

Supplementary Material

Evaluation of a Human 3D Multicellular Hepatic Spheroid Model as a Platform for Studying Hepatic Transporters

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Supplemental Table S1. Cryopreserved Transporter Certified™ human hepatocyte donor demographics and nonparenchymal cell donor information for each MHS preparation (3 donors).

Hepatocyte Donor Information			
Demographics	WID	JEL	BXW
Age (years)	71	27	73
Sex (M/F)	M	F	F
Race	Caucasian	African American	Caucasian
BMI (kg/m²)	25.7	28.2	32.6
COD	Head Trauma/Blunt Injury/MVA	Anoxia	CVA/Stroke; 2nd to ICB/ICH
Medical History	Hypertension x 15 yrs	Respiratory disease & quadriplegic	Hypertension x 20 yrs High cholesterol x 1-2 yrs
Medication Use	None reported	None reported	Cyclobenzaprine, Eliquis, Losartan, Madox, Metoprolol, omeprazole, premarin, Tricor, Vitamin D3
Social History	None reported	None reported	None reported
Nonparenchymal Cell Donor Information			
	Source	Lot and/or (Catalog No.)	
Kupffer Cells	Xenotech	H1292	
	Gibco	HK8176	
	BioIVT	UMQ	
	BioIVT	OJF	
	Lonza	HKC210471 (Catalog No. HLKC-200K)	
Stellate Cells	Lonza	HSC201951	
	BioIVT	BFL	
	ScienCell	34022 (Catalog No. 5300)	
	ScienCell	27742 (Catalog No. 5300)	
Endothelial Cells	Samsara	HL160017ECp2	
	BioIVT	OORAWMATS	
	ScienCell	(Catalog No. 5000)	

BMI, body-mass index; COD, cause of death; CVA, cerebral vascular accident; F, female; ICB, intracranial bleeding; ICH, intracerebral hemorrhage; M, male; MVA, motor vehicle accident

Supplemental Table S2. Selected signature peptides and multiple reaction monitoring (MRM) transitions for proteomic analysis of transporters and metabolizing enzymes. Bold letters show amino acids labeled with stable isotope (^{13}C and ^{15}N). Trypsin does not cleave the amide bond between lysine (K) and proline (P) as highlighted in red (KP).

Protein	Signature Peptide	MRM (Analyte; A, Internal Standard; IS)
BSEP	BSEP.TVQVALDK	A: 437.26/673.39 (y6), 437.26/545.33 (y5); IS: 441.26/681.40 (y6), 441.26/553.34 (y5)
OAT2	OAT2(H,M).NVALLALPR	A: 483.81/753.50 (y7), 483.81/569.38 (y5); IS: 488.81/763.51 (y7), 488.81/579.39 (y5)
OAT7	OAT7(H).DTLTLEILK	A: 523.31/716.46 (y6), 523.31/829.54 (y7); IS: 527.32/724.47 (y6), 527.32/837.55 (y7)
OCT1	OCT1.LSPSFADLFR	A: 576.81/476.75 (y8), 576.81/855.44 (y7); IS: 581.81/481.75 (y8), 581.81/865.44 (y7)
OST- α	OST-alpha.YTADLLEVLK	A: 582.83/900.54 (y8), 582.83/829.50 (y7); IS: 586.84/908.55 (y8), 586.84/837.52 (y7)
OST- β	OST-beta.DHNSLNNLR	A: 361.52/516.29 (y4), 361.52/454.17 (b4); IS: 364.85/526.30 (y4), 364.85/454.17 (b4)
MRP2	MRP2(H,M,D,R).LTIPQDPILFSGSLR	A: 885.51/441.31 (b4), 885.51/989.58 (y9); IS: 890.52/441.31 (b4), 890.52/999.59 (y9)
MRP3	MRP3(H,M,D,R,Ms).GALVAVVGPVGC GK	A: 642.36/674.33 (y7), 642.36/773.40 (y8); IS: 646.37/682.34 (y7), 646.37/781.41 (y8)
MRP4	MRP4(H,M,R,Ms).SSLISALFR	A: 497.29/593.34 (y5), 497.29/706.42 (y6); IS: 502.29/603.35 (y5), 502.29/716.43 (y6)
Na ⁺ /K ⁺ -ATPase	NaK ATPase.VDNSSLTGESEPQTR	A: 540.59/501.28 (y4), 810.38/501.28 (y4); IS: 543.92/511.29 (y4), 815.38/511.29 (y4)
P-gp	P-gp(H,M,D).IIDNKP SIDSYSK	A: 493.93/627.30 (y11), 493.93/896.44 (y8); IS: 496.60/631.31 (y11), 496.60/904.45 (y8)
OATP1B1	SLCO1B1,OATP1B1.NVTGFFQSFK	A: 587.80/961.48 (y8), 587.80/860.43 (y7);

		IS: 591.81/969.49 (y8), 591.81/868.44 (y7)
OATP1B3	SLCO1B3,OATP1B3.IYNSVFFGR	A: 551.79/826.42 (y7), 551.79/712.38 (y6); IS: 556.79/836.43 (y7), 556.79/722.39 (y6)
OATP2B1	SLCO2B1,OATP2B1.SSPAVEQQLLVSGPGK	A: 798.93/711.90 (y14), 532.96/544.31 (y6); IS: 802.94/715.91 (y14), 535.63/552.32 (y6)
CYP3A4	CYP3A4.LSLGGLLQPEKPVVLK	A: 564.35/689.43 (y13), 564.35/660.92 (y12); IS: 567.03/693.43 (y13), 567.03/664.92 (y12)
CYP3A5	CYP3A5.DTINFLSK	A: 469.25/217.08 (b2), 469.25/721.42 (y6); IS: 473.26/217.08 (b2), 473.26/729.44 (y6)
UGT1A1	UGT1A1.DGAFYTLK	A: 457.73/671.38 (y5), 457.73/524.31 (y4); IS: 462.75/681.40 (y5), 462.75/524.31 (y4)
Aldehyde Oxidase	Aldehyde Oxidase.HLGTLAGSQIR	A: 384.89/560.32 (y5), 384.89/522.30 (b5); IS: 388.22/570.32 (y5), 388.22/522.30 (b5)
CYP2C9	CYP2C9.SLVDPK	A: 329.69/458.26 (y4), 329.69/ 244.17 (y2); IS: 333.70/466.28 (y4), 333.70/252.18 (y2)
CYP2C19	CYP2C19.GHFPLAER	A: 309.50/439.21 (b4), 309.50/375.20 (y3); IS: 312.84/439.21 (b4), 312.84/385.21 (y3)

BSEP, bile salt export pump; CYP, cytochrome P450; MRP, multidrug resistance-associated protein; Na⁺/K⁺-ATPase, sodium-potassium pump; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; OST, organic solute transporter; P-gp, P-glycoprotein; UGT, UDP-glucuronosyltransferase

Supplemental Table S3. Source and dilution of primary and secondary antibodies used for immunofluorescent analysis.

Antibody/Protein	Source (Catalog No.)	Host	Dilution	Antibody type/target class
NTCP	GeneTex (GTX17693)	Rabbit	1:30	Primary/transporter
OATP1B3	Custom-made; provided by Dr. Wei Yue	Rabbit	1:250	Primary/transporter
BSEP	Santa Cruz Biotechnology (F-6, sc-74500)	Mouse	1:40	Primary/transporter
MRP2	Abcam (ab172630)	Rabbit	1:250	Primary/transporter
Na ⁺ /K ⁺ -ATPase	Santa Cruz Biotechnology (sc-48345)	Mouse	1:100	Primary/membrane marker
Na ⁺ /K ⁺ -ATPase	Abcam (ab185065)	Rabbit	1:200	Primary/membrane marker
ZO1	Abcam (ab221547)	Rabbit	1:100	Primary/membrane marker
ZO1	ThermoFisher (33-9100)	Mouse	1:100	Primary/membrane marker
CD-13	Abcam (ab108382)	Rabbit	1:750	Primary/membrane marker
Goat anti-mouse IgG Alexa Fluor 568	Invitrogen (A11037)	-	1:100	Secondary
Goat anti-rabbit IgG Alexa Fluor 488	Invitrogen (A11008)	-	1:100	Secondary
Goat anti-mouse IgG Alexa Fluor 647	Invitrogen (A21235)	-	1:100	Secondary

BSEP, bile salt export pump; CD-13, aminopeptidase N; MRP, multidrug resistance-associated protein; Na⁺/K⁺-ATPase, sodium-potassium pump; NTCP, sodium-taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide; ZO1, zonula occludens-1

Supplemental Table S4. Gene-specific TaqMan probes used for mRNA analysis.

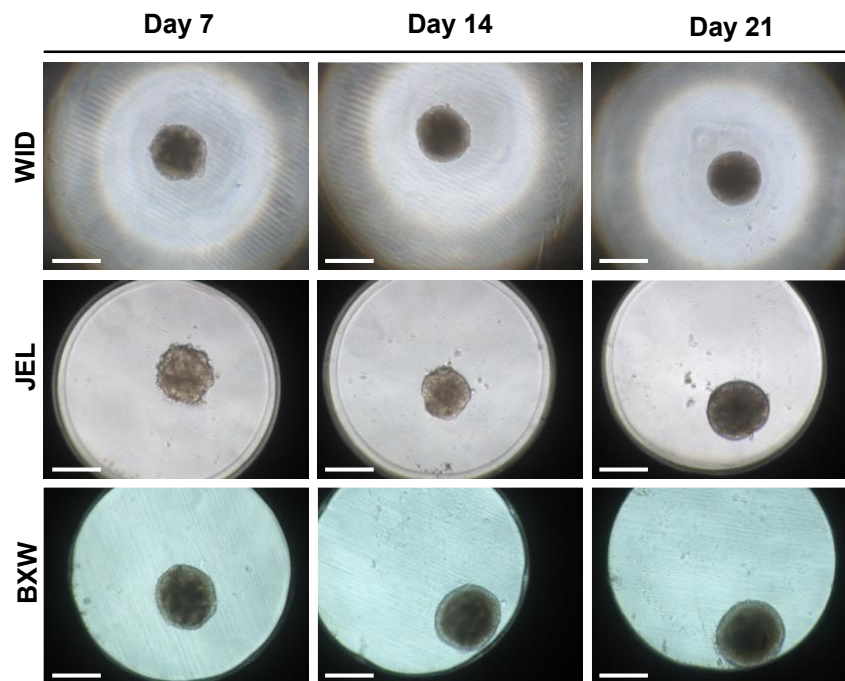
Target Gene	TaqMan Probe	Dye
<i>β-Actin</i>	Hs01060665_g1	FAM-MGB
<i>SLC51A</i> (OST- α)	Hs00380895_m1	FAM-MGB
<i>SLC51B</i> (OST- β)	Hs01057182_m1	FAM-MGB

OST, organic solute transporter; SLC, Solute carrier

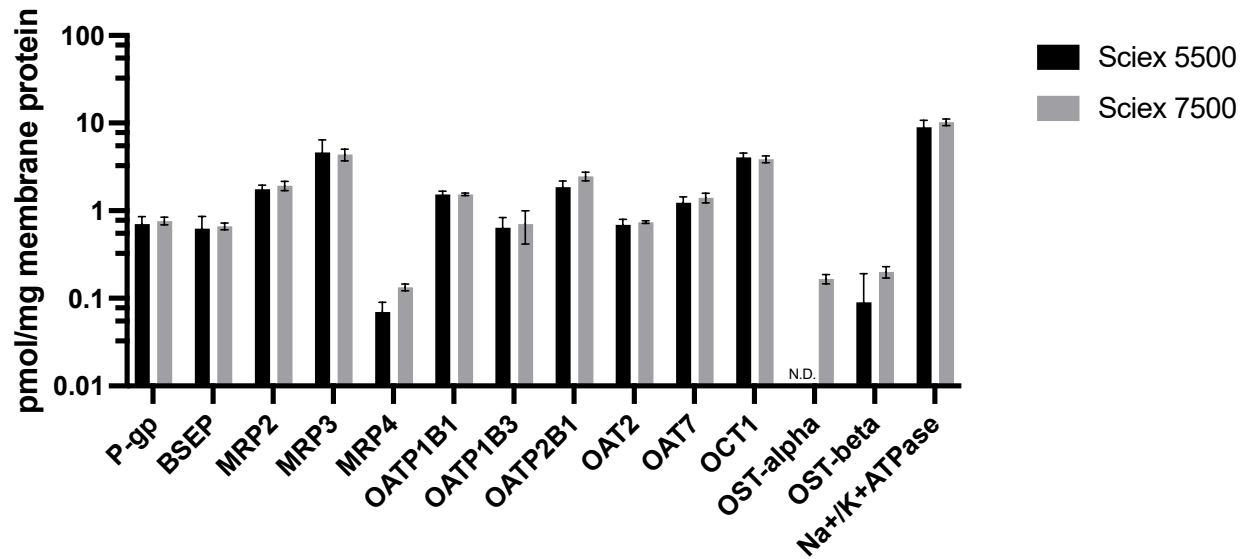
Supplemental Table S5. Multiple reaction monitoring (MRM) transitions, collision energy and source of bile acid species and internal standards (IS) measured using liquid chromatography with tandem mass spectrometry (LC-MS/MS).

Bile Acid Species	Parent ion m/z	Daughter ion m/z	Collision Energy (V)	Retention Time (min)	Source (Catalog No.)
Cholic acid (CA)	407.2	407.2/343.0	-40/-44	17.1	Sigma (1133503)
Taurocholic acid (TCA)	514.9	514.9/79.8	-62/-126	11.9	Sigma (T4009)
Glycocholic acid (GCA)	464.0	73.9	-94	14.0	Sigma (G2878)
Chenodeoxycholic acid (CDCA)	391.2	391.2	-38	18.4	Sigma (C9377)
Taurochenodeoxycholic acid (TCDCA)	498.0	498.0/80.0	-38/-130	14.9	Sigma (T6260)
Glycochenodeoxycholic acid (GCDCA)	448.0	448.0/74.0	-38/-78	17.7	Sigma (G7059)
GCDCA 3-sulfate (GCDCA-S)	528.0/263.6	528.0/74.1	-38/-24	13.8	Toronto Research Chemicals (TRC; G641270)
GCDCA 3-O-glucuronide (GCDCA-3G)	624.2	448.2	-38	14.3	TRC (G641275)
TCA-d5 (IS)	519.2	519.2/79.8	-62/-128	11.9	TRC (T008852)
GCA-d5 (IS)	469.7	74.0	-94	14.0	TRC (G641357)
CDCA-d4 (IS)	395.2	395.2	-38	18.4	TRC (C291902)
TCDCA-d5 (IS)	503.9	80.0	-130	14.9	TRC (T008133)
GCDCA-d7 (IS)	454.8	74.8	-38	17.6	TRC (G641257)

Supplemental Figure S1. Brightfield images of MHS from all three Transporter Certified™ human hepatocyte donors taken with a 10X objective on days 7, 14, and 21 of culture. Scale bar = 200 μ m.

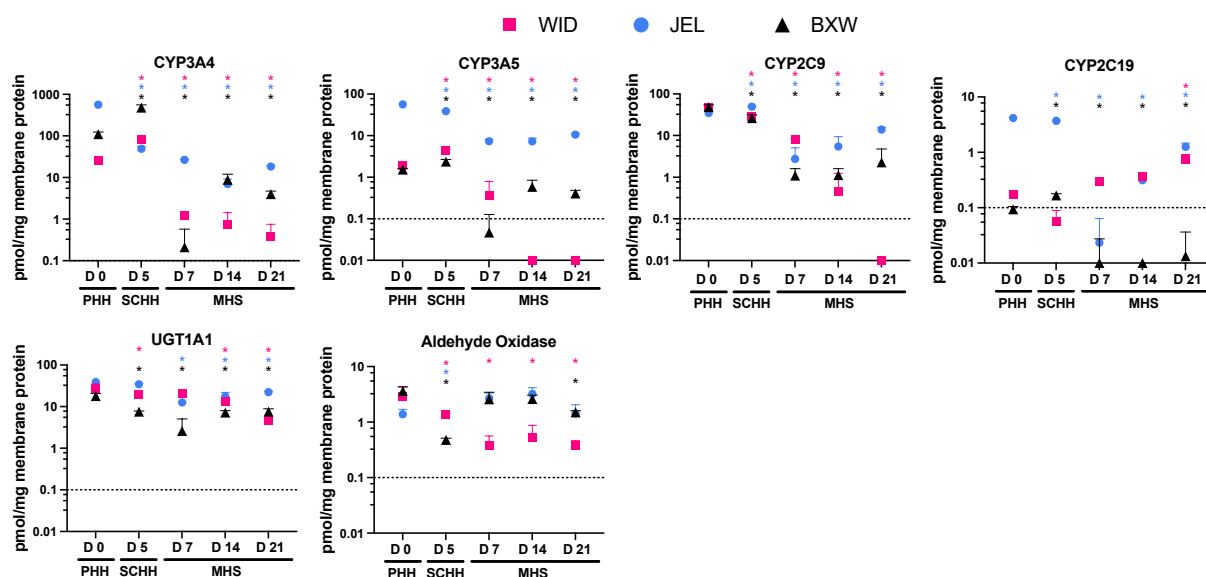


Supplemental Figure S2. Transport protein concentrations of the same triplicate samples (Day 5 SCHH; donor BXW) measured on the Sciex 5500 and the Sciex 7500.



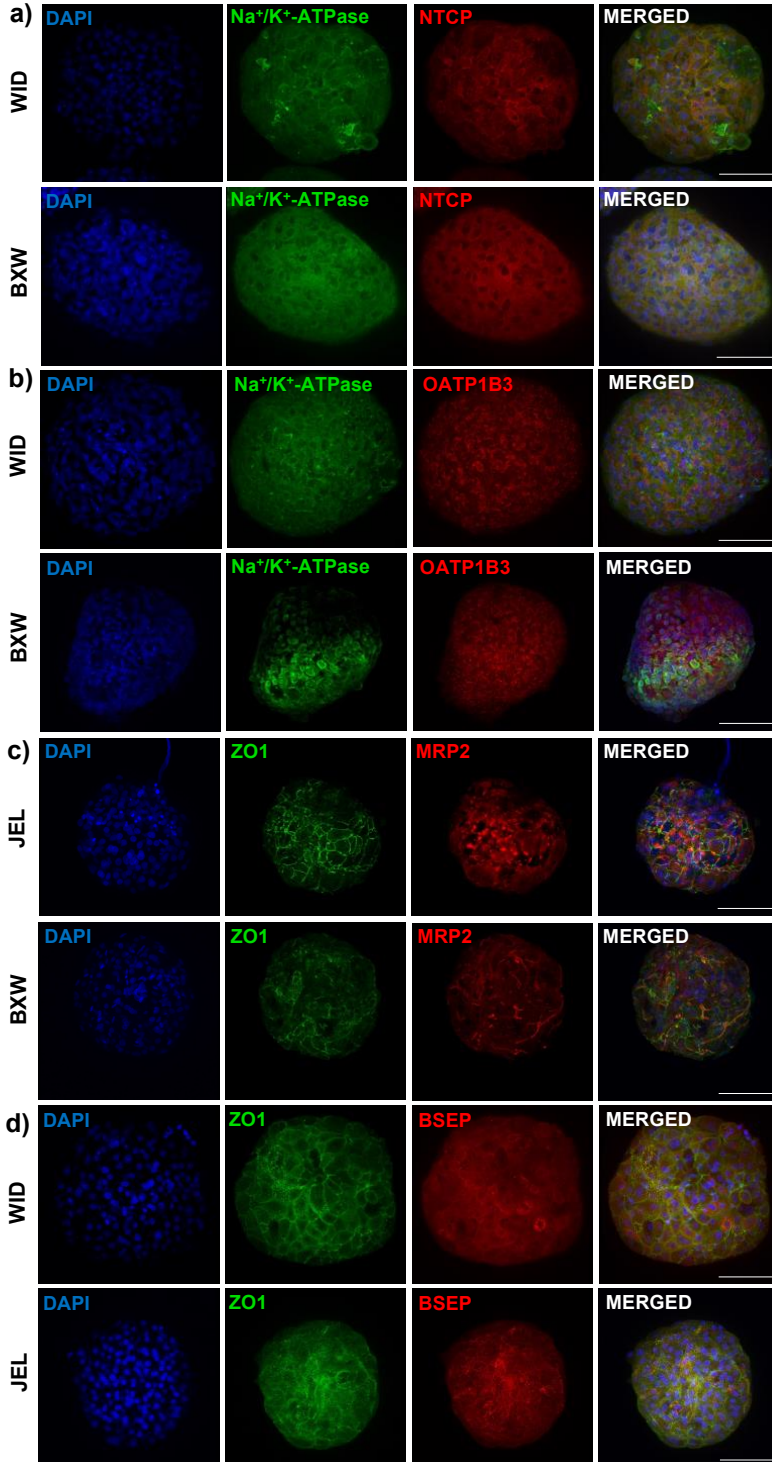
BSEP, bile salt export pump; MRP, multidrug resistance-associated protein; Na⁺/K⁺-ATPase, sodium-potassium pump; N.D., not detected; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; OST, organic solute transporter; P-gp, P-glycoprotein

Supplemental Figure S3. Select drug-metabolizing enzyme protein concentrations in human hepatic multicellular spheroids (MHS) over a 21-day culture compared to sandwich-cultured human hepatocytes (SCHH) on culture day 5 and freshly thawed primary human hepatocytes (PHH) from the same Transporter Certified™ hepatocyte donors. Data represent n=3 technical replicates (mean±SD) at each timepoint for each of 3 hepatocyte donors [WID (pink), JEL (blue), and BXW (black)]. Statistically significant differences were measured using a one-way analysis of variance (ANOVA) by donor, compared to day (D) 0, with Dunnett's multiple comparison [*, P < 0.05]. The dotted line represents the LLOQ of 0.1 pmol/mg membrane protein. When all three replicates were equal to zero, mean values were imputed as 0.01 pmol/mg membrane protein for visualization on a log scale.



CYP, cytochrome P450; UGT, UDP-glucuronosyltransferase

Supplemental Figure S4. Donor variability in cellular localization of transporters in whole human multicellular hepatic spheroids (MHS) on culture day 14. Staining of membrane markers and select transporters **a)** NTCP, **b)** OATP1B3, **c)** MRP2 **d)** BSEP in whole MHS from different donors [WID, JEL, or BXW]. Images were taken with a Zeiss LSM 880 Confocal Laser Scanning Microscope with a 20X objective. DAPI = Nuclei stain. Scale bar = 100 μ m.



BSEP, bile salt export pump; MRP2, multidrug resistance-associated protein 2; Na^+/K^+ -ATPase, sodium-potassium pump; NTCP, sodium-taurocholate cotransporting polypeptide OATP, organic anion transporting polypeptide; ZO1, zonula occludens-1