Supplementary Material and Methods

Cell culture

HepaRG cells were purchased from the Thermo Fisher SCIENTIFIC Company. The cells were cultured in high-glucose Dubelcco's modified Minimum Essential Medium (H-DMEM, Gibco), supplied with 10% fetal bovine serum (FBS, Gibco), 1% nonessential amino acid solution (NEAA, Gibco), 1% penicillin G and streptomycin (Gibco), 1% glutamax (Gibco), 5 μg/ml insulin (Sigma), and 5×10^{-5} mol/L hydrocortisone hemisuccinate (Sigma). Cells were subcultured in a 5% CO_2 incubator at 37°C and harvested by trypsin (0.25%, Invitrogen) dissociation at about 80% confluence. The culture medium was replaced every other day. Ten-donor mixed gender pooled primary human hepatocytes were purchased from Bioreclamationivt. The cells were seeded in complete InVitroGRO CP Medium (Bioreclamationivt), supplied with 2% Torpedo Antibiotic (Bioreclamationivt) at a density of 7×10^5 cells/ml.

Material preparation

Gelatin powder (G1890, Sigma) was dissolved in a 0.9% NaCl solution (w/v) at 20% (w/v). Sodium alginate powder (A0682, Sigma) was dissolved in the 0.9% NaCl solution (w/v) at 4% (w/v). Both solutions were sterilized by heating three times in an oven (70°C) for 30 min. The sterilized materials were subpackaged into 1.5 ml EP tubes, stored at 4°C, and warmed to 37°C before use.

Cell survival

Cell survival in the 3DP-HOs was evaluated immediately after printing to assess the influence of the manufacturing process on cell viability, particularly the hydrogel composition and temperatures of the nozzle and forming space. A fluorescent live/dead assay was carried out to determine cell survival. Briefly, a mixture of calcein-AM (1 μmol/L; Sigma) and propidium iodide (PI) (2 μmol/L; Sigma) was prepared and passed through a 0.22 μm filter prior to staining. 3DP-HOs were gently washed with phosphate buffered saline (PBS) after crosslinking, and immediately incubated in the calcein-

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AM/PI mixture for 15 min at 20-25°C while being protected from light. After incubation, the 3DP-HOs were gently washed with PBS and observed under a laser scanning confocal microscope (C2/C2si, Nikon). Cell viability was calculated by counting the number of cells using ImageJ [(live cell/total cell) ×100%]. Five images of random fields were captured for each sample, and cells in five samples were counted.

2D planar culture and 3D sandwich culture

2D planar culture sample was prepared by seeding HepaRG cells at a density of 2×10^4 cells per cm^2 in a plate containing 3 ml culture medium per well. 3D sandwich culture sample was prepared as followed. HepaRG cells were resuspended in matrigel (Corning) or the bioink mixed with gelatin and sodium alginate solution described above at a density of 1×10^6 cells/ml, then seeded in a 24-well flat bottom cell culture plate. The matrigel became solidified by incubation in a 37°C and 5% CO_2 cell culture incubator for 20 min, and the solidified mixture of bioink and cells can be obtained by low temperature storage for 20 min. All 3D culture samples were covered with 3ml of complete media. The 2D and 3D culture samples were subjected to the same procedure of initial culture, differentiation, and medium changes as the 3DP-HOs. The 2D and 3D culture samples were subjected to the same procedure of initial culture, differentiation, and medium changes as the 3DP-HOs.

Cell proliferation

3DP-HOs at culture days 0, 1, 3, 5 and 7 were incubated in a mixture of culture medium and CCK-8 (Dojindo) at a volume ratio of 10:1 to analyze cell proliferation. After 2 h of incubation at 37°C, fluorescence of the culture medium at 630 nm with 450 nm excitation was detected (Model 680; Bio-Rad). A standard curve of fluorescence to certain number of cells was established by incubation of HepRG cells with CCK-8 containing culture medium in a 6-well plate. The detected fluorescence of 3D sample was then normalized to cell number according to the standard curve.

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ALBUMIN, alpha-1 Antitrypsin, Factor VII and Factor IX ELISAs

To measure ALBUMIN, alpha-1 Antitrypsin, Factor VII and Factor IX secretion, HepaRG cells and 3DP-HOs were cultured for various times. The production of ALBUMIN and AAT in different culture configurations was then normalized to 1 million. Culture supernatants were collected at 24 h after a medium change. Culture supernatants and mouse sera were stored at -80℃ before analysis. A standard curve was drawn based on the optical density detected by CCK8 and cell number to calculate the number of cells.

ELISAs were performed in accordance with the manufacturers' instructions using a human albumin ELISA kit (Bethyl Laboratory), human α-1-Antitrypsin ELISA kit (Bethyl laboratories), and human Coagulation Factor VII and IX ELISA kits (Cloud-clone Corp.). Samples were diluted 10- to 10000-fold to obtain values in the linear range of the standard curve. The amount of secreted protein was calculated according to the standard curve in each experiment, followed by normalization to the theoretical input cell number and time period.

CYP Induction

For assessing CYP induction potential, 3DP-HOs and other cells were treated with 3-methylcholanthrene (25μM) for 48h, rifampicin (25μM) and sodium phenobarbital (2mM) for 72 hours. Total RNA of cells with and without inducer treatment were used to measure the expression of CYP450 genes. The gene expression levels of CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP3A4 and CYP3A11 were measured by qPCR as described above.

CYP 450 enzyme activity

The CYP1A2 and CYP3A4 enzyme activities were evaluated with the P450-Glo™ CYP1A2 Induction/Inhibition Assay and P450-Glo™ CYP3A4 Assay (Luciferin-IPA) (Promega, Mannheim, Germany), respectively. Briefly, 3DP-HOs were dissociated into the single cells that clustered and suspended in medium. CYP1A2(6μM) and CYP3A4

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substrate Luciferin-IPA (3μM) was added on 3DP-HOs and incubated at 37°C, 5% CO_2 for 1 h. Then supernatant was transferred to 96-well plate and incubated with Luciferin detection reagent at room temperature for 20min. The luminescence was measured with a luminometer (Molecular Devices Flexstation 3).

Immunofluorescence

Samples were washed with PBS three times, fixed in 4% paraformaldehyde at room temperature for 20 min, and permeabilized with 0.2% Triton X-100 for 30 min at room temperature. They were then blocked with 3% BSA at room temperature for 1 h and incubated with the following primary antibodies in 1.5% BSA at 4°C overnight: chicken anti-albumin (1:100; Abcam), rabbit anti-cytokeratin 19 (1:200; Abcam), rabbit anti-MRP2 (1:500; Abcam), mouse anti-cytokeratin CK18 (1:100; Abcam), rabbit anti-GST (1:200; Abcam), mouse anti-CYP3A4 (1:50; Cell Signaling), and mouse anti-CD31 (1:100; Abcam). The next day, the following secondary antibodies in 1.5% BSA were applied for 1 h at room temperature: goat anti-rabbit IgG Alexa Fluor 405 (1:500; Abcam), goat anti-mouse IgG Alexa Fluor 488 (1:500; Abcam), goat anti-rabbit IgG Alexa Fluor 488 (1:500; Abcam), goat anti-mouse IgG Alexa Fluor 594 (1:500; Abcam), goat anti- rabbit IgG Alexa Fluor 594 (1:500; Abcam), and goat anti-chicken IgG Alexa Fluor 594 (1:500; Abcam). Then, DAPI (1:10000; Sigma) was applied for 5 mins at room temperature. Samples were washed with PBS three times between each step. The laser scanning confocal microscope was used to observe the stained samples.

Reverse-transcription-polymerase chain reaction and real-time quantitative polymerase chain reaction

Total RNA was extracted from 3DP-HOs and cells using Trizol (Invitrogen), following the manufacturer's instructions. One microgram of RNA was reverse transcribed using a ReverTra Ace qPCR RT Master Mix with gDNA Remover Kit (Toyobo) in a 20 μl reaction. After 20-fold dilution, 4 μl of complementary DNA was used as the template in a 20 μl real-time polymerase chain reaction. For real-time polymerase chain reaction,

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amplification was performed for 40 cycles using EvaGreen 2X qPCR MasterMix (Applied Biological Materials Inc, Canada). Primers were designed using exon junctions to prevent coamplification of genomic complementary DNA (Supplementary Table 1).

PAS and DiI-ac-LDL staining, and the ICG uptake and release assay

The 3DP-HOs were stained with periodic acid-Schiff (PAS; Sigma) and DiI-ac-LDL (Invitrogen), following the manufacturers' instructions. For the indocyanine green (ICG; Sigma) uptake assay, the 3DP-HOs were cultured in medium containing progesterone, pregnenolone-16a-carbonitrile, and 8-bromo cAMP for two days. The 3DP-HOs were then treated with 1 mg/ml ICG at 37℃ for 1 h, followed by washing with PBS three times. For ICG release assay, the 3DP-HOs with ICG uptake were cultured in the medium at 37 ℃ for 6 hours.

***In vivo* liver function analysis**

At 4 weeks after transplantation of 3DP-HOs into F/R mice, blood from each of the surviving F/R mice was collected from the retro-orbital sinus and centrifuged at 300 *g* for 15 min. The serum was frozen at -80℃ until analysis. ALBUMIN, total protein, bilirubin, alanine aminotransferase, aspartate aminotransferase, lactic dehydrogenase, gamma-glutamyl transpeptidase, alkaline phosphatase, cholinesterase, pre-albumin, and albumin/globulin ratio were estimated using an AU 5800 automated analyser (Beckmann) at the clinical laboratory of our hospital.

Amino acid detection

At 4 weeks after transplantation of 3DP-HOs in F/R mice, blood was collected and centrifuged. The plasma thus obtained was mixed with a 10% yellow-based salicylic acid solution at room temperature. After precipitation, the samples were centrifuged at 5000 *g* for 4 min at 4℃ and the supernatants thus obtained were assessed for the

presence of amino acids by an automatic amino acid analyzer (Hitachi L8900).

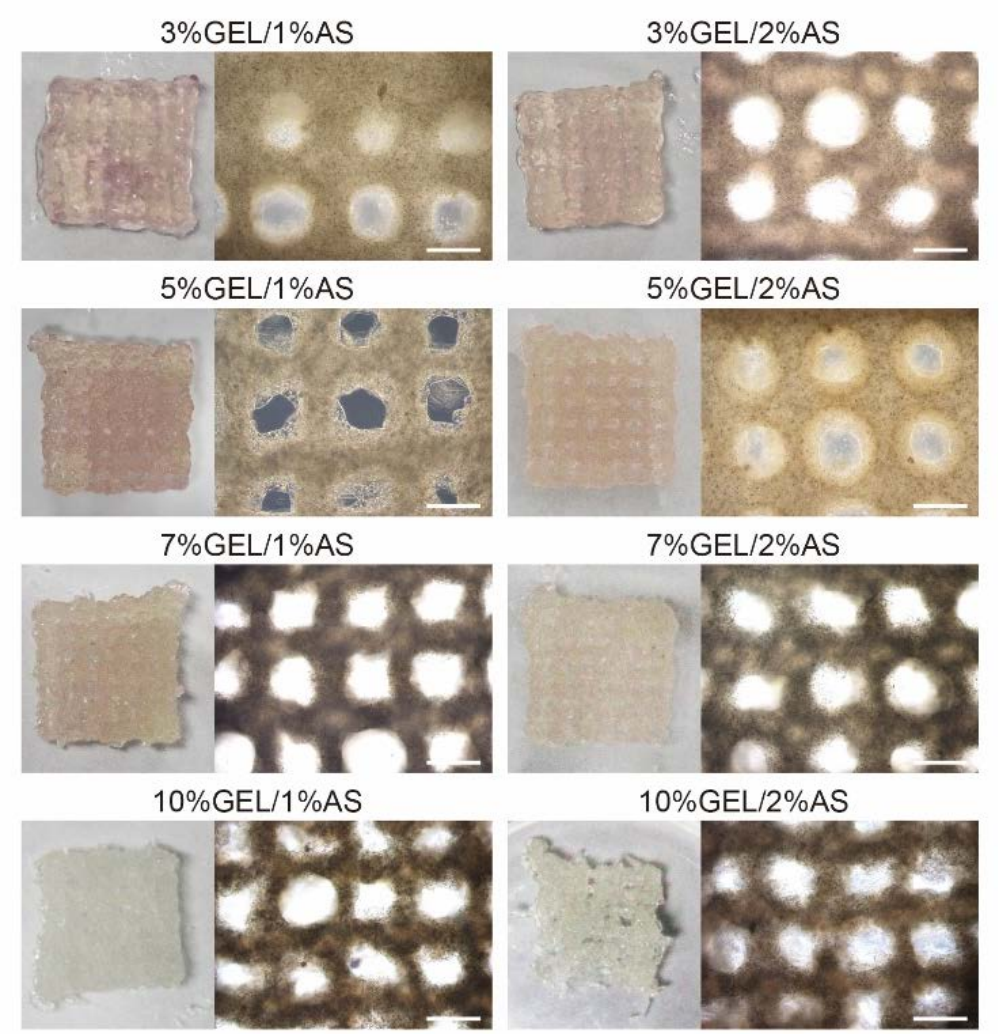
Vessel imaging

At 2 weeks after transplantation of 3DP-HOs into F/R mice, 1% fluorescein-isothiocyanate-conjugated dextran (2,000,000 MW; Sigma) was injected into mice via the tail vein. After 5 min, mice were anesthetized, 3DP-HOs were dissected out, and angiogenesis was observed by confocal microscopy (C2/C2si, Nikon).

Detection of human-specific drug metabolism

Debrisoquine (DEB, Sigma; 2 mg/kg) was orally administrated to Fah-deficient mice transplanted with 3DP-HOs in their abdominal cavities (n=3). Sham-operated Fah-deficient mice were used as control mice. Blood samples were collected after intragastric administration for 1 h. Plasma was separated from blood and stored at -80℃ before analysis. Plasma metabolites were detected by liquid chromatography-tandem mass spectrometry (AB Sciex 4000 QTRAP; AB Sciex). The levels of DEB and 4-hydroxydebrisoquine (4-OH-DEB) were measured, and the 4-OH-DEB/DEB ratio was calculated.

Supplementary Figure



Supplementary Figure 1 Representative optical images of 3DP-HOs structures at day 0 using different gelatin (Gel)/ alginate (AS) concentrations. Scale bar: 1mm.

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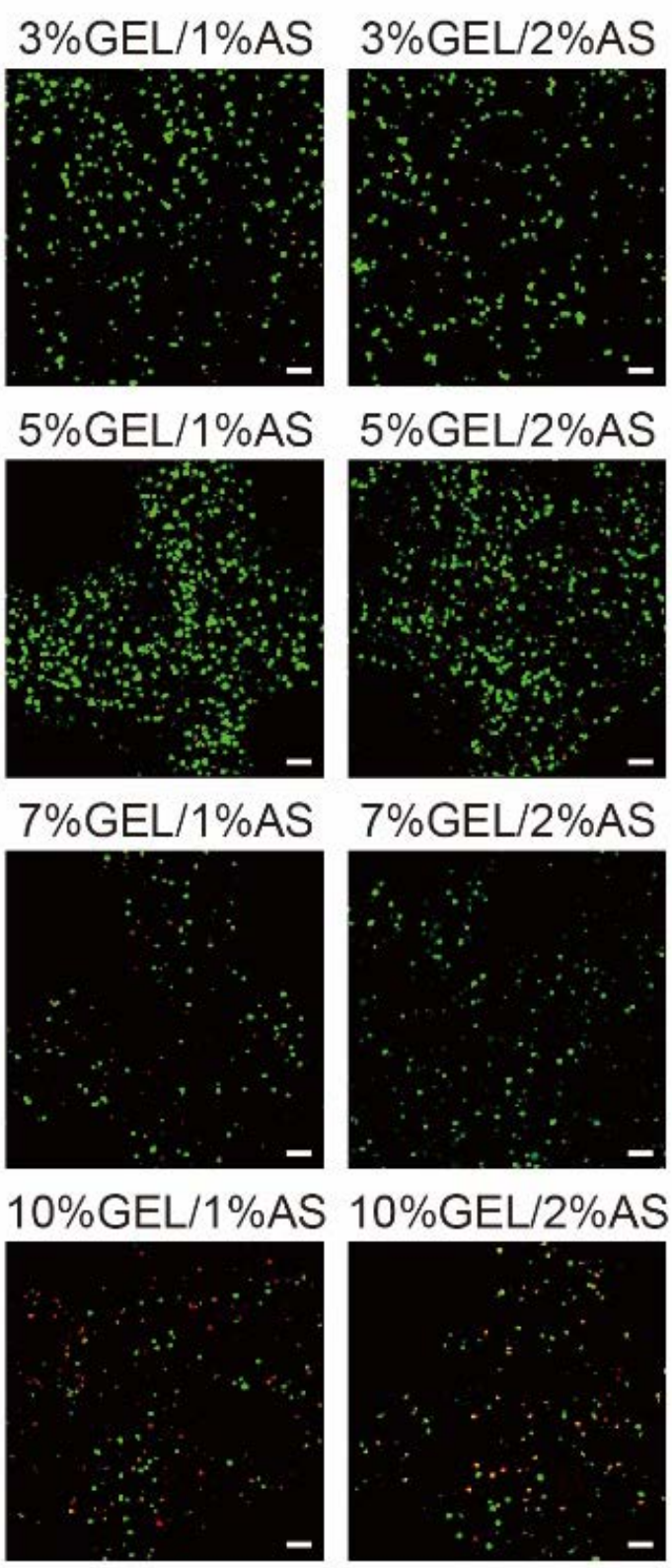
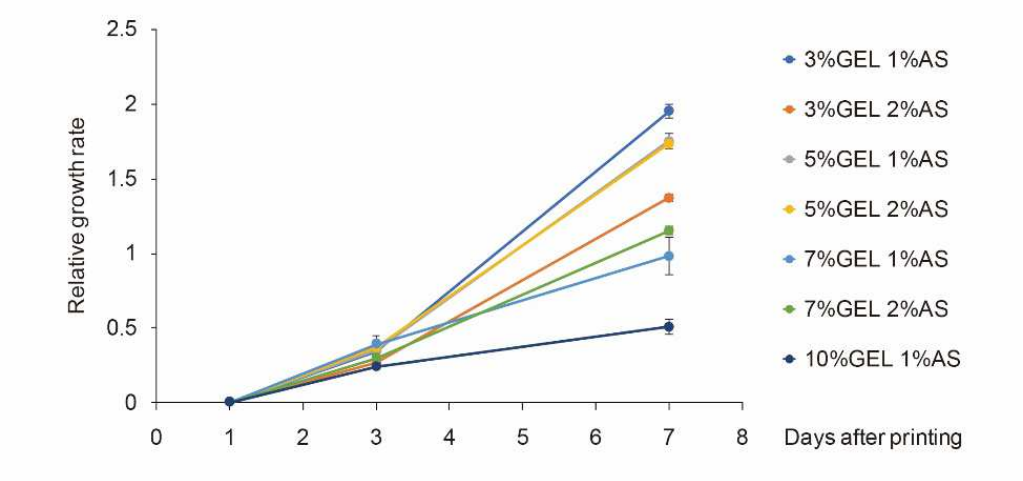
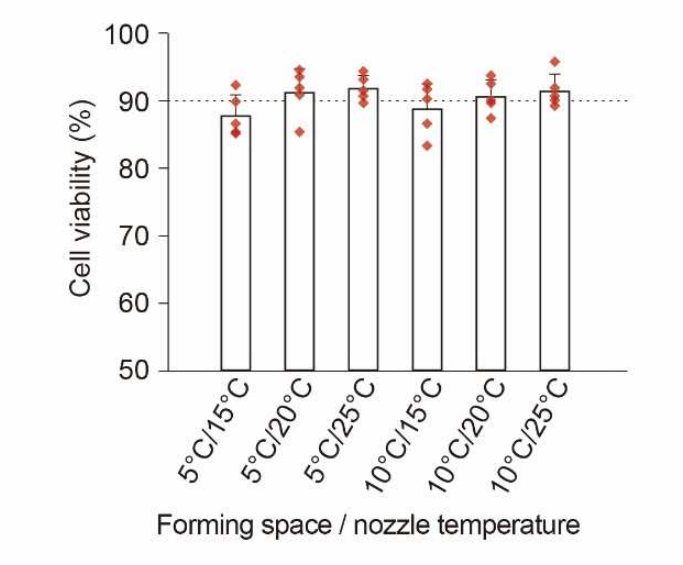


Supplementary Figure 2 Representative live-dead staining images of 3DP-HOs

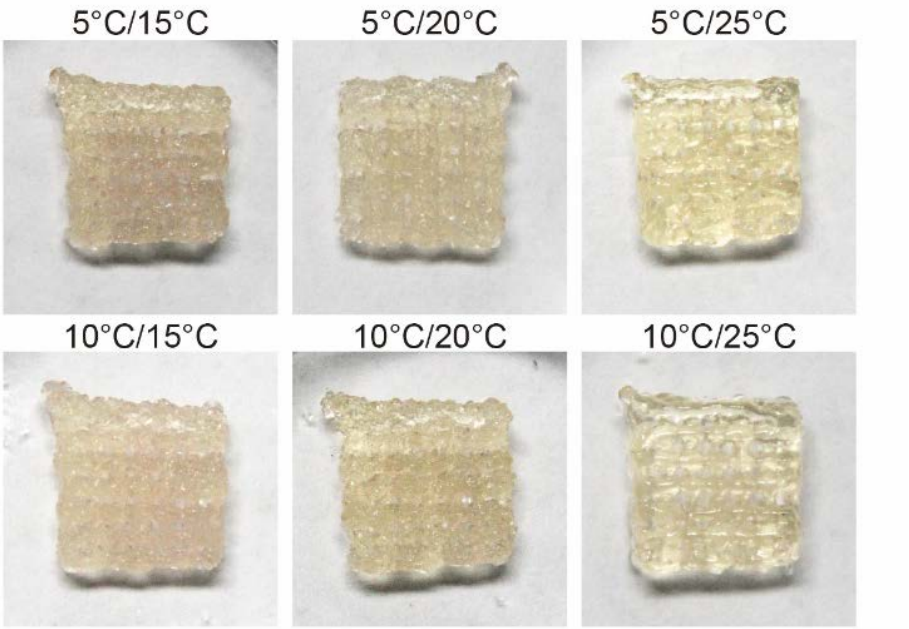
structures at day 1 using different gelatin (Gel)/ alginate (AS) concentrations. Live and dead cells were labelled with calcein-AM (green) and PI (red), respectively. Scale bar: 100μm.



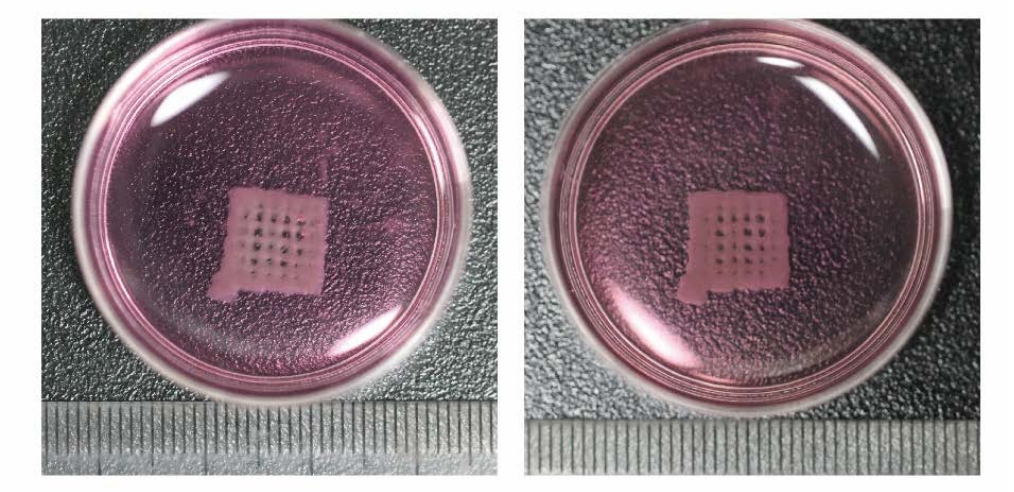
Supplementary Figure 3 Normalized growth rate of HepaRG cells in 3DP-HOs structure using CCK8 assay.



Supplementary Figure 4 Quantified cell viability of 3DP-HOs structures at day 0 under different forming space/nozzle temperatures. Bioink composed of 5% gelatin, 1% alginate and 10^6 cells/ml was used.



Supplementary Figure 5 Representative optical images of 3DP-HOs structures at day 0 under different forming space/nozzle temperatures. Bioink composed of 5% gelatin, 1% alginate and 10^6 cells/ml was used.



Supplementary Figure 6 Optical images of 3DP-HOs structures at 0 day and 7days.

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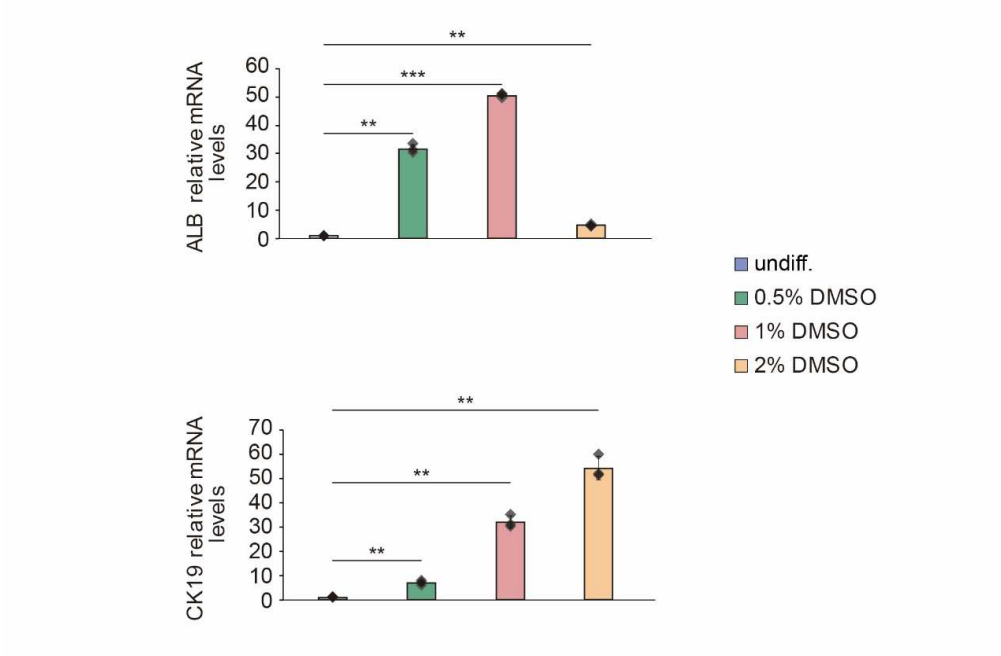
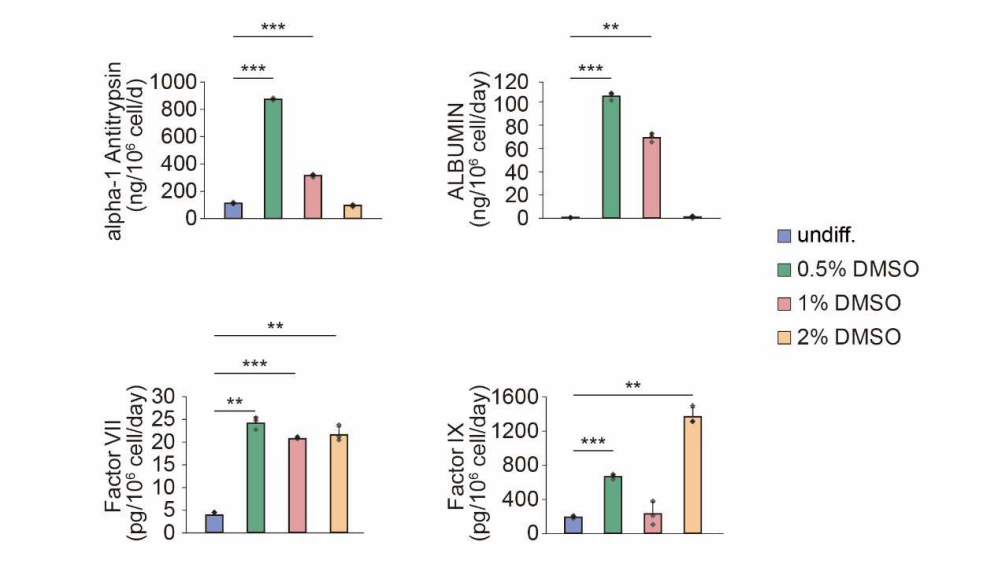
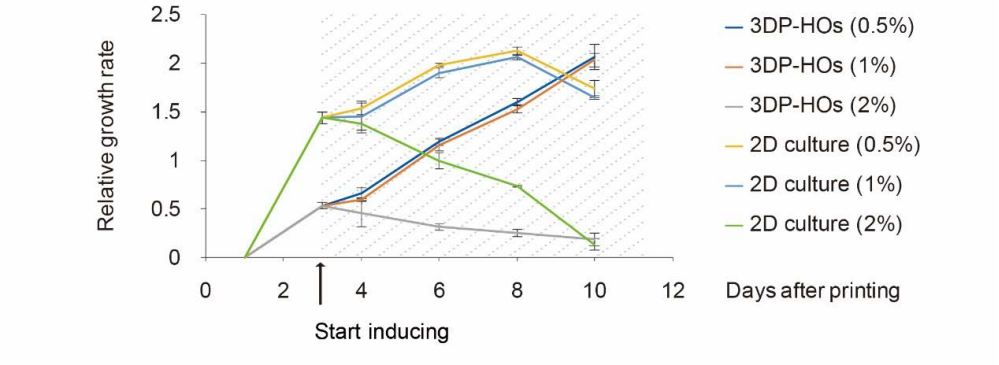


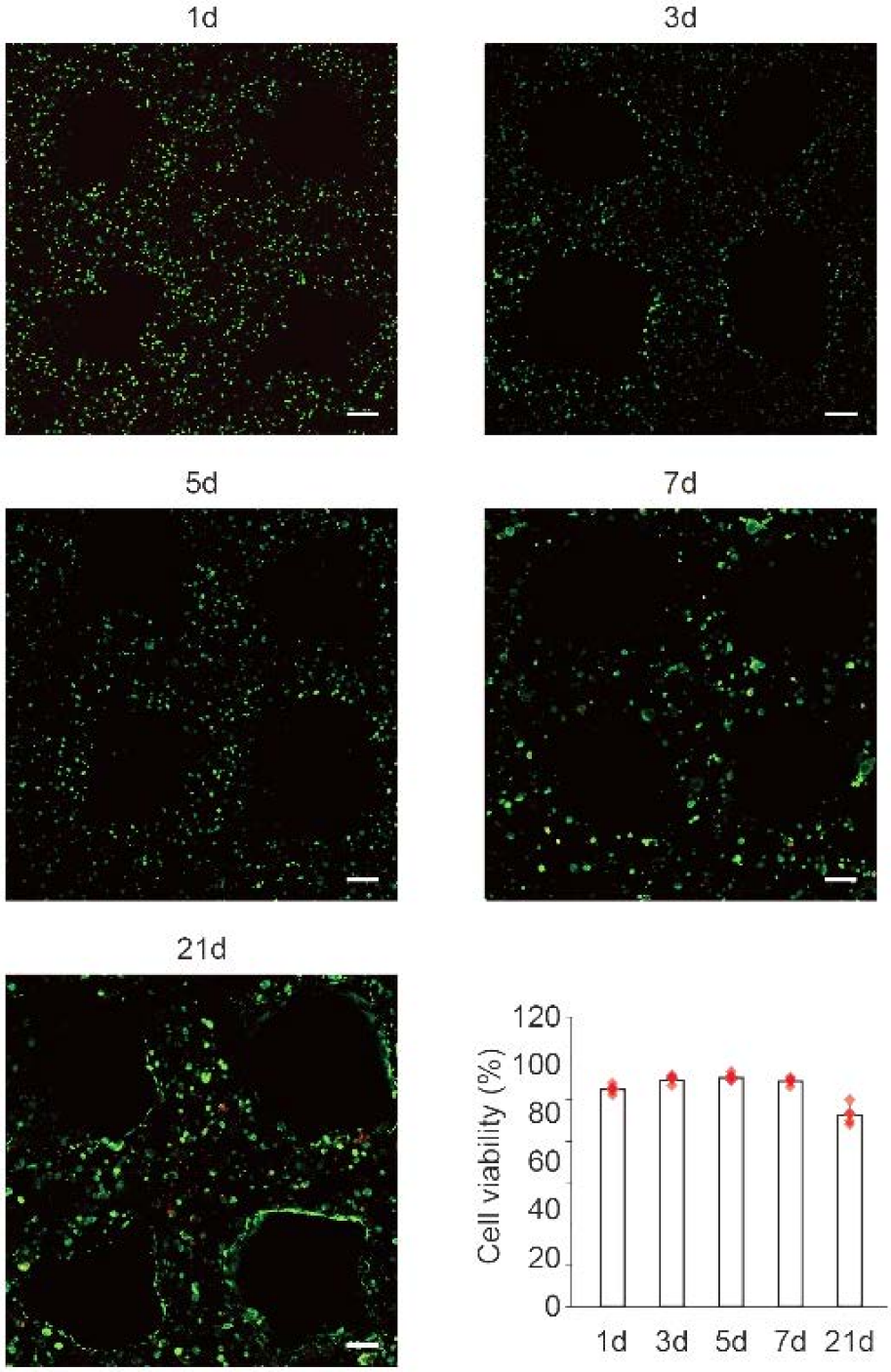
Supplementary Figure 7 Liver function-related gene differences induced under various composition of DMSO. The ALB and CK19 mRNA expression of 3DP-HOs under various induction conditions were evaluate by real-time quantitative PCR. **: P<0.01; ***: P<0.001.



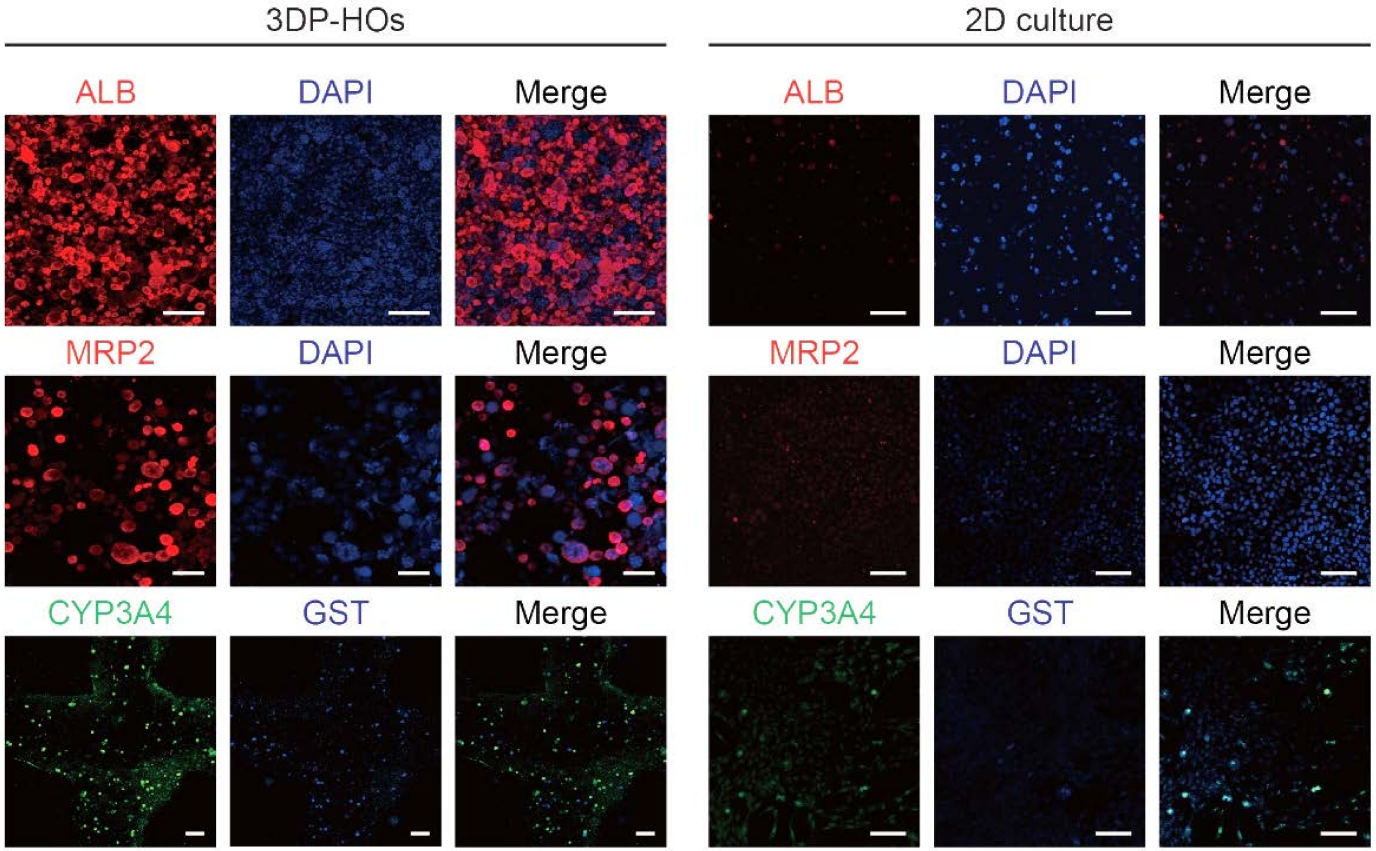
Supplementary Figure 8 Liver function-related proteins expressed differently under the induction of DMSO at various compositions. Concentrations of ALBUMIN, alpha-1 Antitrypsin, Factor VII, and Factor IX in culture supernatants of 3DP-HOs under various induction conditions were evaluate by ELISA sassy. **: P<0.01; ***: P<0.001.



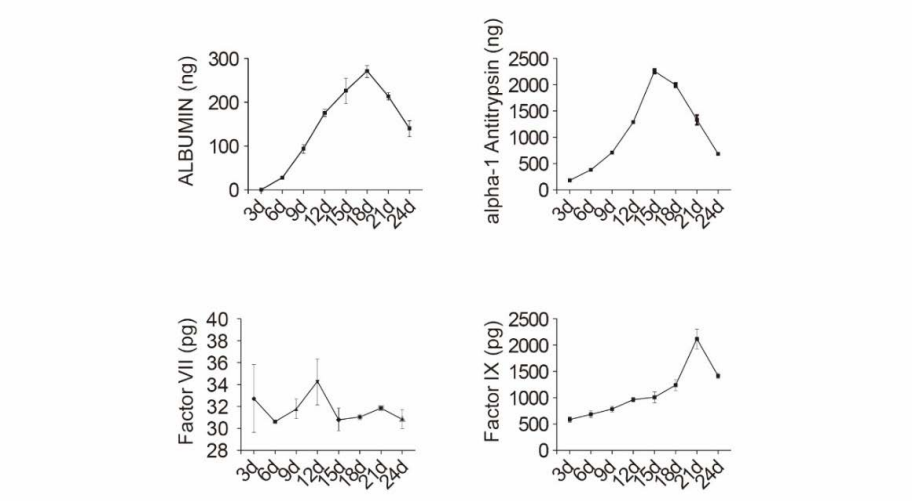
Supplementary Figure 9 Cell proliferation under different induction conditions. The relative growth rate of 3DP-HOs and 2D culture under various induction conditions (0.5% DMSO, 1% DMSO and 2% DMSO) was accessed by CCK8 assay.



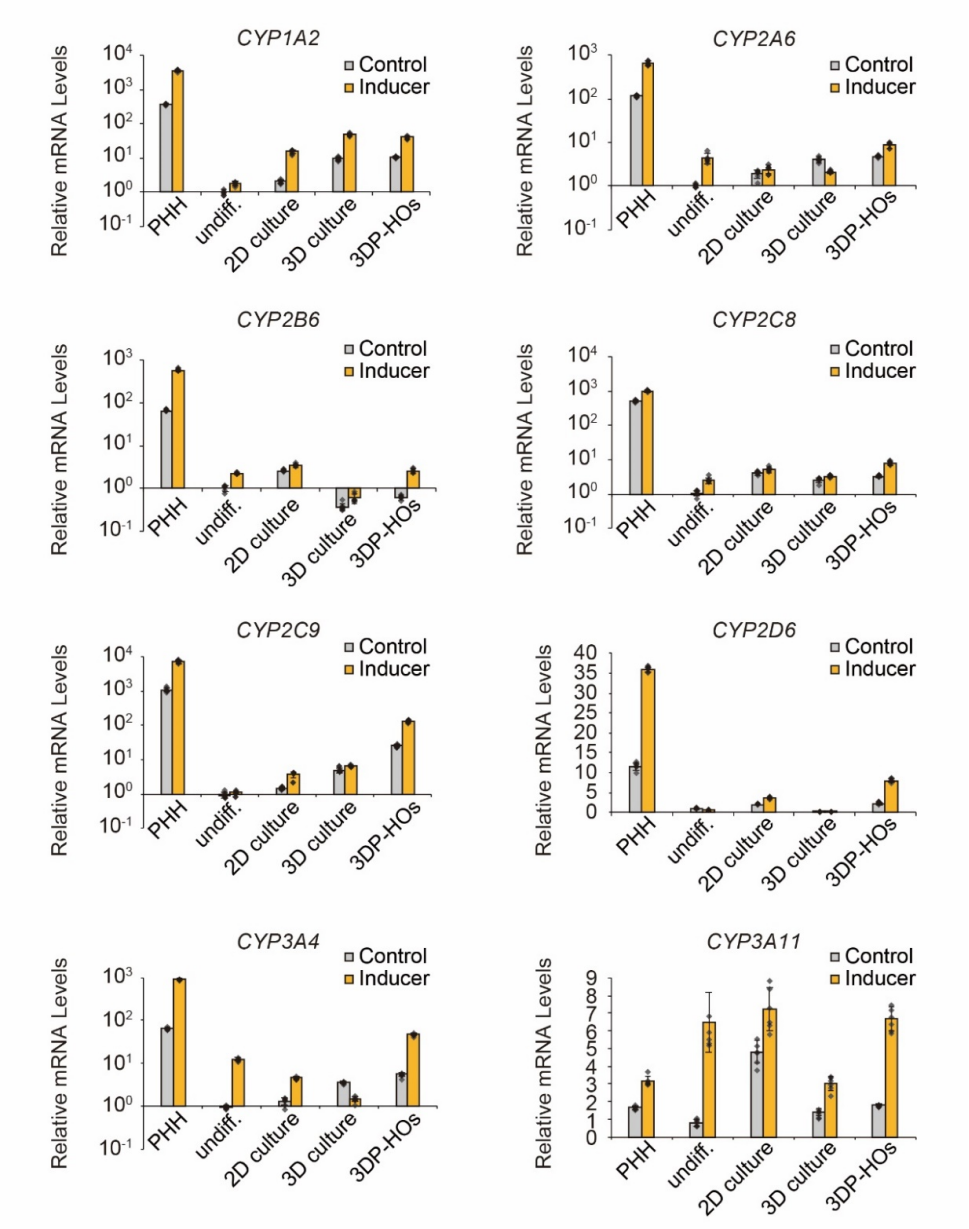
Supplementary Figure 10 Cell viability of 3DP-HOs cultured for various times *in vitro*. Calcein-AM (+) cells in green were live cells and PI (+) cells in red were dead cells. Scale bar, 250 μm.



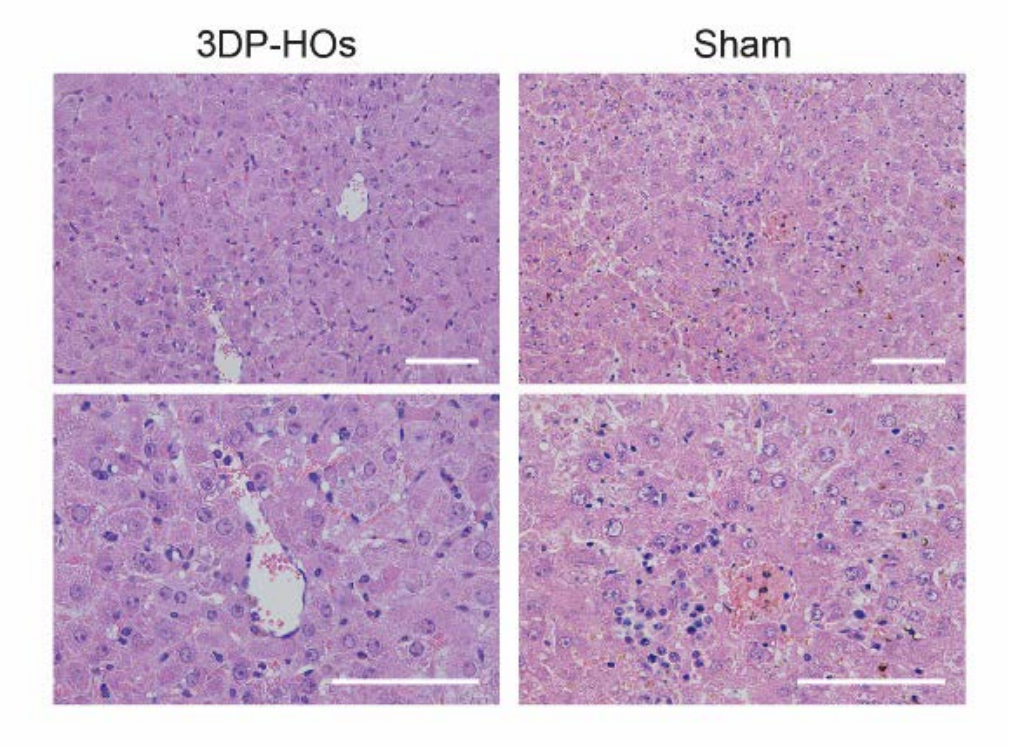
Supplementary Figure 11 Liver function-related proteins expressed differently between 3DP-HOs and 2D cultures. Expressions of ALB, MRP2, GST and CYP3A4 of the HepaRG cells in 3DP-HOs and 2D cultures were detected by immunofluorescence. Scale bar, 100 μm.



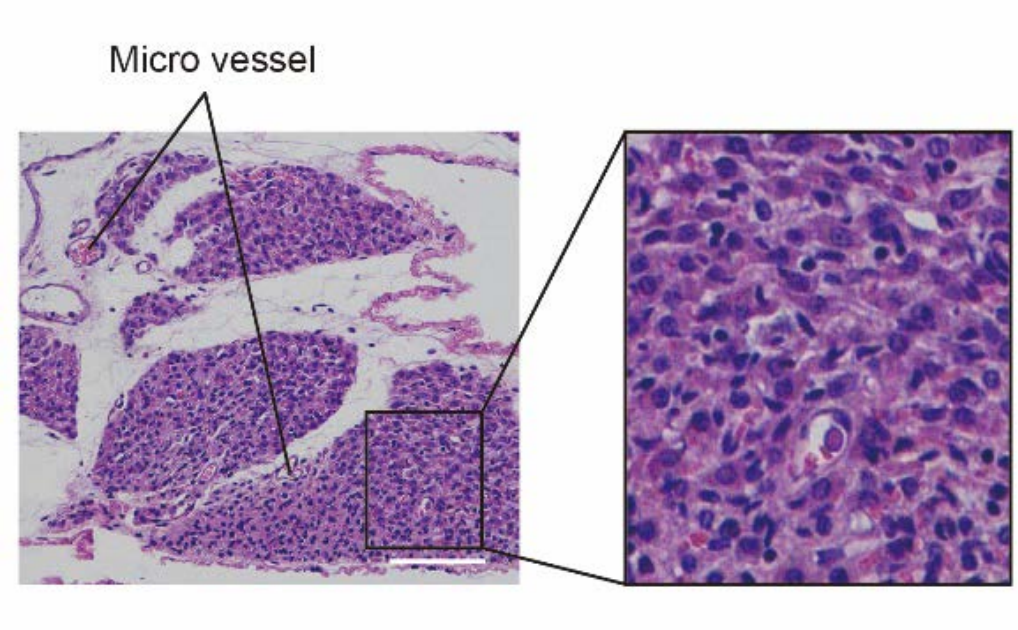
Supplementary Figure 12 Liver function-related protein secretion of 3DP-HOs. Levels of ALBUMIN, alpha-1 Antitrypsin, Factor VII, and Factor IX in culture supernatants of 3DP-HOs cultured for various times *in vitro* were assessed by ELISA sassy.



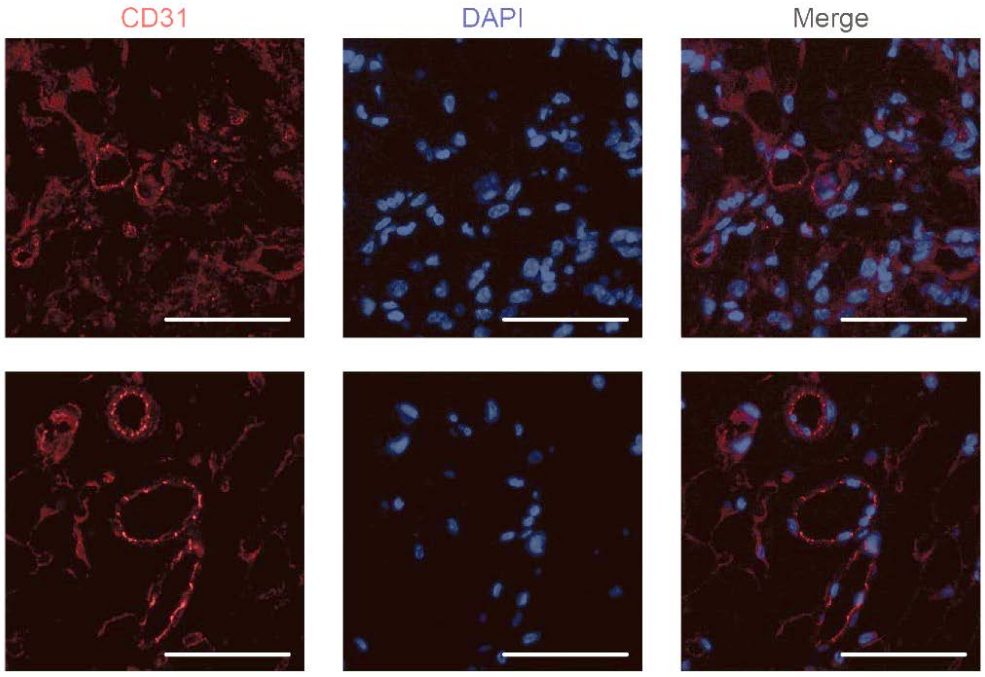
Supplementary Figure 13 The mRNA levels of CYP genes in 3DP-HOs. The mRNA levels of the uninduced CYP and induced CYP enzymes were determined by qPCR. CYP1A2 was induced by 3-methylcholanthrene. CYP2A6, CYP2B6, CYP2C8, CYP2D6, and CYP3A11 were induced by phenobarbital. CYP2C9 and CYP3A4 were induced by rifampicin. Almost all the difference between the uninduced CYP and induced CYP enzymes were significant except CYP2A6 in 2D culture group and CYP2C9 in undiff. group.



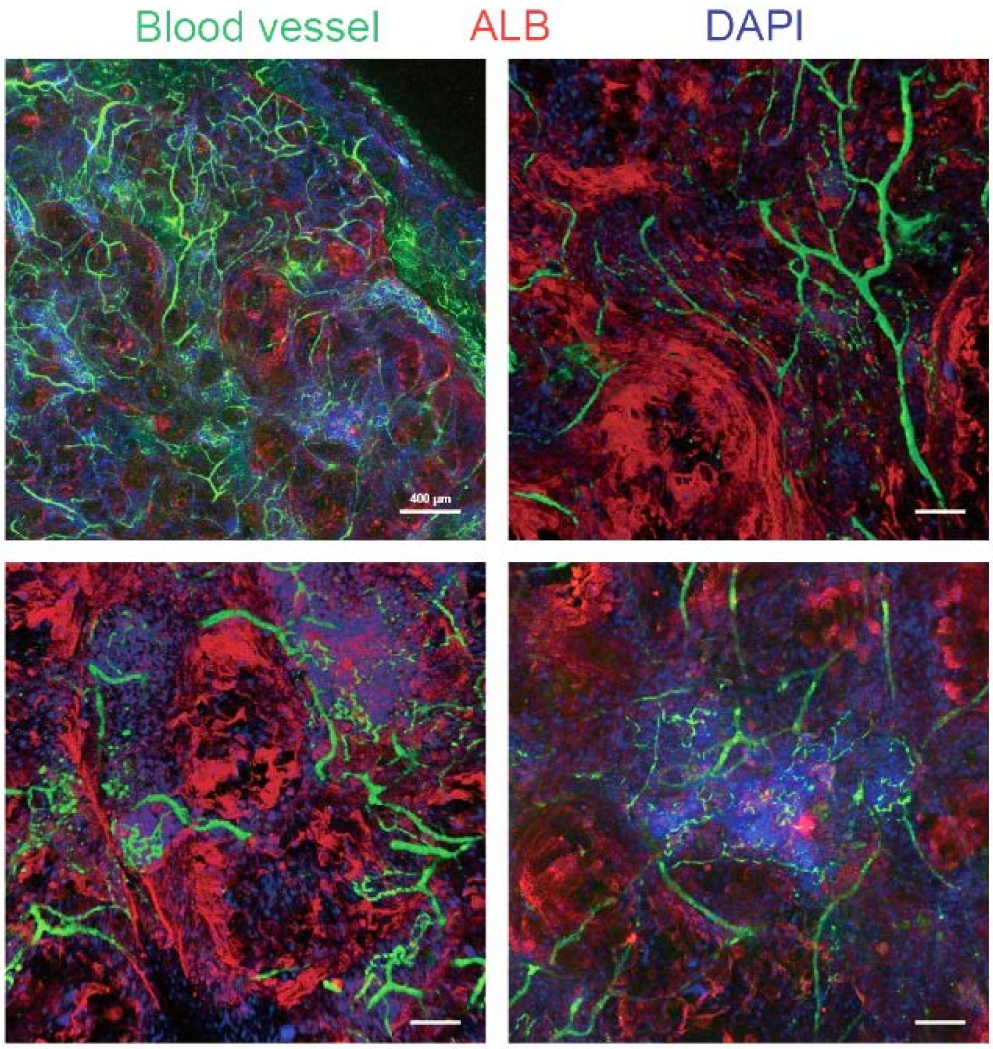
Supplementary Figure 14 Mice liver injury after 3DP-HOs transplantation. Scale bar, 100 μm.



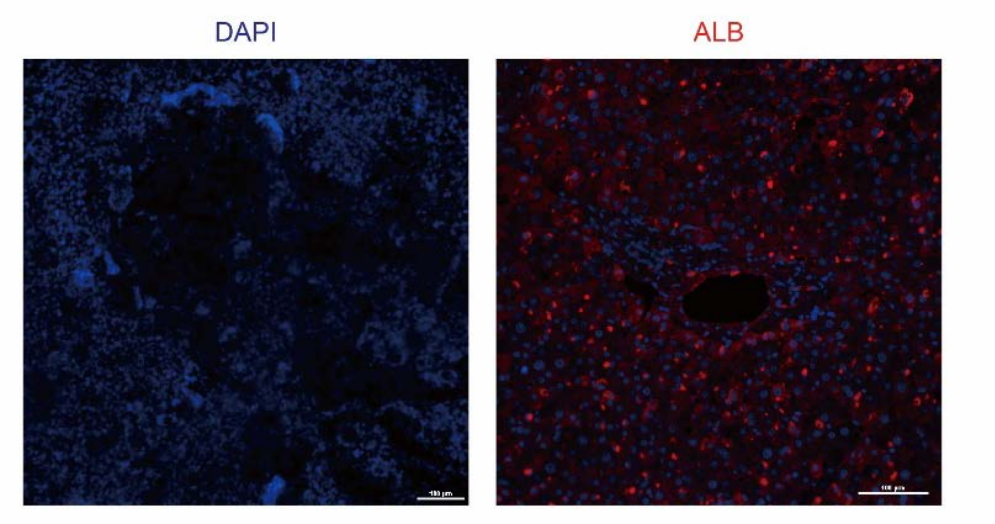
Supplementary Figure 15 Histopathological HE staining of 3DP-HOs transplanted *in vivo*. Scale bar, 100 μm.



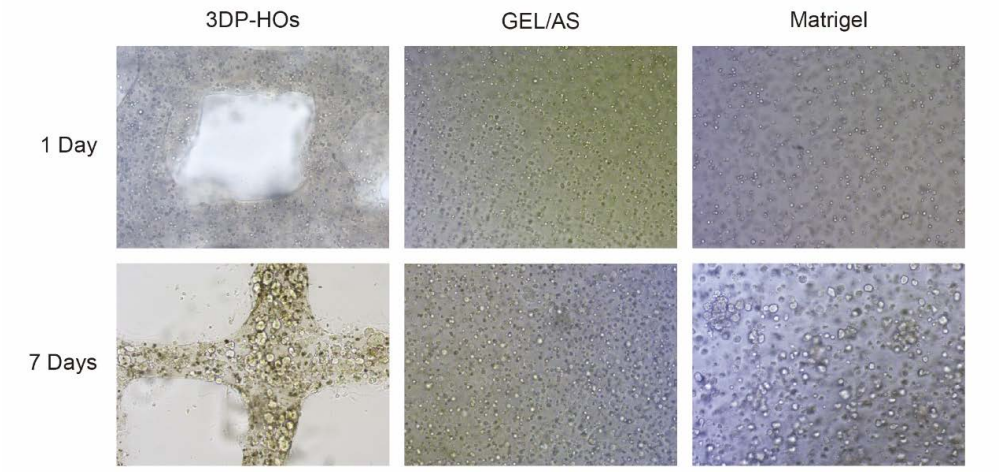
Supplementary Figure 16 Immunofluorescence staining of mouse CD31 in 3DP-HOs. Scale bar, 100 μm.



Supplementary Figure 17 Dextran infusion showing functional vessel formation (green) and red fluorescence showing human ALBUMIN expression (red) at 4 weeks. Scale bar, 100 μm.



Supplementary Figure 18 Negative control and positive control (human liver tissue slide) of 3DP-HOs *in vivo*. Red fluorescence showing human ALBUMIN expression and blue fluorescence showing DAPI at 4 weeks. Scale bar, 100 μm.



Cell cluster diameter	3DP-HOs	GEL/AS	Matrigel
1 Day	12.8 ± 1.8 μm	11.0 ± 2.0 μm	11.1 ±1 .4 μm
7 Days	125.2 ± 13.2 μm	20.7 ± 2.3 μm	27.0 ± 3.0 μm

Supplementary Figure 19 The growth of cell clusters in 3D culture systems constructed in different ways and matrix.

Supplementary Table 1 Serum amino acid concentrations after 3DP-HOs transplantation

Amino acid (μg/ml)	WT	3DP-HOs	Sham
P-Ser	2.83±0.14	3.23±0.61	7.45±5.94
Tau	25.78±3.13	30.40±5.78	41.16±13.13
PEA	/	0.34±0.12	0.43±0.25
Urea	339.85±65.53	414.97±31.80	362.70±66.58
Asp	0.96±0.34	3.36±2.44	8.94±5.69
Thr	11.87±3.80	54.66±14.77	85.41±12.63
Ser	8.98±1.33	37.04±6.88	70.72±23.86
Glu	5.64±1.06	153.24±59.49*	345.98±80.69
Sar	2.00±0.40	0.96±0.25	1.48±0.37
a-AAA	2.77±0.11	1.99±0.93	1.28±0.31
Gly	17.78±3.51	36.39±4.25	64.28±18.67
Ala	23.73±1.08	89.19±28.26	214.85±58.58
Cit	10.92±2.65	11.97±1.07*	15.57±1.83
a-ABA	0.82±0.29	2.81±0.77	4.09±1.70
Val	14.11±6.96	20.02±1.61*	33.83±5.73
Cys	0.41±0.45	7.21±5.45	10.21±2.68
Met	6.42±2.02	21.28±11.45*	59.36±13.61
Cysthi	0.67±0.09	2.40±0.99	1.57±0.55
Ile	7.87±4.15	8.15±0.73*	13.90±2.19
Leu	11.22±6.66	14.19±2.67*	25.42±4.42
Tyr	7.24±0.61	80.33±4.62*	143.92±18.51
Phe	7.79±1.45	25.67±12.98	26.51±4.73
b-Ala	0.27±0.03	1.06±0.36	1.74±1.61
b-AiBA	/	2.54±0.48	1.93±1.16
g-ABA	0.31±0.05	0.75±0.32	0.79±0.28

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EOHNH2	2.24±0.24	3.20±2.17	4.64±0.47
Hylys	/	0.82±0.26	2.26±1.91
Orn	10.33±3.91	35.22±1.36	60.06±16.60
Lys	30.38±5.86	49.64±8.33	89.02±16.78
1Mehis	4.65±0.63	0.55±0.14	1.07±0.44
His	9.59±1.64	40.28±16.94	49.25±5.64
3Mehis	1.16±0.09	0.63±0.15	0.63±0.15
Ans	7.42±1.05	5.87±2.52	5.03±2.17
Car	7.91±0.11	2.67±0.90	2.82±1.74
Arg	9.64±2.35	5.50±4.86	3.88±2.29
Hypro	2.73±0.49	2.70±1.53*	9.09±1.92
Pro	8.45±2.05	14.39±5.50	36.56±11.72

Serum levels of amino acids were measured in wildtype mice (WT group), F/R mice 4 weeks after 3DP-HOs transplantation (3DP-HOs group), and F/R mice underwent the same surgical procedure but without transplantation (Sham group). Data were presented as mean ± S.D.. Asterisks indicate the values were significantly reduced compared with those in Sham group (P<0.05, *t-test*).

Supplementary Table 2 Primer table

Gene	Forward (5'-3')	Reverse (5'-3')
ALBUMIN	AGCATGGGCAGTAGCTCGCCT	AGGTCCGCCCTGTCATCAGCA
CK18	TCGCAAATACTGTGGACAATGC	GCAGTCGTGTGATATTGGTGT
CK19	TCCGAACCAAGTTTGAGACG	CCCTCAGCGTACTGATTTCCT
AAT	TATGATGAAGCGTTTAGGC	CAGTAATGGACAGTTTGGGT
FOXA2	GGAGCAGCTACTATGCAGAGC	CGTGTTCATGCCGTTCATCC
HNF4A	CAACCCAACCTCATCCTC	GTCCCATCTCACCTGCTC
TRANSFERRIN	TGTCTACATAGCGGGCAAGT	GTTCCAGCCAGCGGTTCT
MRP2	AGCGTCCTCTGACACTCG	GGCATCTTGGCTTTGACT
CYP1A2	GCCTTCATCCTGGAGACCTT	AGCGTTGTGTCCCTTGTTG
CYP2A6	GGGCCAAGATGCCCTACATG	CGTCAATGTCCTTAGGTGACTGG
CYP2B6	AAGCGGATTTGTCTTGGTGAA	TGGAGGATGGTGGTGAAGAAG
CYP2C8	CATTACTGACTTCCGTGCTACAT	CTCCTGCACAAATTCGTTTTCC
CYP2C9	TCCTATCATTGATTACTTCCCG	AACTGCAGTGTTTTCCAAGC
CYP2D6	TGAAGGATGAGGCCGTCTGGGAGA	CAGTGGGCACCGAGAAGCTGAAGT
CYP3A4	CAGGAGGAAATTGATGCAGTTTT	GTCAAGATACTCCATCTGTAGCACAGT
CYP3A11	ACA AACAAGCAGGGATGGAC	GGTAGAGGAGCACCAAGCTG
ACTB	GAGCTGCGTGTGGCTCCC	CCAGAGGCGTACAGGGATAGCA