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**Drug transporter expression and activity in cryopreserved human hepatocytes isolated
from chimeric TK-NOG mice with humanized livers**

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Abstract

Chimeric mice with humanized liver are thought to represent a sustainable source of isolated human hepatocytes for *in vitro* studying detoxification of drugs in humans. Because drug transporters are now recognized as key-actors of the hepatic detoxifying process, the present study was designed to characterize mRNA expression and activity of main hepatic drug transporters in cryopreserved human hepatocytes isolated from chimeric TK-NOG mice and termed HepaSH cells. Such cells after thawing were shown to exhibit a profile of hepatic solute carrier (SLC) and ATP-binding cassette (ABC) drug transporter mRNA levels well correlated to those found in cryopreserved primary human hepatocytes or human livers. HepaSH cells used either as suspensions or as 24 h-cultures additionally displayed notable activities of uptake SLCs, including organic anion transporting polypeptides (OATPs), organic anion transporter 2 (OAT2) or sodium-taurocholate co-transporting polypeptide (NTCP). SLC transporter mRNA expression, as well as SLC activities, nevertheless fell in HepaSH cells cultured for 120 h, which may reflect a partial dedifferentiation of these cells with time in culture in the conventional monolayer culture conditions used in the study. These data therefore support the use of cryopreserved HepaSH cells as either suspensions or short-term cultures for drug transport studies.

Keywords

Cell culture; Cell suspension; Drug transporter; Human hepatocytes; Humanized liver mice; Transport activity.

1. Introduction

Chimeric mice with humanized livers represent an attractive emergent model for studying detoxification and toxicity of drugs in humans (Bateman et al., 2014; Kitamura and Sugihara, 2014; Strom et al., 2010; Xu and Peltz, 2016). They are generated by engrafting primary human hepatocytes into the livers of immunodeficient mice, whose native hepatocytes have been previously ablated by genetic modifications that result in toxicity within the murine liver cells (Duncan et al., 2009; Sugahara et al., 2020). Three main models of humanized liver chimeric mice have been developed and are currently commercially available: (i) the urokinase-type plasminogen activator (uPA)/severe combined immunodeficiency (SCID) model (also known as the PhoenixBio/PXB model) (Tateno et al., 2015), (ii) the fumarylacetoacetate hydrolase (Fah)^{-/-}/recombination activating gene 2 protein (Rag2)^{-/-}/interleukin 2 receptor γ (IL2rg)^{-/-} model (also known as the FRG model) (Azuma et al., 2007), and (iii) the herpes simplex virus-1 thymidine kinase (HSVtk)/non-obese diabetic (NOD)/Shi-SCID/IL2rg^{-/-} (NOG) model (called the TK-NOG model) (Hasegawa et al., 2011; Uehara et al., 2022a). The TK-NOG mice express the transgene HSVtk in the liver under the regulation of a mouse albumin enhancer/promoter, resulting in the hepatic formation of toxic metabolites in response to ganciclovir administration; this allows destruction of the mouse liver before engraftment with injected primary human hepatocytes (Hasegawa et al., 2011). Human hepatic drug detoxifying proteins, including phase I and II drug metabolizing enzymes and sinusoidal and canalicular drug transporters, are expressed in the repopulated livers of humanized mice (Kato et al., 2004; Uehara et al., 2022b; Zerdoug et al., 2022). Such mice have been consequently successfully used for *in vivo* analysing pharmacokinetics (Dickie et al., 2017; Miura et al., 2019; Yoshida et al., 2022), human hepatic metabolism and secretion of drugs and pollutants (Bateman et al., 2014; Kato et al., 2020; Uehara et al., 2021) and drug-drug interactions (Uchida et al., 2018).

Chimeric mice with humanized liver constitute a sustainable source of human hepatocytes, after enzymatic dissociation of livers (Harimoto et al., 2018; Wei et al., 2022). Interestingly, these human hepatocytes isolated from chimeric mice have been shown to exhibit substantial levels of human drug detoxifying enzymes, including cytochromes P-450 (CYPs) (Bachour-El Azzi et al., 2022; Ishida et al., 2020; Uehara et al., 2019), making them attractive for *in vitro* drug metabolism and toxicity studies (Kakuni, 2022; Sanoh and Ohta, 2014). Fresh or cryopreserved suspended human hepatocytes from chimeric mice thus display notable activity of various CYPs, as well as UDP-glucuronosyltransferase activity (Yamasaki et al., 2010), whereas their cultured counterparts are fully responsive to main inducers of CYP expression (Kakuni et al., 2013). Besides CYPs, plasma membrane drug transporters, implicated in drug uptake and secretion at the sinusoidal and canalicular pole of hepatocytes, respectively, are recognized as major actors of the hepatic drug detoxification way (Jetter and Kullak-Ublick, 2020; Nies et al., 2022). Some of them, such as the sinusoidal uptake transporters organic anion transporting polypeptide (OATP) 1B1 (*SLCO1B1*) and OATP1B3 (*SLCO1B3*) and the canalicular efflux pumps P-glycoprotein (*ABCB1*), encoded by the gene *MDR1*, and breast cancer resistance protein (*BCRP/ABCG2*), have to be studied during the pharmaceutical development of new molecular entities according to a regulatory point of view (Sudsakorn et al., 2020). Expression of drug transporters in human hepatocytes isolated from chimeric mice remains however rather poorly characterized, even if cultured human hepatocytes from PXB mice display expression of transporters (Kohara et al., 2020), whose levels may be regulated by culture density (Yamasaki et al., 2020). The present study was therefore designed to accurately describe expression and activity of main drug hepatic transporters in cryopreserved human hepatocytes prepared from TK-NOG mice, termed HepaSH cells and used as suspensions or monolayer cultures.

2. Materials and methods

2.1. Chemicals

[³H(G)] taurocholic acid (specific activity = 15.5 Ci/mmol), [6, 7-³H(N)] estrone-3-sulfate (E3S) (specific activity = 51.8 Ci/mmol), [8-³H] cyclic guanosine monophosphate (cGMP) (specific activity = 2.4 mCi/mmol) and [1-¹⁴C] tetra-ethylammonium (TEA) (specific activity = 3.5 mCi/mmol) were purchased from Perkin–Elmer (Boston, MA, USA). Probenecid, verapamil, bromosulphophthalein (BSP) and ketoprofen were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell preparation and culture

Cryopreserved HepaSH cells were prepared at Central Institute for Experimental Animals (Kawasaki, Japan), as previously described (Uehara et al., 2019). A total of 11 vials of HepaSH cells, prepared from TK-NOG mice engrafted with primary human hepatocytes from three human donors, was used in the study; each vial originated from one engrafted TK-NOG mice and was considered as one batch in the present study. Information about the human donors as well as the number of cell batches per donor used in the study are indicated in Table 1. The contamination of these cryopreserved HepaSH cells by mouse cells before cryopreservation was less than 3.0%, as demonstrated by immunolabeling of cells with monoclonal antibodies anti-human leukocyte antigen and anti-mouse H-2kd and subsequent flow cytometry analysis (Uehara et al., 2020) (data not shown). Cryopreserved human hepatocytes, prepared from human liver fragments, were obtained from Biopredic International (Saint-Grégoire, France), Lonza (Basel, Switzerland), Xenotech (Kansas City, KS, USA) or BioIVT (West Sussex, United Kingdom). Characteristics of the donors for these human hepatocytes are shown in Table 1.

Table 1: Donor information for batches of cryopreserved HepaSH cells and primary human hepatocytes used in the study

Donor (Human hepatocytes)	Supplier	Age	Sex	Ethnicity	Cryopreserved human hepatocytes	Cryopreserved HepaSH cells
Lhum17003	Biopredic	31 years old	Male	African	1 batch	1 batch
HUM4282	Lonza	11 months	Male	Hispanic	1 batch	5 batches
HEP187266	Biopredic	5 years old	Male	Caucasian	-	3 batches
CHP161007	Biopredic	6 years old	Male	NA	1 batch	-
HEP187318	Biopredic	39 years old	Female	NA	1 batch	-
HEP187378	Biopredic	74 years old	Female	NA	1 batch	-
HEP187163	Biopredic	69 years old	Female	Caucasian	1 batch	-
HEP187214	Biopredic	38 years old	Male	Caucasian	1 batch	-
HEP187216	Biopredic	39 years old	Female	NA	1 batch	-
HEP187272	Biopredic	8 years old	Male	Caucasian	1 batch	-
HEP187058	Biopredic	69 years old	Female	NA	1 batch	-
HC5-27	Xenotech	21 years old	Male	Caucasian	1 batch	-
HC5-30	Xenotech	21 years old	Male	Caucasian	1 batch	-
ONR	BioIVT	74 years old	Female	Caucasian	1 batch	-

NA, not available; -, not provided.

Vials of cryopreserved HepaSH cells and primary human hepatocytes were thawed quickly at 37 °C in a water bath. The vial content was next emptied into 37°C pre-warmed OneStep PHep Thawing Medium (Biopredic International) and centrifuged at 200 x g for 6 min. Cells were then collected for resuspension in Williams' E medium (Invitrogen, Cergy-Pontoise,

France), supplemented with 10% fetal calf serum (FCS) (Cytiva, Velizy-Villacoublay, France), 5 µg/ml bovine insulin (Sigma–Aldrich), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine (Invitrogen). Cells were either immediately collected for further RNA isolation or transport assays or seeded on rat collagen type-I-coated plates at density of 1.6×10^5 cells/cm² for culturing them under a monolayer 2-D configuration. After 24 h, medium was discarded, and hepatocytes were cultured in maintenance medium composed of Williams' E medium, supplemented with 5 µg/ml bovine insulin, $5 \cdot 10^{-5}$ M hydrocortisone hemisuccinate (Upjohn, Paris La Défense, France), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. The culture medium was routinely renewed every 2-3 days. Phase contrast imaging of cell cultures was performed using an Axiovert 40 C phase contrast microscope (Carl Zeiss, Le Pecq, France).

2.3. Immunocytochemistry

Cultured cells were first fixed with 4% (weight/volume) paraformaldehyde/ phosphate-buffered saline (PBS) solution for 15 min, permeabilized with 0.2% (vol/vol) Triton X-100/PBS for 15 min and then incubated in a 3% (weight/vol) bovine serum albumin/PBS blocking solution for 30 min at room temperature. Cell were next incubated with primary antibodies, *i.e.*, goat monoclonal antibody directed against human albumin (Reference A80-129A, Bethyl laboratories, Montgomery, TX, USA), used at a final dilution of 1:1000, or mouse monoclonal antibodies against H-2Kd/H-2Dd (Reference 34-1-2, SouthernBiotech, Birmingham, AL, USA), used at a final dilution of 1:200, or against human multidrug resistance-associated protein (MRP) 2 (*ABCC2*) (Reference ab172630, Abcam, Cambridge, United Kingdom), used at a final dilution of 1:500. Cells were thereafter incubated with secondary Alexa FluorTM-coupled antibodies (References A-32816, A-21200, and A-21441, Cell Signaling, Leiden, The Netherlands), used at final dilutions of 1:500, 1:200 and 1:1000, respectively. Nuclei were then stained with 4',6-diamidino-2-phenylindole and fluorescent immunolabeling was finally

visualized using a CELENA™ S Digital Imaging System (Logos Biosystems, Villeneuve d'Ascq, France). Mouse cells were identified as mouse H-2Kd/H-2Dd-positive and human albumin-negative cells.

2.4. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays

Total RNAs were extracted from cells using the TRIzol reagent (Invitrogen/Life Technologies), and were then reverse-transcribed to cDNA using the reverse-transcription (RT) kit from Applied Biosystems (Foster City, CA, USA). Quantitative polymerase chain reaction (qPCR) assays were next performed using the fluorescent dye SYBR Green methodology and a CFX384 real-time PCR detection system (Bio-Rad, Marnes-la-Coquette, France), as previously described (Le Vee et al., 2015). Gene primers were exactly as previously described (Le Vee et al., 2015; Moreau et al., 2011). The sequence of the other primers (in the 5' to 3' orientation) were: organic solute transporter (OST) α (*SLC51A*) sense, TACAGCATCCTTTCATTGTC, OST α antisense, TATTCCTCTAAAACCAGGTCTC, OST β (*SLC51B*) sense CTTAGGTTGTTTAGGCTGTTG, OST β antisense GCAGAAAAGAAAAGATGCAG; albumin sense, GGGCATGTTTTTGTATGAAT and albumin antisense, CCCACTTTTCCTAGGTTTCT. Amplification curves were analyzed with the CFX Maestro software (Bio-Rad, Hercules, CA, USA), using the comparative cycle threshold method. Relative quantification of the steady-state target mRNA levels was calculated after normalization of the total amount of cDNA tested to the 18S rRNA endogenous reference, using the $2^{-\Delta C_t}$ method. Data were commonly expressed as arbitrary units (a.u.) comparatively to 18S rRNA, knowing that the amount of 18S rRNA in the sample was arbitrarily set at 10^6 a.u., as previously described (Moreau et al., 2011), or as percentage of values found in suspended hepatocytes (for cultured cells). Variability of transporter mRNA expression among HepaSH cells or primary human hepatocyte batches was assessed through

determining the coefficient of variation for their expression, *i.e.*, the ratio of the standard deviation to the expression mean.

2.5. Expression of drug transporter mRNAs in human livers

Data for transporter mRNA expression in human adult livers (n=226), determined by RNA-sequencing through the Genotype-Tissue Expression (GTEx) project (Melé et al., 2015), were collected via The Human Protein Atlas website (<https://www.proteinatlas.org/>, accessed: 6 December 2022). For each transporter, mRNA expression mean (for ranking transporter expression) and coefficient of variation were considered.

2.6. Drug transport assays

Activity of the sinusoidal uptake transporters, *i.e.*, sodium-taurocholate cotransporting polypeptide (NTCP/*SLC10A1*), OATPs, organic cation transporter 1 (OCT1/*SLC22A1*) and organic anion transporter 2 (OAT2/*SLC22A7*), was determined through measuring accumulation of radiolabeled reference substrates into cells, as previously described (Le Vee et al., 2015), either immediately after thawing cryopreserved cells (assay with cell suspensions) or after 24 h or 120 h of culture (assay with cell cultures). Briefly, for OATP, OCT1 or OAT2 activity measurement, cells were incubated for 5 min at 37°C with transport assay buffer consisting of 5.3 mM KCl, 1.1 mM KH₂PO₄, 0.8 mM MgSO₄, 1.8 mM CaCl₂, 11 mM D-glucose, 10 mM HEPES and 136 mM NaCl (standard sodium-containing buffer), adjusted to pH=7.4, and containing the OATP substrate [³H] E3S (12.9 nM), with or without the OATP inhibitor BSP (100 μM), the OCT1 substrate [¹⁴C] TEA (30 μM), with or without the OCT1 inhibitor verapamil (50 μM), or the OAT2 substrate [³H] cGMP (35 μM), with or without the OAT2 inhibitor ketoprofen (300 μM). For NTCP activity measurement, cells were incubated with the NTCP substrate [³H] taurocholate (12.9 nM), in the sodium-containing transport assay medium described above and also in sodium-free transport assay medium in which NaCl was

replaced by N-methylglucamine. Cells were then centrifuged and washed with ice-cold PBS (for cell suspension assay) or directly washed with ice-cold PBS (for cell culture assay), before being lysed in distilled water. Cellular accumulation of radiolabeled substrates was next determined through scintillation counting and normalized to cellular protein content, measured by Bradford's method (Bradford, 1976). Taurocholate accumulation values in the presence of sodium minus accumulation values in the absence of sodium, E3S uptake values in the absence of BSP minus uptake values in the presence of BSP, TEA uptake values in the absence of verapamil minus uptake values in the presence of verapamil and cGMP uptake values in the absence of ketoprofen minus uptake values in the presence of ketoprofen are thought to represent NTCP-, OATPs-, OCT1- and OAT2-related transport activities (Jigorel et al., 2005; Le Vee et al., 2015).

2.7. Statistical analysis

Experimental data were statistically analysed through unpaired Student's *t* test, ratio paired *t* test, Kruskal-Wallis test followed by the Dunn's post-hoc test, or Spearman's rank correlation, using the Prism 8.4.3 software (GraphPad Software, San Diego, CA, USA). The criterion of significance was $p < 0.05$. For quantifying transporter expression or activity change in HepaSH cells versus primary human hepatocytes, the fold increase, *i.e.*, the ratio expression or activity in HepaSH cells versus that in primary human hepatocytes, and the fold decrease, *i.e.*, the ratio expression or activity in primary human hepatocytes versus that in HepaSH cells, were used.

3. Results

3.1 Expression of drug transporter mRNAs in suspension of cryopreserved HepaSH cells

Expressions of drug transporter mRNAs in suspended HepaSH cells after their thawing are shown in Fig. 1 (for SLC transporters) and Fig. 2 (for ABC transporters). Suspended HepaSH cells displayed very high mRNA expression (mRNA expression >1000 a.u.) for OATP1B1, OATP1B3 and OAT2; they exhibited high levels of mRNAs (400 a.u. < mRNA expression < 1000 a.u.) for NTCP, OCT1, MDR1 and MRP2, whereas they showed intermediate mRNA levels (50 a.u. < mRNA expression < 400 a.u.) for OATP2B1 (*SLCO2B1*), MRP3 (*ABCC3*) and OST β , or low mRNA expression (mRNA expression < 50 a.u.) for OST α , bile salt export pump (BSEP/*ABCB11*), MRP4 (*ABCC4*) and BCRP. When compared to suspended primary human hepatocytes, HepaSH cells displayed similar mRNA levels of NTCP, OATP1B1,

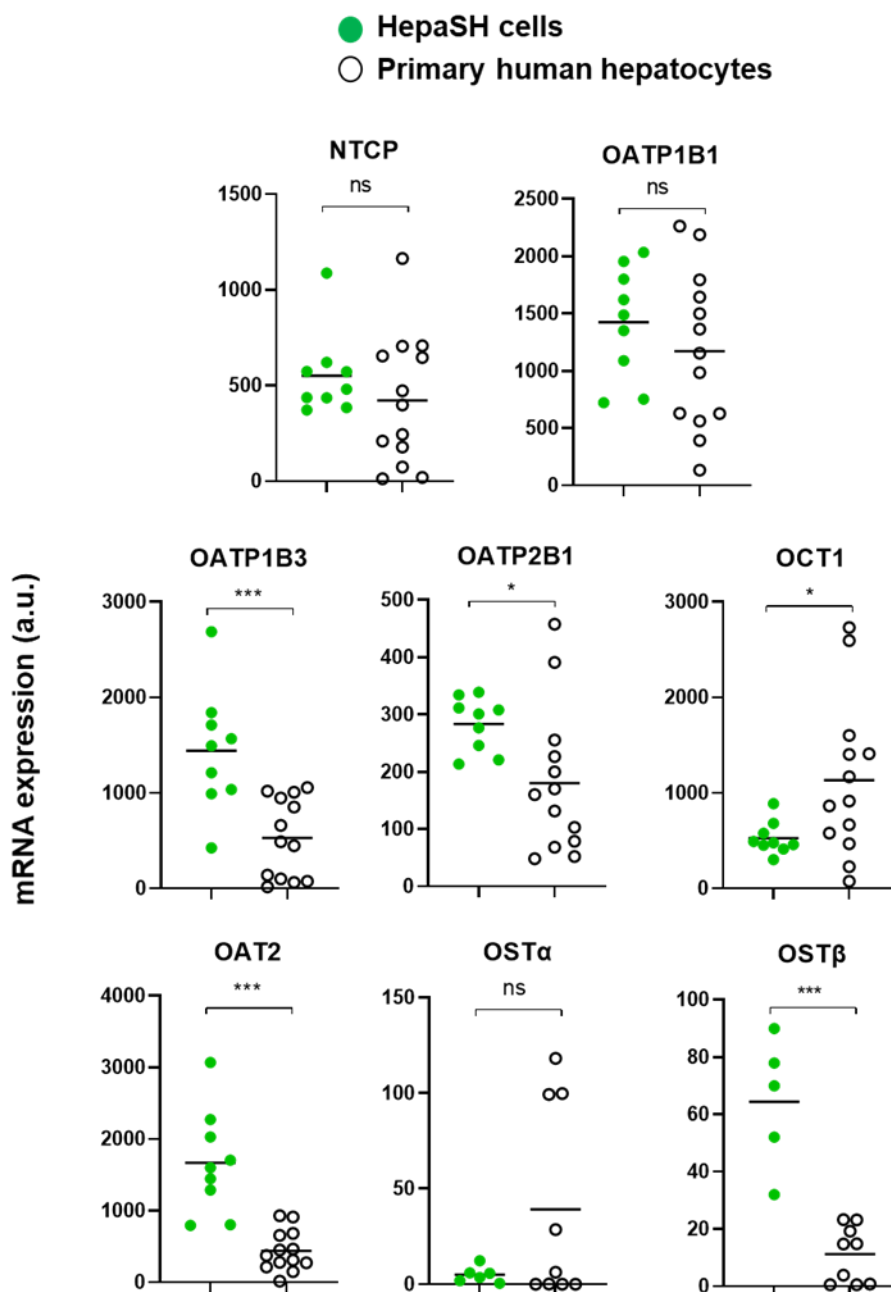


Fig. 1. SLC mRNA expression in suspended HepaSH cells and primary human hepatocytes. Cryopreserved HepaSH cells and primary human hepatocytes were thawed and put in suspension, before RNA extraction and analysis by RT-qPCR for determining SLC mRNA expression. Data are expressed in arbitrary units (a.u.). Black bars on the graphs represents the expression mean of 5-9 batches (HepaSH cells) or 9-13 batches (primary human hepatocytes). *, $p < 0.05$; ***, $p < 0.001$; ns, not statistically significant.

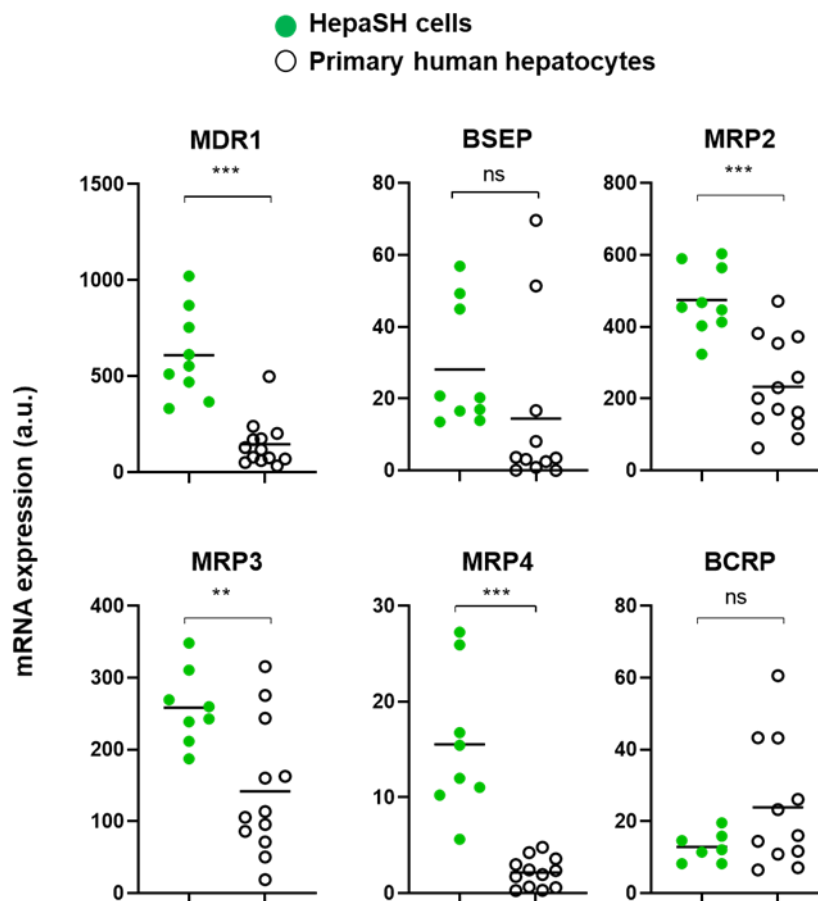


Fig. 2. ABC transporter mRNA expression in suspended HepaSH cells and primary human hepatocytes. Cryopreserved HepaSH cells and primary human hepatocytes were thawed and put in suspension, before RNA extraction and analysis by RT-qPCR for determining ABC transporter mRNA expression. Data are expressed in arbitrary units (a.u.). Black bars on the graphs represents the expression mean of 7-9 batches (HepaSH cells) or 11-13 batches (primary human hepatocytes). **, $p < 0.01$; ***, $p < 0.001$; ns, not statistically significant.

OST α , BSEP and BCRP (Fig. 1 and Fig. 2). mRNA expression of the hepatic reference marker albumin was also similar in suspended HepaSH cells and primary human hepatocytes (Fig. S1A). By contrast, HepaSH cells exhibited reduced mRNA expression of OCT1 (by a factor of 2.1, with respect to expression mean) and enhanced mRNA levels of OATP1B3 (by a factor of 2.7), OATP2B1 (by a factor of 1.6), OAT2 (by a factor of 3.8), OST β (by a factor of 5.7), MDR1 (by a factor of 4.2), MRP2 (by a factor of 2.0), MRP3 (by a factor of 1.8) and MRP4 (by a factor of 7.2), comparatively to primary human hepatocytes (Fig. 1 and Fig. 2). When

specifically comparing transporter expression in one hepatocyte population (HUM4282), used as hepatocyte donor for engrafting mice and getting HepaSH cells, with those found in the 5 independent batches of resulting HepaSH cells, OCT1 mRNA expression mean in HepaSH cells was also decreased when compared to expression in parental human hepatocytes, whereas those of OATP1B3, OATP2B1, OAT2, OST β , MDR1 and MRP4 were increased (Table S1). When considering all HepaSH cells and primary human hepatocyte batches, transporter expression ranking in suspended HepaSH cells (from the most to the less expressed one) was nevertheless highly correlated to that found in suspended human hepatocytes ($r_s = 0.82$) (Fig. 3). It was also correlated to transporter expression ranking in human livers (established from RNA sequencing data from the GTEx project), but more weakly ($r_s = 0.56$). Besides, transporter expression rankings in primary human hepatocytes and human livers were highly correlated ($r_s = 0.81$) (Fig. 3).

The variability of transporter expression in different HepaSH cell batches deriving from the same hepatocyte population is likely a parameter to consider. We have therefore determined coefficients of variation for transporter mRNA expression in the five independent HepaSH cell batches obtained with the same human hepatocyte population (HUM4282). As indicated in Table S2, the coefficients of variation for transporter expression in these HepaSH cells ranged from 12.7% to 63.0%. They were weaker than those reported for primary human hepatocytes batches or for human liver samples ($p < 0.0001$) (Table S2).

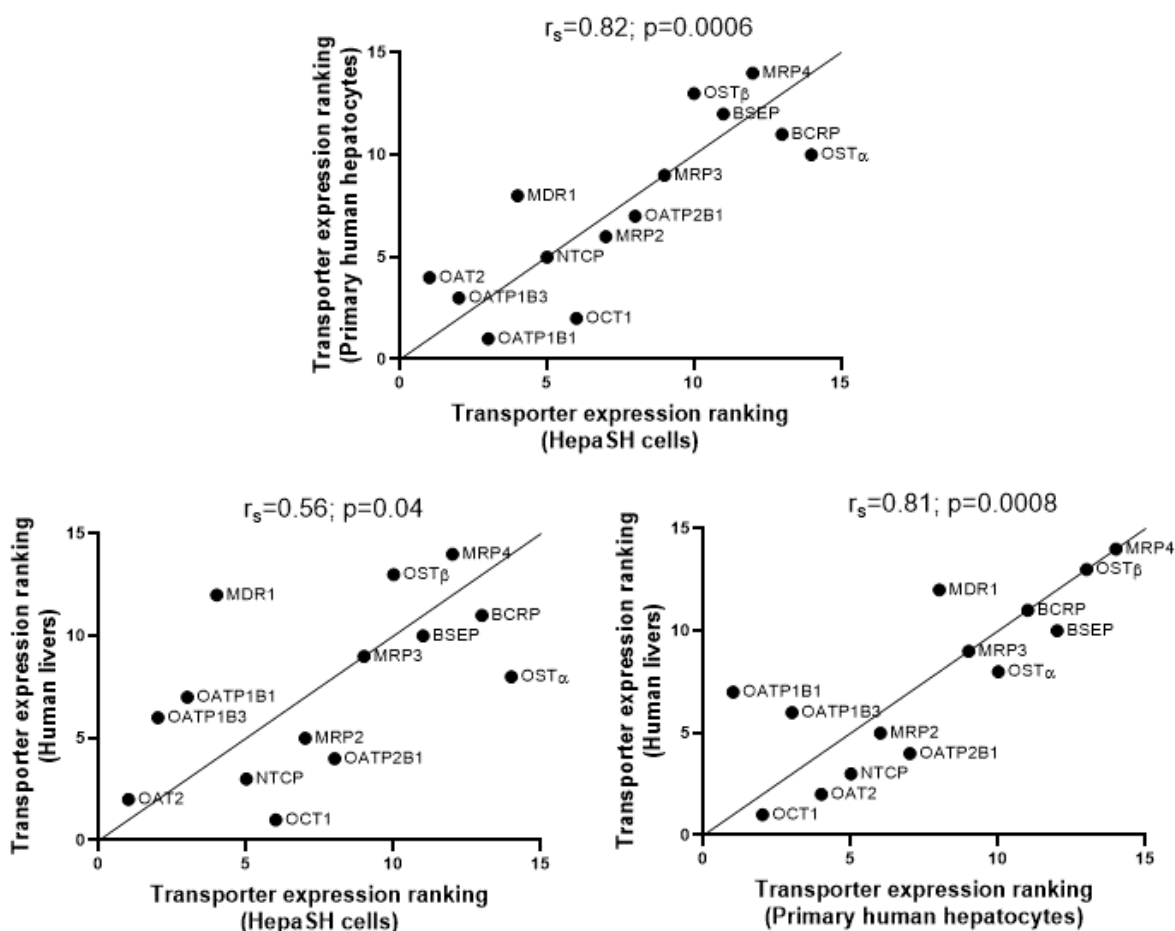


Fig. 3. Rank correlation analysis of transporter mRNA expression in HepaSH cells, primary human hepatocytes and human livers. Drug transporters were ranked according to their mRNA expression mean, with data from Fig. 1 and Fig. 2 (for HepaSH cells and primary human hepatocytes) or from the Human Protein Atlas website (for human livers), from the most to the less expressed one. Correlations were analyzed using the Spearman's rank correlation method. Spearman's rank coefficients (r_s) and p values are provided on the top of each correlation graph.

3.2 Transporter activities in suspension of cryopreserved HepaSH cells

Activities of SLC transporters were investigated in suspended HepaSH cells and primary human hepatocytes, using reference substrates, in the presence or absence of sodium (for NTCP) or reference inhibitors (for OATPs, OCT1 and OAT2), as previously described (Jigrel et al., 2005; Le Vee et al., 2015). As shown in Fig. 4A, withdrawal of sodium and addition of BSP, verapamil or ketoprofen significantly reduced accumulation of the NTCP

substrate taurocholate, the OATP substrate E3S, the OCT1 substrate TEA and the OAT2 substrate cGMP, in suspended HepaSH cells and human hepatocytes, thus demonstrating that both cryopreserved HepaSH cells and human hepatocytes exhibited NTCP, OATP, OCT1 and OAT2 activities when used in suspension after their thawing. Comparison of these transporter activities, expressed as sodium-dependent or reference inhibitor-sensitive accumulations of substrates, in suspended HepaSH cells and human hepatocytes is shown in Fig. 4B. NTCP and OCT1 activities were decreased in HepaSH cells when compared to human hepatocytes (by factors of 1.5 and 1.7, respectively), whereas OATP activity was increased (by a factor of 1.8); none of these changes of transporter activities however reached a significant level. By contrast, OAT2 activity was significantly increased by a factor of 2.7 in HepaSH cells when compared to human hepatocytes (Fig. 4B).

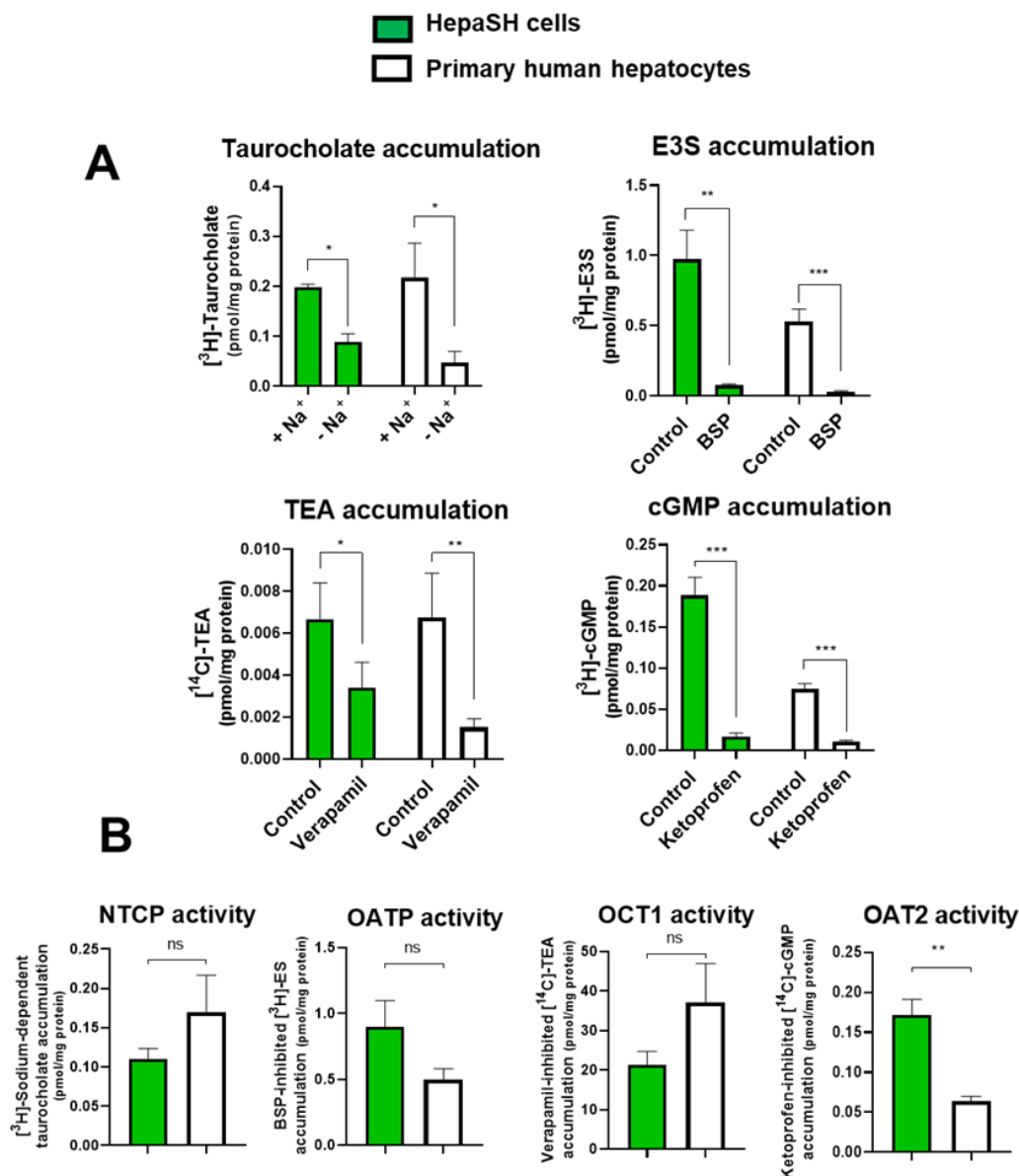


Fig. 4. SLC transport activities in suspended HepaSH cells and primary human hepatocytes. Cryopreserved HepaSH cells and primary human hepatocytes were thawed and put in suspension, before measurement of accumulation of the following reference SLC substrates for 5 min at 37°C: Taurocholate (substrate for NTCP) in the absence or presence of sodium, E3S (substrate for OATPs) in the absence (control) or presence of BSP, TEA (substrate for OCT1) in the absence (control) or presence of verapamil and cGMP (substrate for OAT2) in the absence (control) or presence of ketoprofen. Data are expressed as (A) the amount of accumulated reference substrates normalised to protein content or as (B) transporter activity, *i.e.*, the sodium-dependent accumulation of taurocholate (for NTCP activity) or inhibitor-sensitive accumulation of E3S, TEA or cGMP (for OATP, OCT1 or OAT2 activity); they are the means \pm SEM of three independent assays, each one being performed with one batch of HepaSH cells or primary human hepatocytes. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not statistically significant.

3.3 Transporter mRNA expression in cultures of cryopreserved HepaSH cells

Transporter mRNA levels were analysed in cryopreserved HepaSH cells and primary human hepatocytes cultured for 24 h, 72 h and 120 h under a monolayer configuration. Contaminating mouse cells represented less than 0.1% of cultured cells, for both 24 h-old and 120 h-old cultured HepaSH cells (Table S3), thus ruling out any proliferation of mouse cells during the period of HepaSH cell culture, in agreement with previous data (Bachour-El Azzi et al., 2022). Examination of cell cultures by phase contrast light microscopy indicated that cultured HepaSH cells exhibited a flattened morphology, rather distinct from the cobblestone-like hepatocyte morphology shown by primary human hepatocytes, especially after 120 h of culture (Fig. S2); moreover, cultured HepaSH cells displayed no or only few refractive bile canaliculi-like structures, which were, by contrast, clearly observed in 120 h-cultured human hepatocytes (Fig. S2).

Expressions of transporters in cultured HepaSH cells and primary human hepatocytes, expressed as percentages of those found in freshly thawed suspended counterparts, are shown in Fig. 5 (for SLC transporters) and Fig. 6 (for ABC transporters). Expressions of SLC transporters, *i.e.*, NTCP, OAT2, OATP1B1, OATP1B3, OATP2B1 and OCT1, were found to rapidly fall in cultured HepaSH cells, as soon as 24 h after plating (Fig. 5), resulting in rather low level of expression, ranging after 120 h of culture from 2.9% (for OAT2) to 31.3% (for OCT1) of values found in freshly-thawed HepaSH cells. Levels of SLC transporter mRNAs similarly decreased in human hepatocytes cultured for 24 h, but this down-regulation was rather transient, because SLC mRNA expression re-increased with time in culture, to reach levels similar to those found in freshly-thawed human hepatocytes (for OAT2, OATP1B1, OATP1B3, OATP2B1 and OCT1) or even higher (for NTCP), after 120 h of culture (Fig. 5).

With respect to ABC transporters, expressions of MDR1 and MRP2 mRNAs remained rather stable in HepaSH cells along the culture, whereas, by contrast, they were up-regulated in

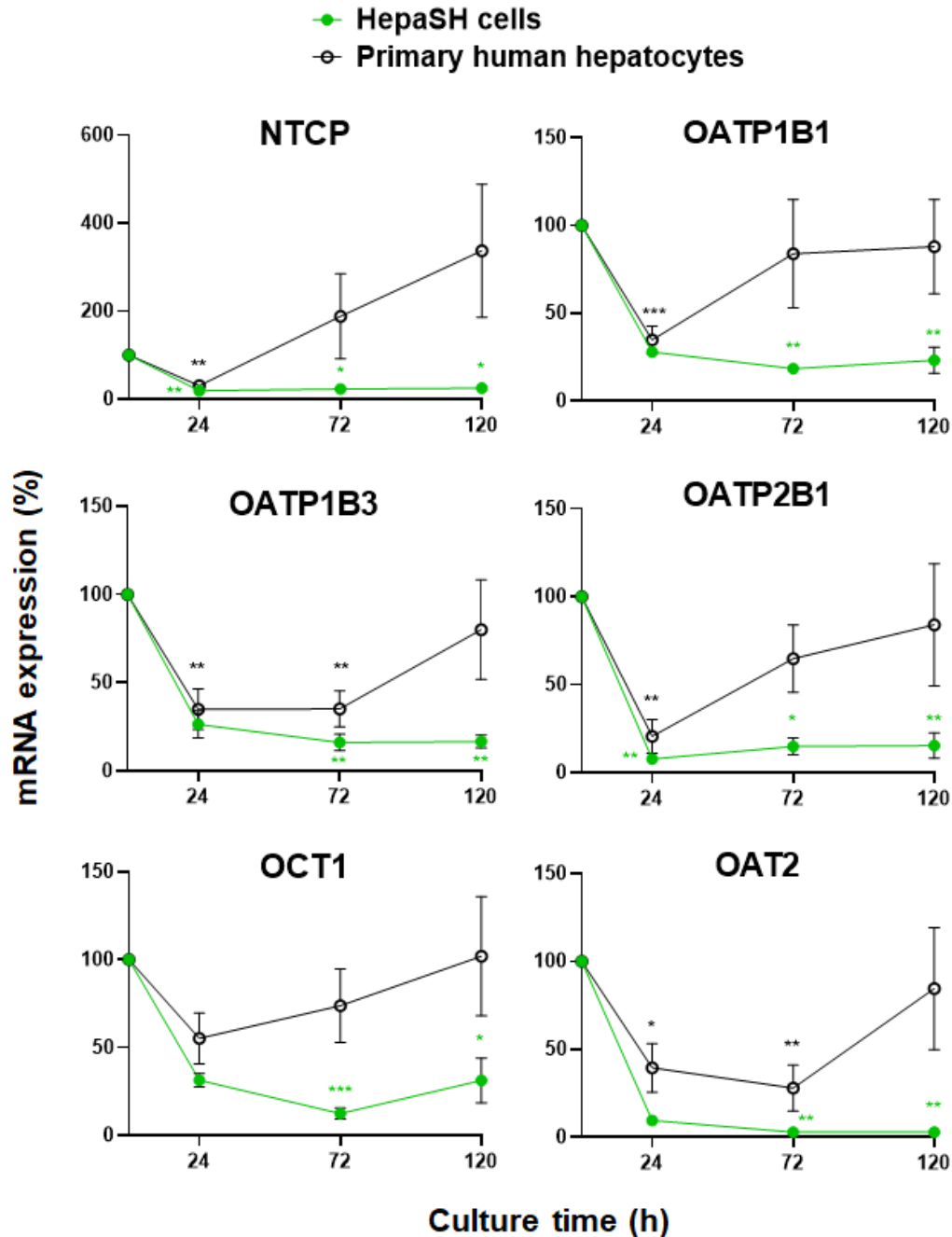


Fig. 5. SLC mRNA expression in cultured HepaSH cells and primary human hepatocytes. Cryopreserved HepaSH cells and primary human hepatocytes were thawed and put in monolayer culture for 24-120 h, before RNA extraction and analysis by RT-qPCR for determining SLC mRNA expression. Data are expressed as percentages of transporter expression found in thawed cells before plating. They are the means \pm SEM of 5-6 batches (for HepaSH cells) or 8-10 batches (for primary human hepatocytes). *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$ when compared to expression levels in thawed cells before plating.

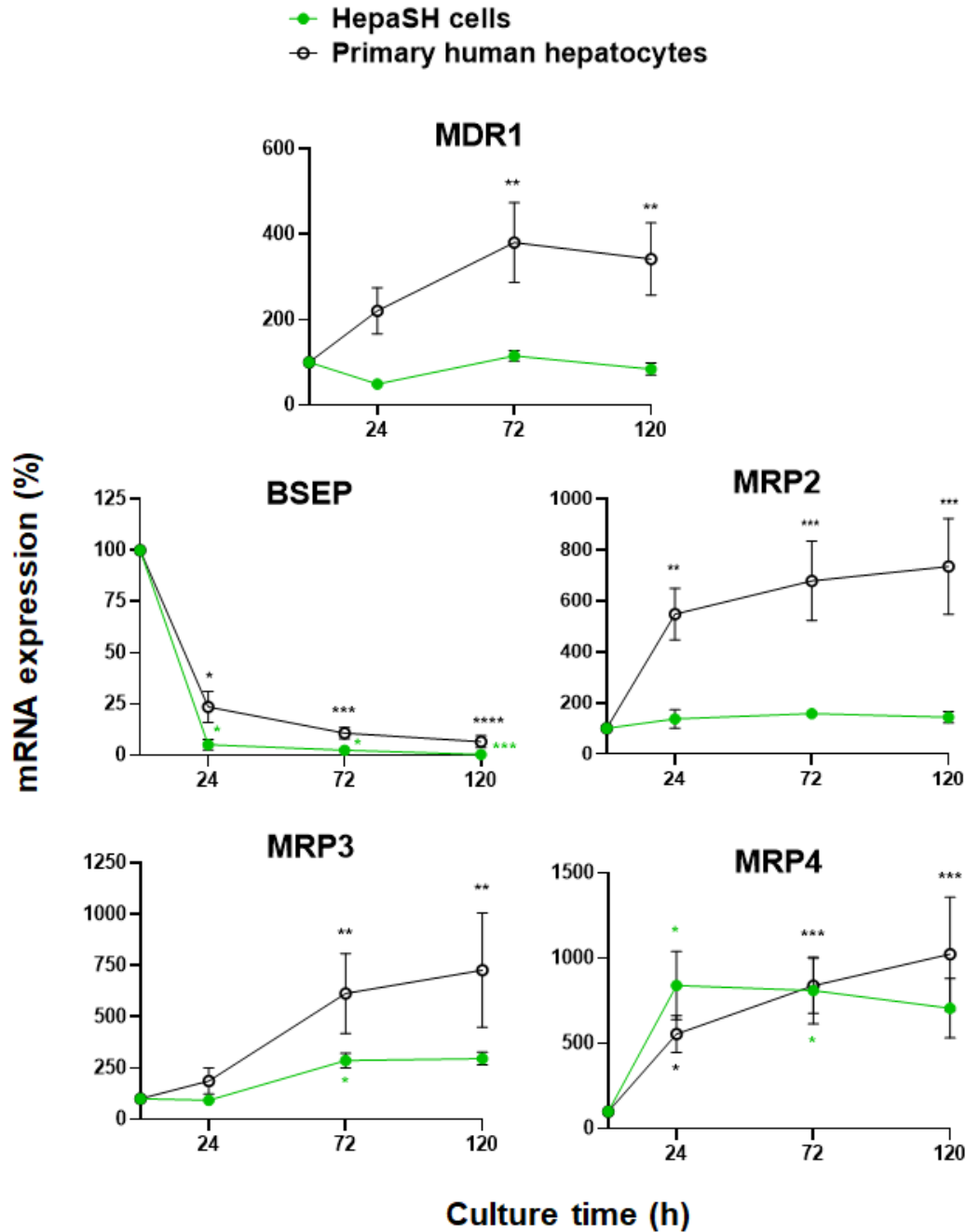


Fig. 6. ABC transporter mRNA expression in cultured HepaSH cells and primary human hepatocytes. Cryopreserved HepaSH cells and primary human hepatocytes were thawed and put in monolayer culture for 24-120 h, before RNA extraction and analysis by RT-qPCR for determining ABC transporter mRNA expression. Data are expressed as percentages of transporter expression found in thawed cells before plating. They are the means \pm SEM of 3-6 batches (for HepaSH cells) or 8-10 batches (for primary human hepatocytes). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ and ****, $p < 0.0001$ when compared to expression levels in thawed cells before plating.

cultured primary human hepatocytes (Fig. 6). MRP2 protein was nevertheless rather poorly expressed in 72 h-old cultured HepaSH cells, with exhibited only few MRP2-positive biliary-like structures, in contrast to primary human hepatocytes (Fig. S3). BSEP mRNA levels were rapidly and markedly down-regulated in cultures of both HepaSH cells and human hepatocytes (Fig. 6); after 120 h of culture, BSEP mRNA levels in HepaSH cells and human hepatocytes represented thus only 0.4 % and 6.6%, respectively, of values found in corresponding thawed cells before plating. MRP3 and MRP4 mRNA expression increased with time in culture in HepaSH cells, reaching after 120 h of culture 295.9% (for MRP3) and 707.3% (for MRP4) of values found in freshly thawed counterparts. MRP3 and MRP4 mRNAs were also strongly up-regulated in cultured human hepatocytes (Fig. 6). Levels of SLC and ABC transporter mRNAs, expressed as a.u., in 120 h-old cultured HepaSH cells and primary human hepatocytes confirmed the decreased expression of NTCP (by a factor of 2.5), OATP1B1 (by a factor of 2.1), OCT1 (by a factor of 6.7), OAT2 (by a factor of 10.3) and MRP2 (by a factor of 2.0) mRNAs in cultured HepaSH cells when compared to cultured primary human hepatocytes (Fig. S4).

Similarly to SLC transporter mRNA levels, those of albumin were rapidly and permanently decreased in cultured HepaSH cells, reaching 26.5% of initial albumin mRNA level, after 120 h of culture (Fig. S1B). By contrast, after an initial fall after 24 h of culture, albumin mRNA levels re-increased in cultured human hepatocytes with time in culture, resulting in levels similar to that found in freshly-thawed hepatocytes after 120 h of culture (Fig. S1B).

3.4 Transporter activities in cultures of cryopreserved HepaSH cells

SLC transporter activities, expressed as sodium-dependent or reference inhibitor-sensitive accumulations of reference substrates, were clearly detected in 24 h-old cultured HepaSH cells and primary human hepatocytes (Fig. 7); NTCP, OATP and OAT2 activities were

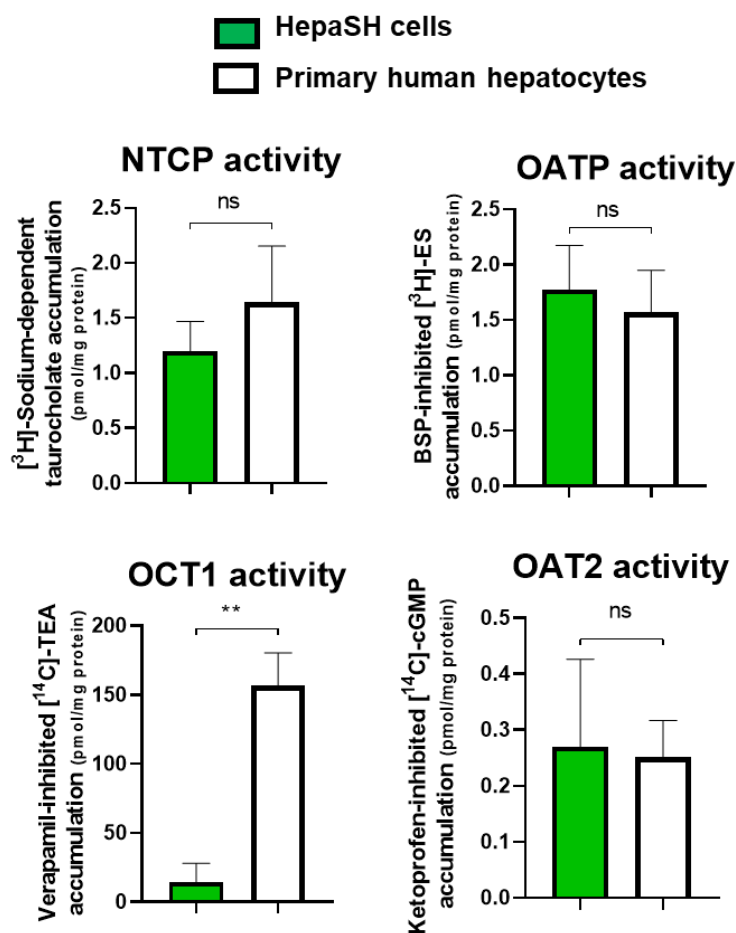


Fig. 7. SLC transporter activities in 24 h-cultured HepaSH cells and primary human hepatocytes. Cryopreserved HepaSH cells and primary human hepatocytes were thawed and put in monolayer culture for 24 h. Measurement of SLC transporter activities was then performed using reference substrates, as described in the legend of Fig. 4. Data are expressed as the sodium-dependent accumulation of taurocholate (for NTCP activity) or inhibitor-sensitive accumulation of E3S, TEA or cGMP (for OATP, OCT1 or OAT2 activity). Data are the means \pm SEM of 3-6 (HepaSH cells) or 6-7 (primary human hepatocytes) independent assays, each one being performed with one batch of HepaSH cells or human hepatocytes. **, $p < 0.01$; ns, not statistically significant.

similar in these HepaSH cells and human hepatocytes cultured for 24 h, whereas, by contrast, OCT1 activity was much lower in HepaSH cells than in human hepatocytes (Fig. 7). After 120 h of culture, NTCP, OATP and OAT2 activities fell in cultured HepaSH cells, to less than 20% of transporter activities found in 24 h-cultured counterparts (Fig. S5); OCT1 activity appeared to be also reduced in 120 h-cultured HepaSH cells (Fig. S5), knowing that OCT1 activity was

already low in 24 h-old cultured HepaSH cells (Fig. 7). With respect to primary human hepatocytes cultured for 120 h, NTCP, OCT1 and OAT2 activities were unchanged when compared to those found in counterparts cultured for 24 h, whereas that of OATPs was moderately, but significantly, decreased and thus represented 49.6% of activity level found in 24 h-cultured human hepatocytes (Fig. S5).

4. Discussion

Human hepatocytes isolated from chimeric mice with humanized livers have been postulated to represent a sustainable source of human hepatocytes, notably for preclinical studies of drugs (Kakuni, 2022). Indeed, they retain expression, activity and regulatory pathways of main hepatic drug metabolizing enzymes, especially of CYPs, and can consequently be used as suspensions or cultures for *in vitro* characterizing drug metabolism and CYP induction (Bachour-El Azzi et al., 2022; Uehara et al., 2019; Yamasaki et al., 2020; Yamasaki et al., 2010). The data reported in the present study indicate that such human hepatocytes, isolated from chimeric TK-NOG mice, *i.e.*, HepaSH cells, also display notable functional expression of drug transporters.

When using as cell suspensions after their thawing, cryopreserved HepaSH cells displayed a mRNA expression pattern for SLC and ABC transporters well correlated to those found in cryopreserved primary human hepatocytes and human livers. They additionally exhibited notable activities of main sinusoidal uptake transporters, similar (for NTCP, OCT1 and OATPs) or even higher (for OAT2) than those observed in suspended cryopreserved primary human hepatocytes. Short-term cultured HepaSH cells (for 24 h) also showed NTCP-, OATPs- and OAT2-related transport activity close to those found in cultured primary human hepatocytes. Taken together, these transporters-related data concerning HepaSH cells, together with those previously published about cultured PXB cells (Kohara et al., 2020; Yamasaki et al., 2020), highlight the interest of human hepatocytes isolated from chimeric mice as a source of

sustainable hepatocytes for studying interactions of drugs with hepatic transporters, especially SLC uptake transporters. In particular, inhibition of OATPs, or transport by these SLCs, can likely be considered for drugs in development using suspended or 24 h-cultured HepaSH cells. This point is noteworthy owing to the fact that OATP1B1, OATP1B3 and OATP2B1 correspond to key hepatic transporters that have to be studied during the pharmaceutical development of new molecular entities according to drug agencies or the International Transporter Consortium (Sudsakorn et al., 2020; Zamek-Gliszczynski et al., 2022). OCT1 and OAT2 also merit consideration (Shen et al., 2017; Zamek-Gliszczynski et al., 2018), even if evaluation of these transporters is not formally required according to a regulatory point of view; in this context, suspended HepaSH cells exhibiting OCT1 and OAT2 activities may be useful for investigating interactions of drugs with these hepatic transporters of emerging relevance.

If the profile of transporter expression in suspended thawed HepaSH cells is correlated to those found in primary human hepatocytes or human livers, some differences have however to be considered. Thus, suspended HepaSH cells exhibited reduced OCT1 mRNA levels, notably observed for each of the five HepaSH cell batches deriving from the same hepatocyte donor (Table S1); other transporters, especially OATP1B3, OAT2, OST β , MDR1 and MRP4, displayed increased mRNA expression in suspended HepaSH cells when compared to human hepatocytes. The enhanced OAT2 mRNA expression in HepaSH cells likely deserves special interest, because it is associated with increased OAT2 activity, which may have therefore to be kept in mind if considering transport data generated with suspended HepaSH cells for OAT2 substrates. The reason of such differences in transporter mRNA expression between suspended HepaSH cells and primary human hepatocytes is unclear. It may be due to the fact that HepaSH cells in the humanized liver of chimeric mice are placed in an immunodeficient murine environment; they are thus exposed to murine cytokines, hormones or growth factors, that may exert specific regulation towards human transporters expressed by HepaSH cells. Therefore,

the changes in transporter mRNA levels observed in HepaSH cells may testify this imprint of murine factors, exacerbated by the immunodeficiency status of the chimeric mice. In addition, the miscommunication between human hepatocytes, mouse liver stromal cells and mouse intestinal cells in chimeric mice with humanized livers is likely to cause dysregulation of bile acid homeostasis, as demonstrated in FRG mice (Chow et al., 2017). The resulting enhanced levels of circulating bile acids may activate the farnesoid X receptor (FXR) and subsequently the targets of this receptor, including OST β (Landrier et al., 2006), whose mRNA expression is increased in HepaSH cells. Alternatively, changes in transporter expression in suspended HepaSH cells when compared to primary human hepatocytes may reflect inherent variability due to the generation of chimeric mice, the known inter-individual variation in transporter mRNA expression between human liver tissue samples, or differences in the genetic backgrounds of the donor hepatocytes (Zerdoug et al., 2022). The fact that transporter mRNA expressions vary among livers of chimeric mice, *i.e.*, for example, OCT1 mRNA levels have been reported to be either increased (by a 5.6-fold factor) or decreased (by a 2.5-fold factor) in the humanized livers of TK-NOG mice (Hasegawa et al., 2011; Uehara et al., 2022c; Zerdoug et al., 2022), supports this hypothesis.

With respect to transporter mRNA expressions in cultured HepaSH cells, those of SLCs as well as that of BSEP fell as soon as 24 h after plating and remained low thereafter (until 120 h of culture), whereas, by contrast, MRP2 and MDR1 mRNA expression remained constant, thus ruling out any unspecific global repression of transporters in cultured HepaSH cells. OATP1B1, OATP1B3 and BSEP, but not MRP2, have been similarly reported to be rather poorly expressed in PXB cells cultured under a monolayer configuration (Yamasaki et al., 2020). SLC and BSEP transporter mRNA expressions also decreased in 24 h-cultured human hepatocytes, but next re-augmented with time in culture, excepted for BSEP, to reach levels similar to those found in suspended counterparts before plating. SLC transport activities are

concomitantly maintained at a notable level in 120 h-cultured human hepatocytes, but not in 120 h-cultured HepaSH cells, in agreement with mRNA expressions. Such a discrepancy between cultured HepaSH cells and primary human hepatocytes with respect to mRNA expression and activities of SLCs such as NTCP, OATP1B1, OATP1B3 and OAT2, considered to be liver-specific markers (Jetter and Kullak-Ublick, 2020), may reflect a partial dedifferentiated status of HepaSH cells with time in culture in the conventional monolayer 2-D culture conditions used in the present study. The fact that 120 h-cultured HepaSH cells poorly exhibited morphological features of cultured human hepatocytes, such as polygonal shape and refractive bile canaliculi-like structures, as well as their reduced mRNA expression of the liver master gene albumin, likely argue in favour of this hypothesis. The dedifferentiated status of 120-h cultured HepaSH cells is additionally attested by their low expression of canalicular MRP2 protein, as demonstrated by immunocytochemistry assays, which suggests a compromised polarized status of these cells, and otherwise precludes the analysis of canalicular ABC transporter activities in the present study. The cause of the dedifferentiation of HepaSH cells with time in culture remains to be identified. This may be related to the initial murine environment/imprint of these cells, as reported above, and may reflect a particular susceptibility of HepaSH cells to factors known to compromise the *in vitro* expression of liver-specific functions, such as the mechanical stress resulting from collagenase perfusion for cell isolation, and cryoinjury associated with the freeze–thaw cycle for the preservation of hepatocytes (Godoy et al., 2013). In this context, it is noteworthy that various culture conditions have been proposed for keeping a high level of differentiation/polarity in cultured human hepatocytes (Ruoß et al., 2020), including the use of improved culture media for monolayer 2-D hepatocyte cultures (Chen et al., 2021; Katsuda et al., 2020; Xiang et al., 2019) or that of 3-D cultures under a sandwich or spheroid configuration (Gijbels et al., 2019; Rose et al., 2021). Testing these culture conditions may be welcome for improving the maintenance of transporter

expression and activity in cultured HepaSH cells. The fact that PXB cells maintained under a 3-D sandwich configuration exhibited a polarized status, with functional canalicular transport of the fluorescent bile acid-derivative cholyl-L-lysyl-fluorescein (Kohara et al., 2020), agrees with this proposition, even if BSEP and OATP1B1 mRNA expression remained low in such 3-D cultured PXB cells (Zerdoug et al., 2022). Finally, it is noteworthy that MRP3 and MRP4 mRNA expressions were up-regulated in cultures of both cryopreserved HepaSH cells and primary human hepatocytes; they are also induced in primary cultures of freshly isolated human hepatocytes (Le Vee et al., 2015), suggesting that the up-regulation of these sinusoidal ABC transporters, known to be overexpressed during cholestasis (Pérez-Pineda et al., 2021), may represent a key-hallmark of cultured hepatocytes. This may be related to the cholestatic nature of cultured hepatocytes (Rippin et al., 2001) and has likely to be taken into account for drug transport studies.

Inter-individual variability of drug detoxifying proteins, including those of transporters (Nies et al., 2022; Pan, 2019; Yang et al., 2013), is likely an important parameter to consider when using primary human hepatocytes for *in vitro* drug metabolism and transport studies (Jigorel et al., 2006), notably because it may make difficult to compare data from different hepatocyte batches. In this context, batches of HepaSH cells obtained with one human hepatocyte donor may be expected to exhibit a low variability with respect to transporter expression. In agreement with this hypothesis, inter-batch variability in HepaSH cells deriving from the same human hepatocyte population, evaluated by the coefficients of variation of transporter expression, was lower than those reported for batches of primary human hepatocytes or for human liver samples (Table S2). This suggests that HepaSH cells generated from the same human donor represent a relatively homogenous population of human hepatocytes with respect to transporter expression, which likely avoids conventional drawbacks such as inter-individual variability or batch-to-batch differences commonly observed with primary human

hepatocytes. Nevertheless, the inter-batch variability for transporter expression for the five batches of HepaSH cells originated from mice engrafted with hepatocytes from the same donor was not negligible, *i.e.*, the coefficients of variation range from 12.7% to 63.0% (Table S2).

Such variations cannot be due to inter-individual genetic polymorphism, because all batches of these HepaSH cells derive from the same donor. They can rather reflect the inherent variability caused by the generation of chimeric mice and the isolation and cryopreservation of HepaSH cells.

In summary, cryopreserved human HepaSH cells isolated from chimeric TK-NOG mice with humanized liver displayed a profile of SLC and ABC transporter mRNA expression correlated to those found in cryopreserved primary human hepatocytes or human livers. Moreover, HepaSH cells used as suspensions or as 24 h-cultures exhibited activities of SLC uptake transporters similar to those displayed by primary human hepatocytes. Such data therefore suggest that cryopreserved human hepatocytes isolated from chimeric mice with humanized liver likely represent a valuable and sustainable alternative to the use of primary human hepatocytes for *in vitro* drug transport studies. In this context, the availability of HepaSH cells, expected to be much higher than that of primary human hepatocytes, is likely a key-point to consider. The use of HepaSH cells may therefore permit to increase the amount and the validity of *in vitro* data generated with relevant human hepatic models in the toxicological field, and by this way, to minimize animal experimentation.

CRedit authorship contribution statement

Anna Zerdoug: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Marc Le Vee:** Conceptualization, Data curation, Formal analysis, Methodology, Writing – review & editing,

Supervision. **Shotaro Uehara**: Resources, Writing – review & editing. **Agnes Jamin**: Methodology, Writing – review & editing. **Yuichiro Higuchi**: Resources, Writing – review & editing. **Nao Yoneda**: Resources. **Beatrice Lopez**: Methodology, Writing – review & editing, Supervision. **Christophe Chesné**: Writing – review & editing, Supervision. **Hiroshi Suemizu**: Resources, Writing – review & editing. **Olivier Fardel**: Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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Declaration of interests

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Highlights

- HepaSH cells are human hepatocytes isolated from humanized liver TK-NOG mice
- Drug transporter mRNA levels are close in HepaSH cells and primary human hepatocytes
- Suspended and 24 h-cultured HepaSH cells exhibit notable activities of transporters
- HepaSH cells are a sustainable source of cells for *in vitro* drug transport studies