), 50 ng/mL HGF (R&D Systems, Minneapolis, MN), 1% DMSO, 0.5 µM Dexamethasone (Sigma-Aldrich, Saint Louis, MO), 0.1% of Bovine Serum Albumin Free of Fatty Acids, 0.1% of Hydrocortisone, 0.1% of Transferrin, 0.1% of Insulin (HCM Bullet Kit, ThermoFisher Scientific, Waltham, MA), 100 µM of Ursodeoxyxylic acid (Sigma-Aldrich, Saint Louis, MO), 20 µM of Palmitic Acid (Sigma-Aldrich, Saint Louis, MO), 30 µM of Oleic Acid (Sigma-Aldrich, Saint Louis, MO), 1X of Cholesterol (ThermoFisher Scientific, Waltham, MA) and with or without 20 µM of Rifampicin (Sigma-Aldrich, Saint Louis, Missouri), accordingly with the experiment (Stage 4, hepatic maturation). At this time the cells were exposed to 1 µg/mL of doxycycline for the dCas9Gain activation. In the following day the cells were transduced with a gRNA pool delivered by lentivirus for the activation of CYP3A4 (gRNA1: 5’-TGGAA GAGGCTTCTCCACCT-3’ and gRNA2: 5’-ACTCAAGGAGGTCAGTGAG-3’) or UGT1A1 (gRNA1: 5’-TGAACCTCCTGCATCCTGTGA-3’ or UGT1A1 (gRNA1: 5’-TGAACCTCCTGCATCCTGTGA-3’; gRNA2: 5’-ATAGGCAACACAGTTGAAC-3’ and gRNA3: 5’-ACTTTCA GAGATAAAAGAGG-3’) (Vector Builder, Chicago, IL). To enhance the transduction efficiency 8 µg/mL of Sequabrene (Sigma-Aldrich, Saint Louis, Missouri) was added in the medium and the cells were centrifuged at 300g for 1 hour at 37°C. All the construction carries an RFP protein to check transducer efficacy and an empty construction has been used as a control in some experiments (gRNA-RFP). On the following day, the medium was replaced for fresh Stage 4 medium and 48 hours later, samples were collected for further analysis or seeded into the Haemobricks (Cellbricks, Berlin, Germany) to make the Human synthetic liverGain tissue.
Quantitative real-time PCR
Total RNA was isolated from human cells using RNeasy Mini kits (QIAGEN, Hilden, Germany) and reverse transcribed using SuperScript III (Invitrogen, Carlsbad, CA) following the manufacturers’ instructions. We performed qPCR with a StepOnePlus system (Applied Biosystems, Foster City, CA) using TaqMan Fast Advanced Master Mix (Life Technologies, Waltham, MA). The probes used are listed in the key resources table and Table S1. Relative gene expression was normalized to β-actin (ACTB) mRNA. Relative expression was calculated using the ΔΔCT method.

Western blot
All the samples were incubated with RIPA lysis buffer (Sigma-Aldrich, Saint Louis, Missouri), 1x Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA) and incubated for 30 minutes at 4 °C. Samples were centrifuged at 13,000g for 10 minutes at 4 °C. The supernatant from each sample was then transferred to a new microfuge tube and was used as the whole cell lysate. Protein concentrations were determined by comparison with a known concentration of bovine serum albumin using a Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA). 30 μg of lysate were loaded per lane into 10% MiniPROTEAN TGX™ gel (BioRad). Next, proteins were transferred onto PVDF Transfer Membrane (Thermo Fisher Scientific, Waltham, MA). Membranes were incubated with a primary antibody solution overnight and then washed. Membranes were incubated for 1 hour in secondary antibody solution and then washed. Target antigens were finally detected using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA). Images were scanned and analyzed using ImageJ software. The antibodies used are listed in the key resources table.

Immunofluorescence
Samples were washed once with PBS, fixed with 4% PFA for 15 minutes, and washed another 3 times with PBS. Samples were washed 3 times with wash buffer (PBS, 0.1% BSA, and 0.1% Tween 20) for 5 minutes and then blocked and permeabilized in blocking buffer (PBS, 10% normal donkey or goat serum, 1% BSA, 0.1% Tween 20, and 0.1% Triton X-100) for 1 hour at room temperature. Samples were then incubated with primary antibody in blocking buffer overnight at 4 °C. Samples were washed 3 times with wash buffer for 5 minutes and incubated with secondary antibody in blocking buffer for 1 hour in the dark at room temperature. Then, the samples were washed 3 times with a wash buffer for 5 minutes, 3 times with PBS, and then counterstained with 1 μg/mL of DAPI (Sigma Aldrich) for 1 minute at room temperature in the dark. Samples were washed 3 times with PBS and stored in the dark at 4 °C. Antibodies specific for antigens were acquired for immunofluorescence and list of the antibodies used can be found in key resources table. Pictures were taken using Nikon Inverted Research Fluorescence Microscope ECLIPSE Ti. Images were subsequently analyzed with ImageJ software, by generating RGB stack, pre-processed to equalize the illumination within the stack, thresholded and measured.

Transcription profiling by RT² profiler PCR array
RNA from Human iHep® transduced with the gRNA-CYP3A4 pool exposed and no exposed to doxycycline were isolated with RNeasy Mini kits (QIAGEN, Hilden, Germany) and reverse transcribed using SuperScript III (Invitrogen, Carlsbad, CA) following the manufacturers’ instructions. 84 key genes involved in the drug metabolism process were simultaneously assayed with the RT² Profiler PCR Array Human Drug Metabolism (PAHS-002ZC-6) (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions and analyzed with the Data Analysis Center (QIAGEN Hilden, Germany). The list of 84 genes analyzed can be found in Table S3.

CYP3A4 functional analysis
CYP3A4 activity was measured using P450-Glo CYP3A4 Assay (Luciferin-IPA) (Promega Corporation, Madison, WI, USA, Cat# V9001) according to the manufacturer’s instructions. For induction of CYP3A4 the Human iHep® was exposed to 20 μM of Rifampicin (Sigma-Aldrich, Saint Louis, Missouri) for 48 hours on the day after the gRNA-CYP3A4 lentivirus transduction. Adult human hepatocytes (HAH) were used as controls.

UGT1A1 functional analysis
The UGT1A1 gene activation was determined by mass spectroscopy. Briefly, the culture medium was supplemented with 10 μM 7-hydroxycoumarin (Toronto Research Chemicals, Toronto, ON) for 0–48 hours. A 40 μL aliquot of the medium was collected prior to treatment and then at 6, 24, and 48 hours of treatment.
The aliquots were extracted for 7-hydroxywarfarin and 7-hydroxywarfarin glucuronide by the addition of 80 µL acetonitrile followed by 1 min of vortexing. A protein-free supernatant was collected by centrifugation in a Labnet MK-2 refrigerated centrifuge (14,000 RPM, 4°C, 5 min). The protein-free supernatant was further diluted 1:10 into acetonitrile/H2O (20/80 v/v) for injection into the mass spectrometer. Detection was achieved in the positive mode with a ThermoFisher TSQ Quantum Ultra Mass Spectrometer, interfaced via electrospray ionization (ESI) probe with the Waters UPLC Acquity solvent delivery system. Transitions used for analysis, peak identification, and quantification of the test samples were determined by infusion and injection of pure 7-Hydroxywarfarin and the 7-Hydroxywarfarin-glucuronide (Santa Cruz Biotechnology, Dallas, TX) standards prior to sample testing. Human adult hepatocytes were used as control.

Human synthetic livergain tissue
The day after the gRNA-CYP3A4 or gRNA-UGT1A1 lentivirus transduction, the iHepgain were detached and seeded into the Haemobricks (Cellbricks, Berlin, Germany) to mimic a liver tissue (representing 85%). Other support cells were also seeded into the 3D model: human vascular endothelial cells (representing 10%), human mesenchymal stem cells (representing 2.5%), and human fibroblast (representing 2.5%). 48 hours after the cell seeding, the Human synthetic livergain tissue was fixed in 4% paraformaldehyde for 12 h and 70% ethanol overnight at 4°C, and then immunolabeled. For the CYP3A4 functional analysis, 20 µM of Rifampicin (Sigma-Aldrich, Saint Louis, Missouri) was added in the medium in the day of cell seeding. After 48 hours, CYP3A4 activity was measured as previously described. To generate the 3D multicellular system, 0.25x10⁶ human iHeps were cultured in a 24-wells low attachment plate in the presence of the LV-gRNA-CYP3A4 for 5 hours. The human vascular cells, human fibroblast, and human mesenchymal stem cells were added to the human iHepgain. The system was exposed to Dox and after 4 days samples were collected for CYP3A4 protein expression and activity and fixed with 4% paraformaldehyde for 12 h and 70% ethanol overnight at 4°C. ZO-1 and ITGB1 were stained in the 3D multicellular system and human adult liver tissue.

QUANTIFICATION AND STATISTICAL ANALYSIS
Data from at least three sets of samples were used for statistical analysis. All statistical analyses were performed with Prism 6.0 Software (GraphPad Software, La Jolla, CA). All experiments were independently performed three times in triplicate. Due to the relatively small sample size, normality testing was not feasible. Data are expressed as mean ± standard deviation (SD) and were compared using an analysis of the Wilcoxon test. Values of p < 0.05 were considered statistically significant.