Stem Cell Reports

Supplemental Information

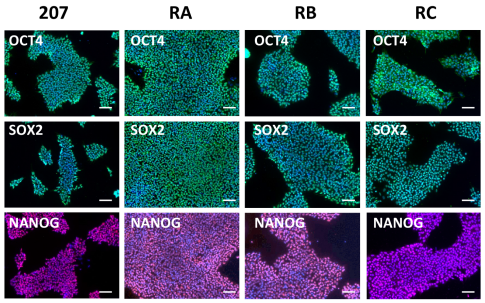
Small-Molecule-Driven Hepatocyte Differentiation of Human Pluripotent Stem Cells

Richard Siller, Sebastian Greenhough, Elena Naumovska, and Gareth J. Sullivan

Supplemental Data

Figure S1. Related to Figure 1. (Sullivan)

A



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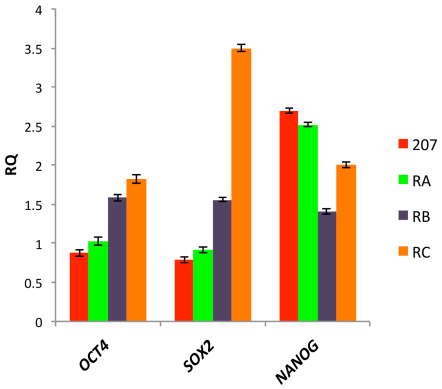


C

1 Day	1 Day	5 Days	10 Days
RPMI-B27 +/- INSULIN + 3-4 μM CHIR99021	RPMI-B27	KOSR + 1% DMSO	L-15 + 100 nM Dihexa + 100 nM DEX

D

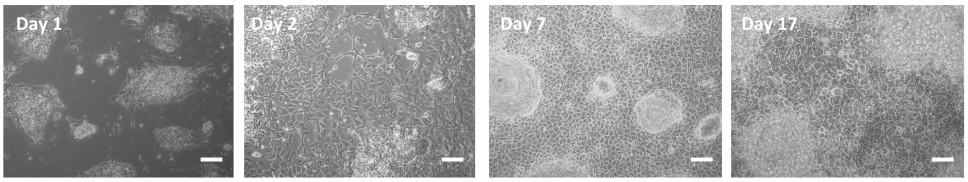
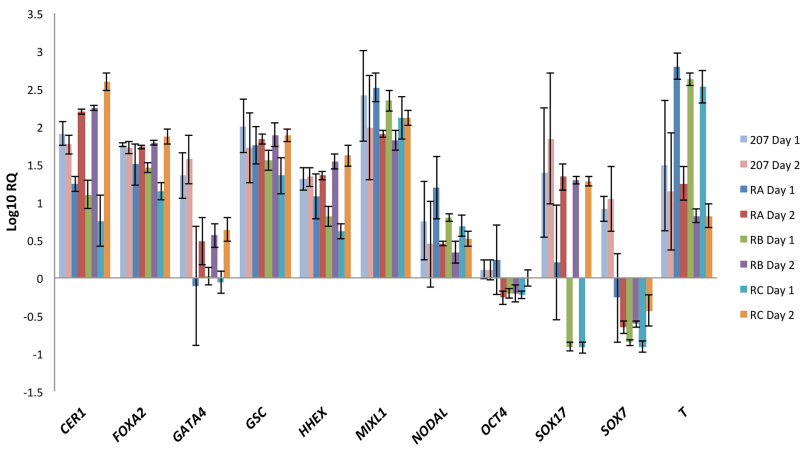


Figure S2. Related to Figure 2. (Sullivan)

A



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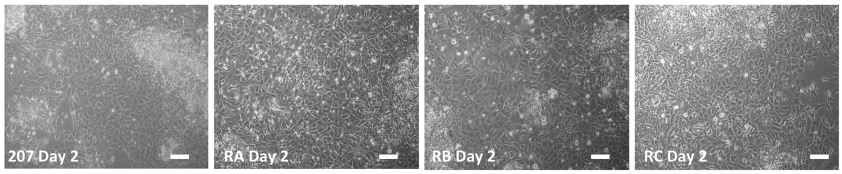

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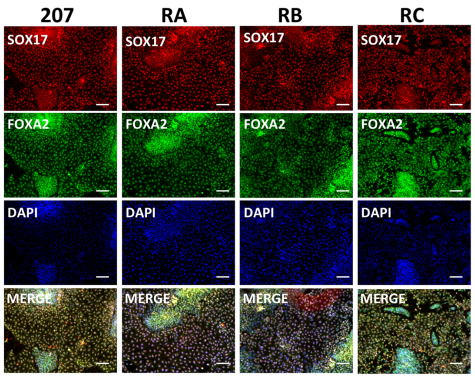


D

Line	FOXA2	SOX17
207	81.9% +/- 8.7	82.8% +/- 7.4
RA	84.2% +/- 7.2	81.3% +/- 6.9
RB	82.1% +/- 8.0	82.9% +/- 6.8
RC	82.2% +/- 5.1	79.9% +/- 7.8

Figure S3. Related to Figure 3. (Sullivan)

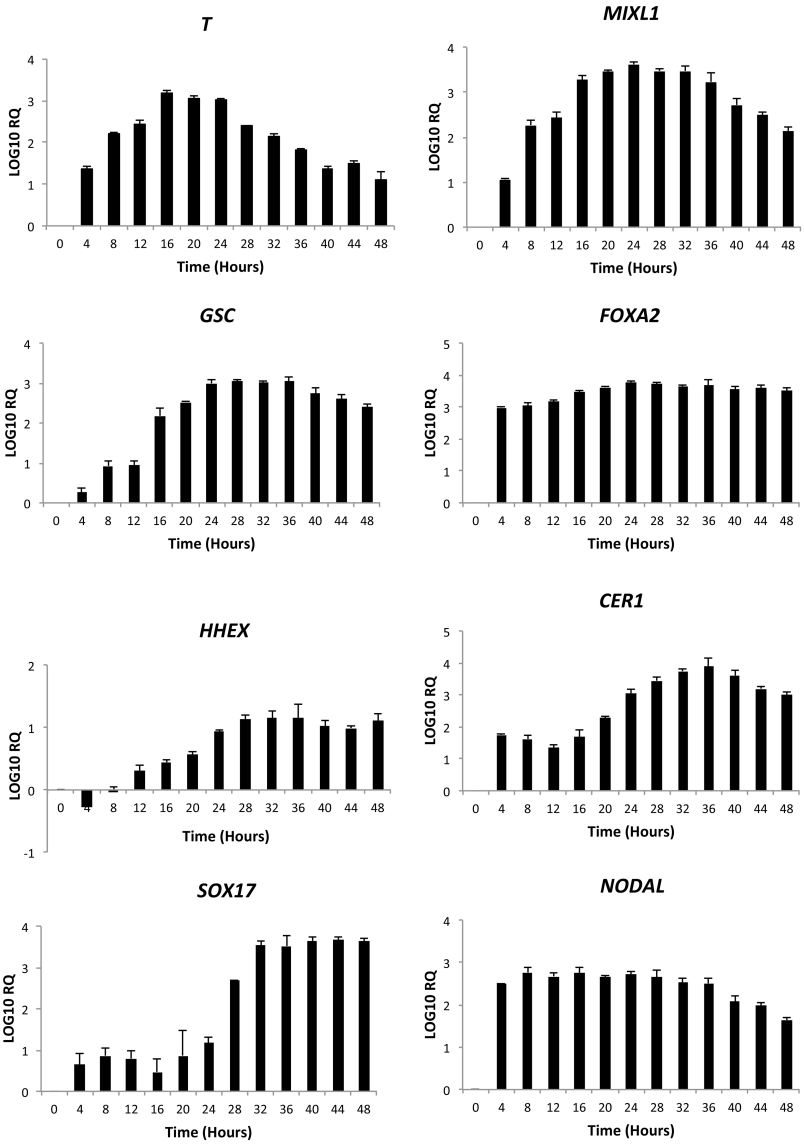
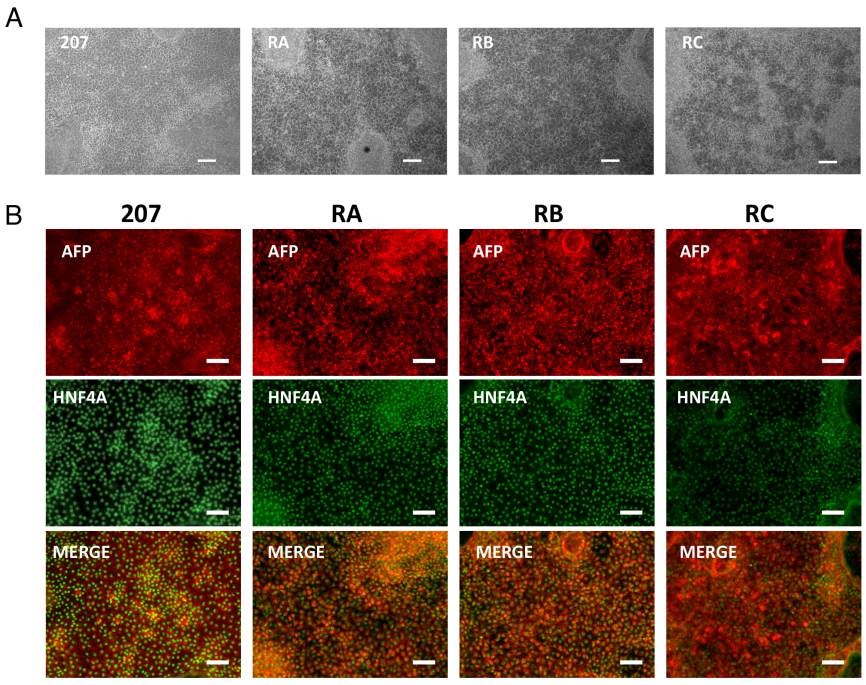


Figure S4. Related to Figure 4. (Sullivan)



C

Line	HNF4A / AFP
207	89.2% +/- 2.4
RA	86.1% +/- 3.6
RB	87.8% +/- 3.1
RC	88.7% +/- 2.7

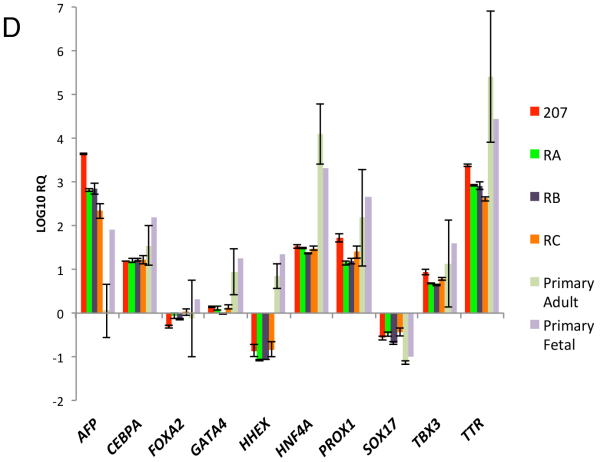


Figure S5. Related to Figure 5. (Sullivan)

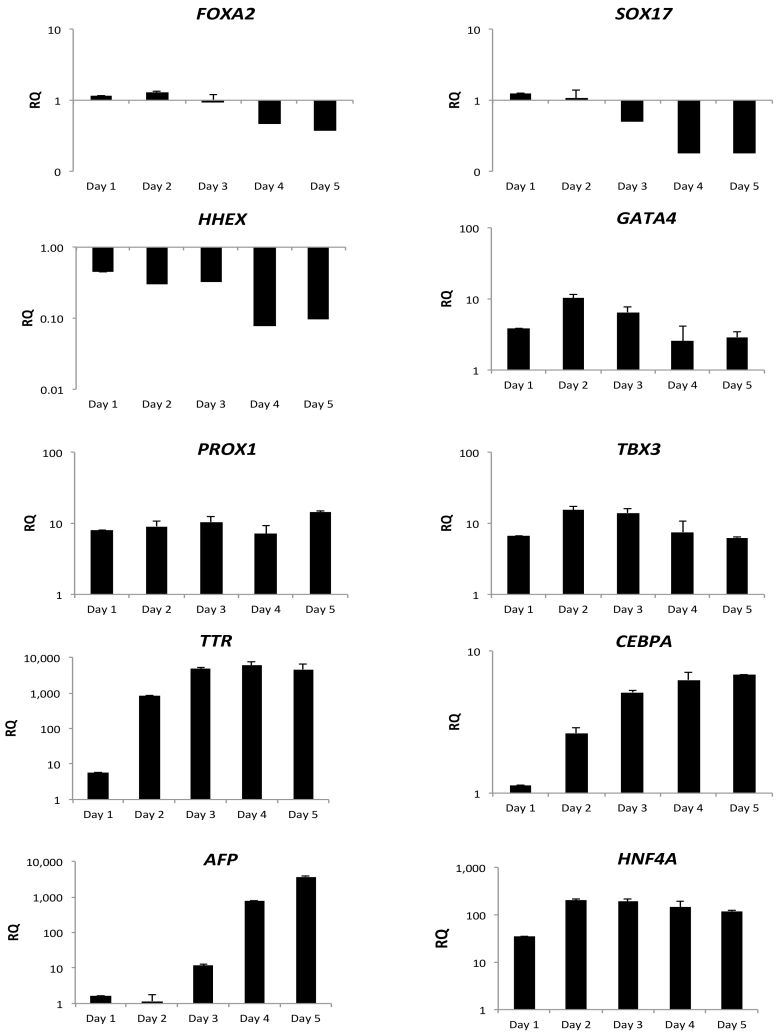


Figure S6. Related to Figure 6. (Sullivan)

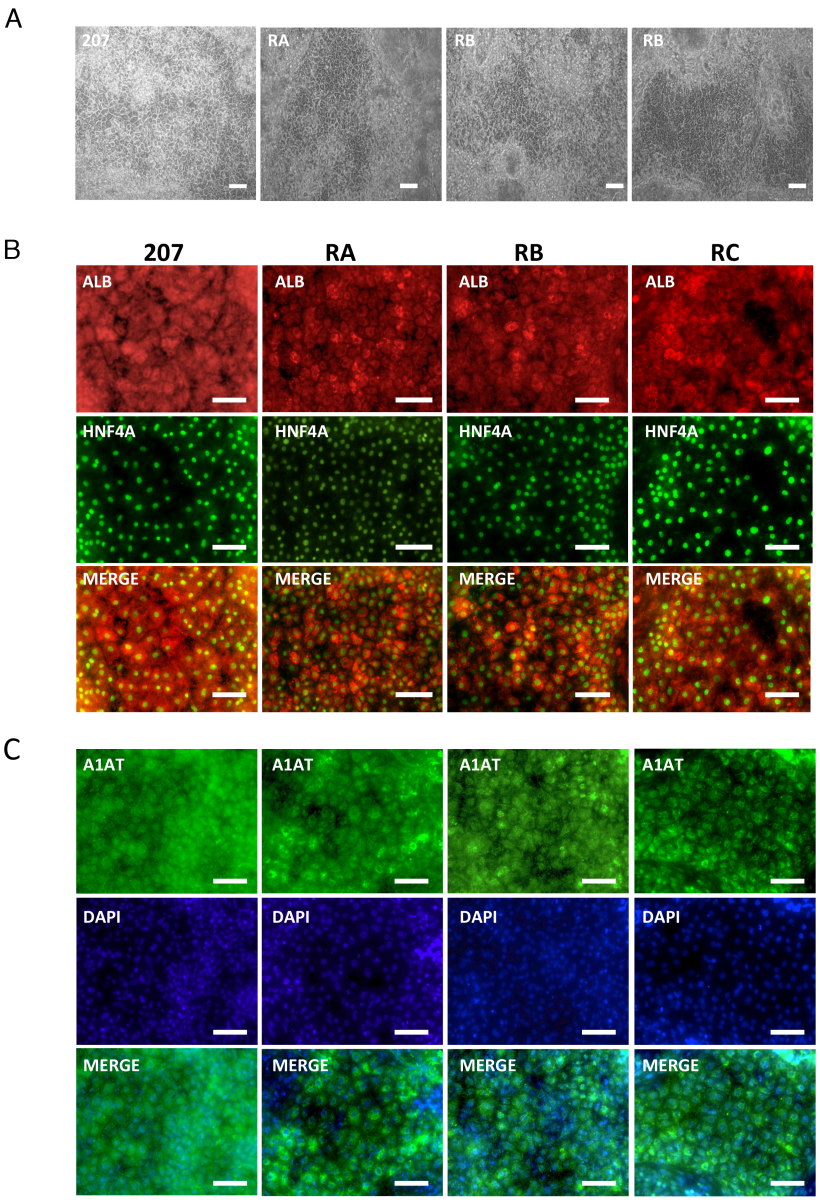


Figure S7. Related to Figure 7. (Sullivan)

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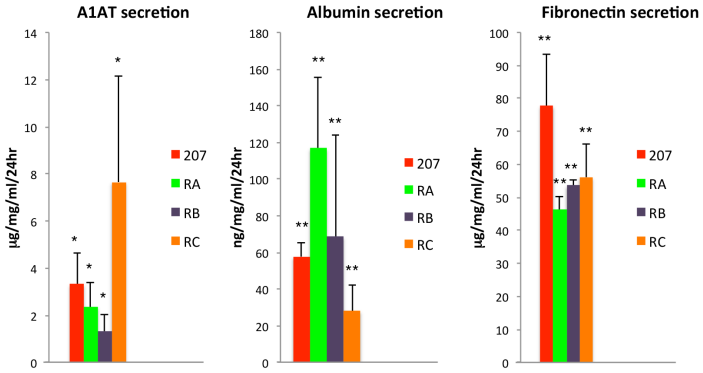

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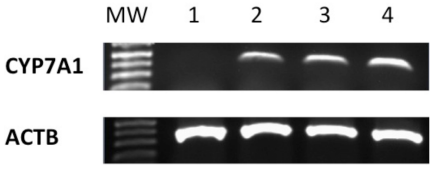

C

Line	ALB / HNF4	A1AT
207	79.4% +/- 7.2	91.9% +/- 2.8
RA	72.1% +/- 6.9	90.1% +/- 1.4
RB	73.8% +/- 7.9	92.7% +/- 2.9
RC	75.9% +/- 8.3	88.7% +/- 4.6

D

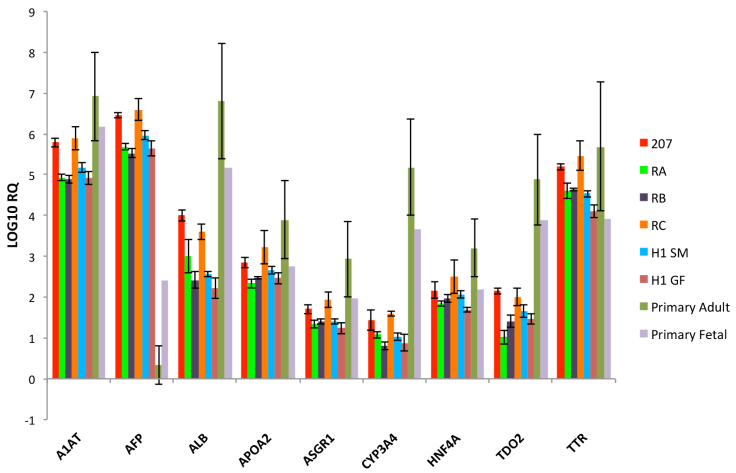

Figure S1. hiPSC Characterisation and differentiation scheme. A) Expression of OCT4, SOX2, and NANOG, imaged using fluorescent microscopy. Cell line identity is indicated at the head of each column. Scale bars = 100 μm. B) Gene expression for key pluripotency genes *OCT4* (*POU5F1*), *SOX2*, and *NANOG* by RT-qPCR, normalised to H1 undifferentiated control. C) Schematic of the small molecule differentiation process on other pluripotent stem cell lines showing optimisation of CHIR99021 concentrations and base media compositions (+/-INS). D) Representative images of morphology throughout the differentiation procedure on Detroit hiPSC lines. Imaged using phase contrast microscopy. Scale bars = 100 μm.

Figure S2. Characterisation of Phase I differentiation on multiple pluripotent lines. A) Gene expression changes during Phase I of differentiation assessed by RT-qPCR. All lines were differentiated using RPMI / B27 (minus insulin) supplemented with 4 μM CHIR99021. Cells were collected at days 1 and 2 and analysed by TaqMan. Data presented as the mean of 3 independent experiments; error bars represent standard deviation. B) Typical morphology of small molecule derived definitive endoderm end points taken using phase contrast microscopy (10x). Line identity located bottom right corner of each plate. Scale bars = 100 μm. C) Expression of FOXA2 and SOX17 at Phase I endpoint after treatment with CHIR99021 imaged using fluorescent microscopy. Line identity located at the head of each plate. Scale bars = 100 μm. D) Efficiency of Phase I differentiation for each line was determined by counting FOXA2 positive cells and SOX17 positive cells. Efficiencies are presented as the percentage of positive cells plus or minus the standard deviation of all fields counted.

Figure S3. Phase I 48 hour time course to assess transcriptional developmental trajectory. The human induced pluripotent stem cell line Detroit RA was differentiated in RPMI-B27 (minus insulin) supplemented with 4 μM CHIR99021. The expression profiles of key genes were examined to establish the dynamics of the differentiation process. We monitored developmentally relevant markers of primitive streak (*T, MIXL1, GSC, FOXA2*), mesendoderm (*T*, *FOXA2*), and definitive endoderm (*HHEX, CER1, SOX17, FOXA2*). Cells were collected for analysis every 4 hours for 48 hours (Phase I - DE stage). Data presented as the mean of 3 independent experiments; error bars represent standard deviation. The X-axis represents the time (in hours) after the start of differentiation. The Y-axis represents the Log10 RQ values from the TaqMan analysis.

Figure S4. Characterisation of Phase II on multiple pluripotent lines - hepatic specification. A) Typical morphology observed at Phase II endpoint, photographed using phase contrast microscopy at 10x. Line identity located top left corner of each plate. Scale bars = 100 μM. B) Expression of AFP and HNF4A at Phase II endpoint of small molecule treated cells, imaged using fluorescent microscopy. The line identity is above each column. Scale bars = 100 μM. C) Efficiency of Phase II differentiation was determined for each line by counting HNF4A and AFP double positive cells. Efficiencies are presented as the percentage of positive cells plus or minus the standard deviation of all fields counted. D) Expression of *AFP, CEBPA, FOXA2, GATA4, HHEX, HNF4A, PROX1, SOX17, TBX3, and TTR* at Phase II endpoint, measured by TaqMan. Normalised to *ACTB* and small molecule derived definitive endoderm. Data presented as the mean of 3 independent experiments; error bars represent standard deviation.

Figure S5. Phase II 5 day time course. The human induced pluripotent stem cell line Detroit RA was differentiated to definitive endoderm using the small molecule protocol. Definitive endoderm was treated with 1% DMSO to differentiate to hepatic progenitors. Cells were collected every 24 hours and gene expression of developmentally relevant hepatic markers was assessed. Data presented as the mean of 3 independent experiments; error bars represent standard deviation. The X axis represents the time (in days) after definitive endoderm. The Y axis represents the RQ values from the TaqMan analysis.

Figure S6. Characterisation of Phase III differentiation to hepatocyte like cells: Morphology and immunofluorescence. A) Morphology of hESC line 207, hiPSC lines Detroit RA, RB and RC at small molecule protocol endpoint (day 17), taken using phase contrast microscopy at 10x. Line identity located top left of each plate Scale bars = 100 μm. B) Expression of albumin and HNF4A at protocol endpoint imaged using fluorescent microscopy. Line identity located at the head of each plate. Scale bars = 100 μm. C) Expression of alpha-1-antitrypsin at protocol endpoints, imaged using fluorescent microscopy. Scale bars = 100 μm.

Figure S7. Characterisation of Phase III derived hepatocyte like cells: RT-qPCR and functional analysis. A) Serum protein secretion at endpoint of small molecule protocol for all lines. Data is presented as the mean of 3 independent experiments; error bars represent standard deviation. *$p < 0.02$, **$p < 0.003$ compared to control. B) Assessment of RT-PCR of *CYP7A1* gene expression of hiPSC derived smHLCs. Lane1 = hESC H1 control, lane 2= Detroit RA, Lane 3 = Detroit RB, Lane 4 = Detroit RC day 17 smHLCs *CYP7A1* expression, lower panel = *ACTB* loading control. C) Efficiency of Phase III differentiation, determined by counting albumin and HNF4A double positive cells and A1AT positive cells. Efficiencies are presented as the percentage of positive cells plus or minus the standard deviation of all fields counted. D) Expression of *A1AT (SERPINA1),*

AFP, ALB, APOA2, ASGR1, CYP3A4, HNF4A, TDO2 and TTR at endpoint of small molecule protocol, as well as growth factor derived HLCs, primary adult and fetal hepatocyte controls, as measured by TaqMan. All normalised to *ACTB* small molecule derived definitive endoderm. Data is presented as the mean of 3 independent experiments; error bars represent standard deviation. Only 1 primary fetal control was run therefore no error bars present.

Table S1. Primer details for RT - qPCR

Table S2. Antibody details for immunofluorescence

Table S3. Primer sequence details

Table S4. Small molecule details

Table S1.

Target	Manufacturer	Reference
OCT4	Life Technologies	Hs00999634_gH
SOX2	Life Technologies	Hs01053049_s1
NANOG	Life Technologies	Hs04260366_g1
NODAL	Life Technologies	Hs00415443_m1
MIXL1	Life Technologies	Hs00430824_g1
T	Life Technologies	Hs00610080_m1
GSC	Life Technologies	Hs00906630_g1
SOX7	Life Technologies	Hs00846731_s1
GATA4	Life Technologies	Hs00171403_m1
FOXA2	Life Technologies	Hs00232764_m1
SOX17	Life Technologies	Hs00751752_s1
HHEX	Life Technologies	Hs00242160_m1
CER1	Life Technologies	Hs00193796_m1
AFP	Life Technologies	Hs00173490_m1
HNF4A	Life Technologies	Hs00230853_m1
CEBPA	Life Technologies	Hs 00269972_s1
PROX1	Life Technologies	Hs00896294_m1
TBX3	Life Technologies	Hs00195612_m1
ASGR1	Life Technologies	Hs01005019_m1
TDO2	Life Technologies	Hs00199611_m1
APOA2	Life Technologies	Hs00952079_g1
ALB	Life Technologies	Hs00910225_m1
CYP3A4	Life Technologies	Hs00604506_m1
TTR	Life Technologies	Hs00174914_m1
A1AT (SERPINA1)	Life Technologies	Hs01097800_m1
ACTB	Life Technologies	N/A

Table S2.

Target	Manufacturer	Catalogue Number	Species	Dilution
OCT4	**Stemgent**	**09-0023**	**Rabbit**	**1:100**
SOX2	**Stemgent**	**09-0024**	**Rabbit**	**1:100**
NANOG	**Stemgent**	**09-0020**	**Rabbit**	**1:100**
FOXA2	**AbCam**	**ab40874**	**Rabbit**	**1:1000**
SOX17	**AbCam**	**ab84990**	**Mouse**	**1:100**
AFP	**Sigma-Aldrich**	**A8452**	**Mouse**	**1:500**
HNF4A	**Santa Cruz**	**sc8987**	**Rabbit**	**1:100**
ALBUMIN	**Sigma-Aldrich**	**A6684**	**Mouse**	**1:500**
Alpha-1-antitrypsin	**Santa Cruz**	**sc30121**	**Rabbit**	**1:50**
Alexaflour 488 anti rabbit	**Life Technologies**	**A21206**	**Donkey**	**1:400**
Alexafluor 488 anti mouse	**Life Technologies**	**A11059**	**Rabbit**	**1:400**
Alexafluor 594 anti mouse	**Life Technologies**	**A11005**	**Goat**	**1:400**

Table S3.

Target	**Forward sequence 5´-3´**	**Reverse sequence 5´-3´**
ACTB	TCACCACCACGGCCGAGCG	TCTCCTTCTGCATCCTGTCG
CYP7A1	CTGCCAATCCTCTTGAGTTCC	ACTCGGTAGCAGAAAGAATACATC

Table S4.

Name	Source	Purity	Solvent	Phase of protocol used	Final concentration used
CHIR99021	STEMGENT	>95%	DMSO	Phase I	3-4 μM
BIO	Tocris	>98%	DMSO	Alternative Phase I	1 μM
DMSO	Sigma-Aldrich	≥99.9%	N/A	Phase II	1% by volume
Dexamethasone	Sigma-Aldrich	≥97%	DMSO	Phase III	100 nM
Dihexa	Kind gift of Prof. Joseph Harding Washing State University	93%	DMSO	Phase III	100 nM

Supplemental Experimental Procedures

Human induced pluripotent stem cell derivation and characterisation.

Detroit 551 fibroblasts were obtained from the American Type Culture Collection (ATCC CCL-110). hOCT4, hSOX2, hcMYC, and hKLF4, retrovirus viral particles were generated by Vectalys and transduced at an MOI of 5 as described by Vallier and colleagues (Vallier et al., 2009). On appearance, hiPSCs were picked and expanded feeder free on Matrigel (Sigma-Aldrich) in E8 Medium (Life Technologies). We verified the iPSC lines expressed human embryonic stem cell markers by immunocytochemistry for NANOG, SOX2 and OCT4 expression (Figure S1A). We also used RT-qPCR to confirm that iPSCs expressed *NANOG, SOX2* and *OCT4* (Figure S1B) and that they had silenced the exogenous genes that were used for reprograming (data not shown). We karyotyped the iPSC lines using KaryoLite BoBs (Perkin Elmer) and demonstrated they were normal (performed by Finnish Microarray and Sequencing Centre (FMSC)). Finally we demonstrated that the derived hiPSCs were able to generate all three germ layers: ectoderm (neurons), mesoderm (cardiomyocytes) and endoderm (hepatocytes) (data not shown), indicating that the iPSCs generated are pluripotent.

RNA isolation and RT-qPCR.

RNA was isolated from cells using TRIzol according to manufacturer's instructions and quantified using a spectrophotometer (NanoDrop). cDNA was prepared using the High Capacity Reverse Transcription kit and a thermal cycler (both from Life Technologies). RT-qPCR was performed using a TaqMan ViiA7 Real Time PCR System with TaqMan Gene Expression Master Mix (Life Technologies). TaqMan assays were used to assess markers of interest and *ACTB* was used as an endogenous control (Life Technologies); see Table S1 for details. Expression levels were quantified relative to *ACTB* and normalised to undifferentiated pluripotent control samples or definitive endoderm cells

as specified. Results are shown as the mean of 3 independent experiments; error bars represent standard deviation.

Immunofluorescence.

Cells were washed with PBS before being fixed with a 10 minute treatment of ice cold methanol. Fixed cells were washed in 0.1% PBS-T: PBS containing 0.1% Tween 20 (Sigma-Aldrich). Cells were blocked for 1 hour in 10% normal goat serum (Life Technologies) made up in 0.1% PBS-T. Cells were then washed twice before being treated with primary antibodies overnight at 4°C; see Table S2 for antibody details. All primary antibodies were made up in 1% normal goat serum in 0.1% PBS-T. Secondary antibody only controls were also included. Following primary incubations, all cells were washed twice and treated with Alexafluor secondary antibodies (Life Technologies) for 1 hour at room temperature. The secondary antibodies were made up in PBS. Cells were then washed twice in PBS-T and twice in PBS before being mounted using Fluoroshield with DAPI (Sigma-Aldrich) and glass coverslips. Cells were imaged using a Zeiss Observer Fluorescence Microscope and Axiovision imaging software. The scale bars represent 100 μm.

Glycogen storage, periodic acid–Schiff staining assay and uptake of indocyanine green.

In order to assess glycogen storage, differentiated cells were fixed and treated with a periodic acid-Schiff staining kit (Sigma-Aldrich) in accordance with manufacturer's instructions and imaged using a Zeiss phase contrast microscope and ZEN software. The scale bars represent 100 μm. We also assessed the cellular uptake of indocyanine green, briefly, ICG (Sigma-Aldrich) was reconstituted in water and used at a final concentration of 1 mg/ml. Cells were incubated in media supplemented with ICG for 1 hour, the cells

were then washed with PBS and imaged using a Zeiss phase contrast microscope and ZEN software.

Cytochrome P450 Induction and analysis.

Induction of cytochrome P450 activity was assessed in both small molecule and growth factor derived HLCs. CYP1A2 activity was detected using the P450-Glo CYP1A2 Induction/Inhibition Assay kit (Promega, Cat. no. V8422). CYP3A4 activity was detected using the P450-Glo CYP3A4 (Luciferin-PFBE) Cell-Based/Biochemical Assay (Promega V8902). Assays were performed according to the manufacturer's instructions for non-lytic P450-Glo assays using cultured cells in monolayers. Cytochrome P450 inductions were performed using the following inducers: for CYP3A4, Rifampicin (25 μM) and CYP1A2, Omeprazole (100 μM) (both purchased from Sigma). Briefly, cells were cultured to day 20 of the differentiation protocol and Rifampicin or Omeprazole was added to L-15 culture medium, which was formulated as described above, but without dexamethasone or hydrocortisone. Medium was replaced daily for 72 hours. After 72 hours, the cells were washed 4 times with PBS (calcium/ magnesium free) and then assayed. Briefly, for CYP1A2 the substrate Luciferin-1A2 was diluted to 6 μM in PBS (calcium/ magnesium free) containing 3 mM freshly prepared salicylamide (Sigma). The Luciferin substrate was added to each well (1 ml per well of a 6 well plate), and incubated for 60 minutes followed by detection. For CYP3A4, the substrate Luciferin-PFBE was diluted in L-15 culture medium (formulated as described above), to a final concentration of 50 μM. After washing the cells, 1 mL of Luciferin-PFBE containing media was added to each well and incubated for 4 hours followed by detection. We assessed basal activity as above with the omission of inducers. In addition, no cell media controls were included. Finally as a negative control, the pluripotent hESC line H1 was assayed for CYP activity as described above. All data was normalised to total protein content in each well. Data is presented as the mean values of 6 independent experiments; error bars represent standard deviation.

Serum protein production.

Cells were incubated for 24 hours in 1 ml of media. ELISA kits were then used to detect human albumin (Alpha Diagnostics), fibronectin (AbCam) and alpha-1-antitrypsin (AbCam) in the supernatants according to manufacturer's instructions. Negative control incubations without cells were included as blanks. Results are normalised to protein weight, and given as the mean of 3 independent experiments; error bars represent standard deviation.

Protein extraction and quantification.

Cells were lysed in 250 µl of SUMO buffer containing 2% sodium dodecyl sulphate (SDS), 50 mM Tris (pH 8), 1 mM EDTA and 10 mM iodoacetamide (Sigma-Aldrich) for 5 minutes at room temperature. Total protein was quantified using a BCA Assay Kit (Pierce) and an absorbance plate reader (Tecan).

PCR and gel electrophoresis.

PCR was carried out using AmpliTaq Gold 360 Master Mix (Life Technologies) supplemented with the relevant oligonucleotide pairs. All assays were run against an *ACTB* control to ensure equivalent amounts of input cDNA; in all cases 5 ng of input cDNA was used. The oligonucleotide sequences are provided in the Table S3. The PCR products were resolved using agarose gel electrophoresis.

Cell Counting.

Immuno-stained cells were quantified for expression of stage specific markers by manual counting. For Phase I, FOXA2 and SOX17 were counted separately. For Phase II, cells were scored positively if the nucleus was stained for HNF4A and the cytoplasm was stained for AFP. For Phase III, cells were scored positively if the nucleus was HNF4A

positive and the cytoplasm was ALB positive. Phase III cells were also quantified for AFP and A1AT staining and were counted as positive if the cytoplasm was stained. In all cases, 3D areas in the image were excluded due to difficulties in counting the nuclei. A minimum of 10 fields of view were quantified, with a minimum of 250 cells counted per field of view. Percentages are presented as the average of all field quantifications, plus or minus the standard deviation across all fields.

Statistical Analysis.

Results were evaluated by performing t tests. $p < 0.09$ was determined significant.

Supplemental References

Vallier, L., Touboul, T., Brown, S., Cho, C., Bilican, B., Alexander, M., Cedervall, J., Chandran, S., Ahrlund-Richter, L., Weber, A., Pedersen, R.A., 2009. Signaling pathways controlling pluripotency and early cell fate decisions of human induced pluripotent stem cells. Stem Cells 27, 2655–2666. doi:10.1002/stem.199