Supplementary Data

Supplementary Materials and Methods

Timelapse imaging of spheroid formation

D20 human embryonic stem cell (hESC) hepatocyte-like progeny were harvested and viable cells counted by Trypan blue exclusion. One percent of the cells was stained with Vybrant DiI (Invitrogen) according to manufacturer's instructions. The labeled cells were combined with the bulk population and seeded in spheroid culture assays. The plate was subsequently placed on a motorized stage in a climate-controlled chamber (37°C, 5% CO_2), and phase contrast and fluorescent images were collected in each of the six wells at 5 z-positions (separated by 10 μm each) every 10 min for 72 h. This is shown in Supplementary Video S1: Timelapse Imaging of Spheroid Formation. Video files were created using ImageJ software.

Viability stain of cells in spheroid cultures

Viability of forming spheroids was examined by staining with Calcein-AM (5 μM; γ_{em} 490 nm, γ_{ex} 515 nm) and propidium iodide (2 μg/mL; γ_{em} 535 nm, γ_{ex} 515 nm) to label live cells and dead cells, respectively, on D20 immediately after centrifugation of the cell suspension and on D21, after 24 h of spheroid formation, respectively. Samples were incubated for 30 min at 37°C before imaging.

Determining the size of aggregates

The average diameter of each aggregate was calculated by averaging the long and short diameters. These were averaged to obtain the mean size of these aggregates which was 149 microns with a standard deviation of 50 μm.

Quantitative real time polymerase chain reaction

Total RNA was isolated from hESC cell lysates using RNAeasy microkit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized from the extracted RNA using the Superscript III reverse transcriptase kit (Invitrogen) according to manufacturer's instructions. The polymerase chain reaction reactions were carried out as previously described; primer sets are listed in our previous study (19). Transcript abundance relative to GAPDH of the same sample is expressed as $\log_2$ (Transcript expression relative to GAPDH) and calculated as $\Delta Ct = Ct$ (gene of interest) $- Ct$ (GAPDH). The obtained transcript abundance value of sample was then normalized to D20 [$\Delta\Delta Ct = \Delta Ct$ (sample) $- \Delta Ct$ (D20)]. The fold changes of gene expression in D26 cells relative to D20 cells (ie, $2^{-\Delta\Delta Ct}$) are shown in Fig. 2. The higher the value represents higher extent of increase in the transcript level.

Cell surface staining for asialoglycoprotein receptor 1 by flow cytometry

Cells were harvested using collagenase followed by trypsin, as described above, washed with PBS thrice, counted, and fixed in 4% paraformaldehyde for 15–20 min at RT. Subsequently, cells were incubated for 30 min with 10 μg/mL asialoglycoprotein receptor 1 antibody (Thermoscientific) or 10 μg/mL mouse IgG_1 isotype control (R&D) in PBS with 2% (w/v) bovine-serum albumin (Sigma), followed by treatment with the secondary antibody, Alexa-fluor 488 labeled anti-mouse IgG_1 (Molecular Probes, 1:500) for 30 min at room temperature (RT) in the dark. Fluorescence activated cell sorting (FACS) was performed on a FACS Calibur (Becton Dickinson) cytometer.

Phosphoenolpyruvate carboxykinase immunofluorescence staining and flow cytometry

Differentiated spheroids from D32 were fixed with 4% paraformaldehyde at room temperature for 20 min. After fixation, the cells were treated with 1 g/L Triton X-100 and 5% FBS at RT for 1 h for blocking, then incubated overnight at 4°C with primary antibody anti-human phosphoenolpyruvate carboxykinase (Santa Cruz, 0.1 μg/mL in blocking solution), followed by treatment with the secondary antibody, Alexa-fluor 488 conjugated anti-rabbit (Molecular Probes, 1:500 dilution) for 30 min, followed by nuclear labeling with 1 mM Hoechst 33258 (Sigma) for an additional 10 min. Imaging was performed with an Olympus FluoView FV100 Inverted confocal laser scanning microscope, and image analysis was performed using ImageJ software. Background intensities were removed based on a control sample (secondary antibody only). FACS analysis was performed using the same protocol, but with dissociation of the spheroids before fixation.

Scanning electron microscopy

D30 differentiated spheroids were fixed with 1 mL glutaraldehyde (8% glutaraldehyde in 0.2 M sodium cacodylate buffer at pH 7.2) for 90 min at RT. The samples were then washed three times with 0.1 M sodium cacodylate buffer for 5, 10, and 10 min, sequentially, followed by treatment with 1 mL of 1% (w/v) osmium tetraoxide [2% (w/v) osmium tetraoxide in 0.2 M sodium cacodylate buffer at pH 7.2]. Samples were then rinsed three times with distilled water for 10, 15, and 15 min, sequentially. The samples were then dried by exposure to increasing concentrations of ethanol: 50% ethanol for 10 min, 75% for 15 min, 100% for 15 min, and dry ethanol twice, once for 20 min and again for 30 min. The samples were then mounted on aluminum stubs using double-sided carbon adhesive tabs, sputter-coated with gold-palladium, and imaged using a scanning electron microscope (S3500N; Hitachi High Technologies America, Inc.) at an accelerating voltage of 5 kV. Sample processing and imaging were performed at the Imaging Center in the College of Biological Sciences at the University of Minnesota.

Transmission electron microscopy

D30 differentiated spheroids were treated thrice for 5 min each with 0.1 M cacodylate buffer and fixed in 2.5% glutaraldehyde with 0.1 M sodium cacodylate buffer (pH 7.2) for 40 min at RT, followed by treatment with 1% osmium

tetroxide and 0.1 M of cacodylate buffer. The samples were subsequently dehydrated in graded series of ethanol followed by propylene oxide treatment and embedded in epoxy resin. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined using a JEOL 1200 EXII electron microscope at the Characterization Facility at the University of Minnesota. Sample processing and electron microscopy was performed by Ms. Fang Zhou, Characterization Facility, University of Minnesota.

Immunohistochemisty on spheroid sections

The spheroids were harvested on D32 and were fixed with 4% paraformaldehyde at room temperature for 20 min. These were embedded in paraffin and sectioned. The sections were stained with CK18 (Abcam, 1:75), CK8 (Abcam, 1:500), alpha-fetoprotein (Neomarkers 1:75) and albumin (Dako, 1:2,000).

Albumin secretion by ELISA

Human albumin was measured using a quantitative ELISA kit (Starters Kit Bethyl E101 and Bethyl E80-129) following the manufacturer's instructions. An internal standard, provided with the kit, was used to obtain a four-parametric logistic fit to enable the estimation of albumin concentration. The albumin concentration in the samples was subtracted from amount present in fresh medium to quantify the amount secreted by the cells in 1 day of incubation. The cell number was estimated and the albumin secretion was reported on a per cell basis.

Cytochrome P450 activity by confocal imaging

For drug treatment conditions, spheroids were incubated for 24 h in differentiation media with or without 50 μM β-Naphthoflavone or 500 μM Phenobarbital as indicated. The spheroids were incubated for 10 min at 37°C with assay media (phenol red free (PRF) Williams' Medium E supplemented with 25 mM dicumarol and 2 mM probenecid at pH 7.4), followed by incubation with ethoxyresorufin (ER; 39 μM in assay media; Sigma) or pentoxyresorufin (PR, 20 μM in assay media; Sigma) for 30 min at 37°C. Resorufin localization within the spheroid was determined by confocal laser scanning microscopy using an Olympus FluoView FV1000 Inverted microscope, and images were processed using ImageJ software. Briefly, z-stacks were collected using a 10× objective with step sizes of 4.36 μm, 10–15 sections for each spheroid, and the final image was prepared by projecting the maximum intensity from each z-slice into a single image. Background fluorescence was subtracted based on negative controls (samples processed identically but without ER or PR).

Biliary excretion visualization by fluorescein staining

Biliary accumulation of cleaved fluorescein from fluorescein diacetate (FDA) was observed on D32 differentiated spheroids. The spheroids were washed in PRF Williams' E medium twice followed by incubation with 3 μg/mL FDA (Molecular probes) in PRF William's E medium at 37°C for 40 min. The samples were imaged with an Olympus FluoView FV100 Inverted confocal laser scanning microscope, and image analysis was performed using ImageJ software. Briefly, z-stacks were obtained at steps of 2 μm, and a stack of 10–15 images (20–30 μm) was selected that spanned representative sections of fluorescein accumulation. The maximum intensities of these images were projected into a single image, and the background intensities were removed based on negative control images (spheroids processed without FDA). Deconvolution was performed to obtain a sharper image using the AutoQuant×3 software. Blind deconvolution was performed to calculate the point spread function iteratively. This was then applied towards the reconstruction of the image enhancing the signal to noise ratio.

Ethoxyresorufin-O-dealkylation and pentoxyresorufin-O-dealkylation activity assay

For drug treatment conditions, spheroids were incubated for 24 h in differentiation media with or without 50 μM β-Naphthoflavone, 500 μM Phenobarbital, or 50 μM Rifampicin as indicated. After the treatment period, spheroids were collected and washed once with assay media (PRF Williams' Medium E supplemented with 25 mM dicumarol and 2 mM probenecid at pH 7.4), followed by incubation for 10 min at 37°C in assay medium. An equal volume of assay medium containing 2×ER (39 μM final concentration) or 2×PR (20 μM final concentration) was added to each well, and the fluorescence within each well was measured every 30 min up to 3 h using a SpectraMax M2 (Molecular Devices) fluorescent spectrometer. The concentration in each well was determined from a standard curve generated with known resorufin concentrations.