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Functional expression and regulation of drug transporters in monolayer- and sandwich-cultured mouse hepatocytes

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Abstract

Primary hepatocyte cultures are now considered as convenient models for *in vitro* analyzing liver drug transport. However, if primary human and rat hepatocytes have been well-characterized with respect to drug transporter expression and regulation, much less is known for primary mouse hepatocytes. The present study was therefore designed to gain insights about this point. The profile of sinusoidal and canalicular drug transporter mRNA expression in short time (4 h)-cultured mouse hepatocytes was found to be highly correlated with that of freshly isolated hepatocytes; by contrast, those of counterparts cultured for a longer time (until 4 days) either in monolayer configurations on plastic or collagen or in sandwich configuration with matrigel were profoundly altered: uptake drug transporters such as Oct1, Oatps and Oat2 were thus down-regulated, whereas most of efflux transporters such as Mdr1a/b, Mrp3, Mrp4 and Bcrp were induced. Moreover, short time-cultured hepatocytes exhibited the highest levels of sinusoidal influx transporter activities. Transporter-mediated drug secretion into canalicular networks was however only observed in sandwich-cultured hepatocytes. Mouse hepatocytes cultured either in monolayer or sandwich configurations were finally shown to exhibit up-regulation of referent transporters in response to exposure to prototypical activators of the drug sensing receptors pregnane X receptor, aryl hydrocarbon receptor or constitutive androstane receptor. Taken together, these data demonstrate the feasibility of using primary mouse hepatocytes for investigating potential interactions of xenobiotics with hepatic transporter activity or regulation, provided that adequate culture conditions are retained.

Key-words

Drug transporters; primary hepatocytes; mouse; monolayer; sandwich; regulation.

1. Introduction

Hepatic drug transporters belonging to the solute carrier (SLC) or the ATP-binding cassette (ABC) transporter superfamilies are involved in hepato-biliary secretion of drugs and drug-drug interactions (Giacomini et al., 2010). They are located at the sinusoidal or canalicular pole of hepatocytes (Funk, 2008; Hirouchi et al., 2009) (See Fig. 1 for a schematic overview of drug transporter expression by mouse hepatocytes). Putative interactions of some hepatic transporters with new molecular entities developed by pharmaceutical companies have now to be characterized (Giacomini et al., 2010; Zhang et al., 2008). For this purpose, primary hepatocyte cultures have emerged as a valuable and useful tool (Ghibellini et al., 2006; Sahi et al., 2010; Soars et al., 2007).

Cultured hepatocytes can be used either in a monolayer standard configuration, i.e., hepatocytes plated on plastic dishes or collagen-coated dishes, or in a sandwich configuration, i.e., hepatocytes plated on collagen-coated dishes and overlaid with a second layer of collagen or matrigel (LeCluyse, 2001). These two configurations have been successfully retained to study drug transporter activity and regulation (Jigorel et al., 2006; Swift et al., 2010). Rat hepatocytes cultured in conventional monolayer conditions however fail to display bile canalicular structures and show a marked down-regulation of sinusoidal influx drug transporters, associated with a concomitant over-expression of the efflux transporter P-glycoprotein (Abcb1) (Fardel et al., 1993; Jigorel et al., 2005; Luttringer et al., 2002). By contrast, sandwich-cultured rat hepatocytes exhibit functional canalicular networks (LeCluyse et al., 1994; Liu et al., 1999b), even if they also display reduced expression of sinusoidal influx transporters with time in culture when compared to freshly isolated hepatocytes (Borlak and Klutcka, 2004; Kotani et al., 2011; Tchaparian et al., 2011). With regard to human hepatocytes cultured either in monolayer or sandwich conditions, expression of drug transporters appears to be much better preserved with time in culture when compared to rat

counterparts (Hoffmaster et al., 2004; Jigorel et al., 2005; Kotani et al., 2011; Li et al., 2009; Schaefer et al., 2012; Takeba et al., 2011).

Unlike primary rat and human hepatocytes, cultured mouse hepatocytes remain poorly characterized with respect to drug transporter expression and activity, even if bile acid transport and multidrug resistance-associated protein (Mrp/Abcc) 4 expression have been recently investigated in sandwich-cultured mouse hepatocytes (Swift and Brouwer, 2010). Primary mouse hepatocytes likely represent an interesting *in vitro* model for studying liver drug transporters, because they can originate from various and already generated knockout mice in which specific liver transporter has been deleted, thus potentially allowing to address the function and the substrates of the disrupted transporter (Klaassen and Lu, 2008). Primary mouse hepatocytes may also served for identifying signalling ways governing transporter expression, through, for example, the use of hepatocytes from transgenic mice deficient in drug-sensing receptors such as pregnane X receptor (PXR), aryl hydrocarbon receptor (AhR) or constitutive androstane receptor (CAR), known to be involved in transporter regulation (Klaassen and Aleksunes, 2010). Before considering the use of primary hepatocytes from transgenic mice for transporter studies, accurate characterization of basal transporter expression in primary wild-type mouse hepatocytes is however required. The present study was therefore designed to carefully analyze expression, activity and regulation of drug transporters in mouse hepatocytes cultured in monolayer or sandwich configurations; the transporters studied in this work correspond to referent hepatic SLC and ABC transporters (Funk, 2008) and their cellular localization is indicated in Fig. 1.

2. Materials and methods

2.1. Chemicals

Rhodamine 123, probenecid, verapamil, phenobarbital and dexamethasone were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France), whereas carboxy-2,7-dichlorofluoresceine (CF) diacetate and 2,3,7,8-tetrachlorodibenzo-p-dioxine (TCDD) were provided by Invitrogen/Life Technologies (Villebon sur Yvette, France) and Cambridge Isotope Laboratories (Andover, MA), respectively. [^{3}H(G)] taurocholic acid (sp. act. 1.19 Ci/mmol), [6, 7-^{3}H(N)] estrone-3-sulfate (E3S) (sp. act. 57.3 Ci/mmol), [1-^{14}C] tetra-ethylammonium (TEA) (sp. act. 2.4 mCi/mmol), p-[glycyl-L-^{3}H] aminohippuric acid (PAH) (sp. act. 4.53 Ci/mmol) and [6.7-^{3}H(N)] estradiol 17β-D glucuronide (sp. act. 41.8 Ci/mmol) were from Perkin-Elmer (Boston, MA). All other chemicals were commercial products of the highest purity available.

2.2. Hepatocyte isolation and culture

Hepatocytes from adult female C57BL/6 mice weighing around 20 g were isolated by perfusion of the liver with a highly active Liberase^{TM} solution (Roche, Meylan, France), as previously described (Fardel et al., 1993). They were then finally suspended in Williams'E medium supplemented with 5 µg/mL insulin, 2 mM glutamine, 10 IU/mL penicillin, 10 µg/mL streptomycin and 10 % (vol/vol) fetal calf serum and immediately seeded on 24-well plastic plates either uncoated or coated with collagen type I (BD Biosciences, Le Pont de Claix, France); the cell density (200000 cells/well) used for seeding has been previously shown to be in the range of optimal ones for culturing mouse hepatocytes (Swift and Brouwer, 2010). Seeding medium was removed after a 4 h-period and replaced by a similar Williams' E medium, except that fetal calf serum was withdrawn and 1 mg/ml bovine serum albumin and 0.1 µM dexamethasone were added. This medium was then changed daily. For sandwich-

configuration primary cultures, collagen-plated hepatocytes were overlaid within 24 h after cell plating with 0.25 mg/ml ice-cold Matrigel (BD Biosciences), as previously described (Annaert et al., 2001). For transporter regulation studies, mouse hepatocytes were daily treated (starting from 4 h after cell seeding) for three days with 10 nM TCDD, 3.2 mM phenobarbital or 10 μM dexamethasone. All protocols were in accordance with the French laws and the institution's guidelines for animal welfare.

2.3. RNA isolation and RT-qPCR analysis

Total RNA was isolated from cells using the TRIzol reagent (Invitrogen/life Technologies). RNA was then subjected to reverse transcription-quantitative polymerase chain reaction (RT-qPCR), using the fluorescent dye SYBR Green methodology, specific gene primers (listed in Supplementary Table 1) and an ABI Prism 7300 detector (Applied Biosystem, Foster City, CA, USA), as previously described (Le Vee et al., 2008). Quantification of the steady-stage target mRNA levels of these genes was calculated after normalization of the total amount of cDNA tested to a 18S RNA endogenous reference, as previously described (Moreau et al., 2011). Data were usually expressed in % of mRNA levels found in freshly isolated hepatocytes or in untreated control hepatocytes, arbitrarily set at 100 %.

2.4. Western-blot analysis

Total cellular extracts were prepared as previously described (Lecureur et al., 2005). Proteins (40 μg) were then separated on polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. After blocking in Tris-buffered saline containing 4% bovine serum albumin, membranes were incubated overnight at 4°C with rat monoclonal antibody M4I-10 directed against Mrp4 (Abcam, Paris, France) or rabbit polyclonal antibodies against bile salt export pump (Bsep/Abcb11) (Cao et al., 2001) or sodium-taurocholate cotransporting

polypeptide (Ntcp/Slc10a1) (Kullak-Ublick et al., 1997). After incubation with horseradish peroxydase-conjugated secondary antibody, signals were detected by chemiluminescence. Gel loading and transfer was checked up through immunolabelling with primary antibody against the heat-shock protein HCS70 (Santa Cruz Technology, Heidelberg, Germany).

2.5. Light and fluorescence microscopy.

Light microscopy and CF fluorescence were employed to monitor the presence of functional canalicular networks in cultured hepatocytes, as previously described (Chandra et al., 2001). For light microscopy studies, primary hepatocytes were observed using an Axiovert microscope (Zeiss, Le Pecq, France). For fluorescence studies, primary hepatocytes were incubated with 3 μM CF diacetate for 10 min at 37°C; cells were then washed with ice-cold phosphate-buffered saline and visualized using a Leica DM IRB microscope (Leica Microsystems, Wetzlar, Germany) equipped with a black/white CoolSNAP ES camera (Roper Scientific, Planegg/Martinsried, Germany). Pictures were processed using the Metaview software.

2.6. Drug transport assays.

2.6.1 Sinusoidal influx assay.

Uptake transport activities due to the sinusoidal transporters Ntcp, organic anion transport polypeptides (Oatps/Slcos), organic cation transporter 1 (Oct1/Slc22a1) and anion organic transporter 2 (Oat2/Slc22a7) were analyzed through measuring, sodium-dependent-intracellular accumulation of the Ntcp substrate taurocholate, probenecid-sensitive uptake of the Oatp substrate E3S, verapamil-sensitive uptake of the Oct1 substrate TEA and probenecid-sensitive uptake of the Oat2 substrate, using a defined transport assay medium consisting of 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM D-

glucose, 10 mM HEPES, and 136 mM *N*-methyl glucamine (Na^+-free buffer) or 136 mM NaCl (standard buffer), as previously described (Jigorel et al., 2005). Briefly, cells were incubated at 37 °C for 5 min in the transport medium supplemented with 43.4 nM [^{3}H] taurocholate in the presence or absence of sodium, 3.4 nM [^{3}H] E3S in the presence or absence of the Oatp inhibitor probenecid (2 mM), 40 µM [^{14}C] TEA in the presence or absence of the Oct1 inhibitor verapamil (50 µM) or 200 nM [^{3}H] PAH in the presence or absence of the Oat2 inhibitor probenecid (10 mM). After washing in phosphate-buffered saline, cells were lyzed and accumulation of radiolabeled substrates was determined through scintillation counting. Taurocholate accumulation values in the presence of sodium minus accumulation values in the absence of sodium, E3S uptake values in the absence of probenecid minus uptake values in the presence of probenecid, TEA uptake values in the absence of verapamil minus uptake values in the presence of verapamil and PAH uptake values in the absence of probenecid minus uptake values in the presence of probenecid are thought to represent Ntcp-, Oatps-, Oct1- and Oat2-related transport activities (Jigorel et al., 2005).

2.6.2 Canalicular secretion assay.

Biliary secretion of drugs was analyzed through determination of drug accumulation into bile canaliculi-like structures as previously reported (Annaert et al., 2001; Turncliff et al., 2006). Briefly, primary hepatocytes were first incubated for 10 min at 37°C with transport assay buffer containing Ca^{2+} described above or with the same buffer, except that 1.8 mM $CaCl_2$ was withdrawed and 100 µM EGTA was added, knowing that incubation with this Ca^{2+}-free buffer promotes disruption of tight junctions and opening of bile canaliculi networks. Buffers were then removed and hepatocytes were further incubated for 10 min at 37°C in transport assay medium containing 43.4 nM [^{3}H] taurocholate, 20 nM [^{3}H] estradiol 17β-D

glucuronide, 5.25 μM rhodamine 123 or 3 μM CF diacetate. After washing with ice-cold phosphate-buffered saline, accumulations of taurocholate, estradiol 17β-D glucuronide and rhodamine 123 into cells + bile canaliculi (Ca^{2+}-containing conditions) and into cells only (Ca^{2+}-free conditions) were determined by scintillation counting or spectrofluorimetry using a SpectraMax Gemini SX spectrofluorometer (Molecular Devices, Sunnyvale, CA) (excitation and emission wavelengths were 485 and 535 nm, respectively). Accumulation of CF into cells + bile canaliculi (Ca^{2+}-containing conditions) and into cells only (Ca^{2+}-free conditions) was analyzed after an additional 20-min incubation efflux period in CFA-free medium using spectrofluorimetry as described above. Biliary excretion index (BEI) was finally calculated using the following equation (Liu et al., 1999a):

$$BEI = \frac{\text{Accumulation (Cells+Bile canaliculi)} - \text{Accumulation (Cells)}}{\text{Accumulation (Cells+Bile canaliculi)}} \text{ X } 100$$

2.6.3. Sinusoidal efflux assay.

Sinusoidal efflux of CF, a know referent substrate for Mrps, including the sinusoidal transporter Mrp3 (Abcc3) (Zamek-Gliszczynski et al., 2003), was investigated through determining probenecid-inhibited efflux of the dye from sandwich-cultured hepatocytes exhibiting intact bile canaliculi. Briefly, cells were first incubated for 10 min at 37°C with transport assay buffer containing Ca^{2+} described above and supplemented with 3 μM CF diacetate. After washing, cells were re-incubated in CF diacetate-free medium for 20 min at 37°C in the absence or presence of 2 mM probenecid, a well-known inhibitor of Mrps (Payen et al., 2000). Retention of CF into cells + bile canaliculi in the presence or absence of probenecid was then determined by spectrofluorimetry as described above, allowing to

determine the amount of CF secreted at sinusoidal pole of cells in a probenecid-inhibitable manner. Sinusoidal excretion index (SEI) was finally calculated using the following equation:

$$SEI = \frac{\text{Accumulation (Cells+Bile canaliculi+Probenecid)} - \text{Accumulation (Cells+Bile Canaliculi)}}{\text{Accumulation (Cells+Bile canaliculi+Probenecid)}} \times 100$$

2.7. Statistical analysis

Quantitative data were usually expressed as means $\pm$ SD. They were statistically analyzed using ANOVA followed by Student-Newman-Keuls or Dunnett post-hoc test, paired Student's t-test or nonparametric Spearman's rank correlation method. The criterion of significance was $p < 0.05$.

3. Results

3.1. Morphology and canalicular network formation.

Primary mouse hepatocytes maintained for 3 or 4 days after seeding either in monolayer on plastic or collagen or in sandwich configuration with collagen and matrigel formed confluent or nearly-confluent cultures (Fig. 2A). Hepatocytes cultured in monolayer exhibited a morphology rather flattener than that of sandwich-cultured counterparts, in agreement with previous data (Richert et al., 2002). Sandwich-cultured hepatocytes, but not monolayer-cultured counterparts, displayed extensive canalicular networks (Fig. 2A). These bile canaliculi-like structures were moreover fully functional since they strongly accumulated the fluorescent dye CF (Fig. 2B).

3.2. Expression of drug transporter mRNAs.

Expressions of SLC drug transporter mRNAs in primary mouse hepatocytes were lower than those found in freshly isolated hepatocytes, whatever the culture time or the culture conditions

(Fig. 3). SLC transporter mRNA levels found in cultured hepatocytes after the 4-h seeding period were thus reduced to approximately 50 % of the values found in freshly isolated counterparts and more significantly fall in day 1-cultured hepatocytes (Fig. 3). Expression of Ntcp, Oatp1b2 (Slco1b2), Oct1, Oat2 and multidrug and toxin extrusion transporter (Mate) 1 (Slc47a1) remained thereafter very low along the culture period (from day 1 to day 4 after seeding), whatever the culture conditions. By contrast, mRNA expression levels of Oatp1a1 (Slco1a1) and Oatp1a4 (Slco1a4) significantly raised up for the period day 2-day 4 of culture when compared to those found in day 1-cultured hepatocytes, without however significant differences between the different culture conditions and without reaching initial value found in freshly isolated hepatocytes (Fig. 3).

With regard to ABC transporters, bile salt export pump (Bsep/Abcb11) and Mrp6 (Abcc6) exhibited a time-dependent mRNA expression profile in cultured hepatocytes similar to that described above for some of SLC transporters, i.e., an early down-regulation not influenced by culture conditions (Fig. 4). By contrast, Mrp2 (Abcc2) mRNA expression remained rather stable during culture (Fig. 4). Other ABC transporters such as multidrug resistance (Mdr) 1a (Abcb1a)/P-glycoprotein, Mdr1b (Abcb1b)/P-glycoprotein, Mrp1 (Abcc1), Mrp4, Mrp5 (Abcc5) and breast cancer resistance protein (Bcrp/Abcg2) exhibited a marked up-regulation of mRNA expression with time in culture, in all of the culture conditions tested. Three days-old sandwich-cultured hepatocytes displayed however rather lower levels of induction of these transporters when compared to their counterparts cultured in monolayer/plastic or monolayer/collagen configuration (Fig. 4). Mrp3 (Abcc3) mRNA expression was also induced with culture time for the three used culture conditions, even if this up-regulation reached a significant level only for 2 days- and 4 days-old hepatocytes maintained in monolayer/plastic conditions (Fig. 4).

To more completely analyze the effects of culture time on drug transporter expression, drug transporters were ranked from the most expressed to the less expressed according to mRNA level of expression and mRNA expression profiles were then compared. As shown in Fig. 5, expression profile of drug transporters in freshly isolated hepatocytes is correlated with that of 4-h cultured hepatocytes, but not with that of 4 days-old hepatocytes, whatever the used culture conditions. By contrast, mRNA expression profiles of 4 days-old hepatocytes cultured in monolayer/plastic, monolayer/collagen or sandwich/matrigel conditions were highly correlated (Fig. 5).

3.3. Expression of drug transporter proteins.

To investigate whether some of the alterations of drug transporter mRNA expression reported above in cultured mouse hepatocytes parallel changes in transporter protein expression, we analyzed protein levels of Ntcp, Bsep and Mrp4 by Western-blotting. As indicated in Fig. 6, expressions of Ntcp and Bsep were similar in freshly isolated hepatocytes and 4-h cultured hepatocytes, but strongly and similarly reduced in 3 days-old hepatocytes cultured either in monolayer/plastic, monolayer/collagen or sandwich/matrigel conditions. By contrast, Mrp4 protein was not detected in freshly isolated hepatocytes and 4 h-cultured hepatocytes, but was strongly induced in 3 days-old hepatocytes cultured in monolayer conditions; it was also up-regulated in sandwich-cultured hepatocytes, but to a much lesser extent (Fig. 6), likely reflecting the lower induction of Mrp4 mRNAs in this culture configuration (Fig. 3).

3.4. Activity of drug transporters.

Activity of sinusoidal uptake transporter was first investigated in 4-h cultured hepatocytes. As shown in Fig. 7A, intracellular accumulations of the Ntcp substrate taurocholate, of the Oatp substrate E3S or of the Oct1 substrate TEA were strongly inhibited by withdrawal of sodium

(inhibition by a 5.5-fold-factor) or addition of the Oatp inhibitor probenecid or of the Oct1 inhibitor verapamil (inhibition by a 7.1- and a 3.7-fold factor, respectively), demonstrating that these 4-h cultured hepatocytes exhibited robust Ntcp, Oatp and Oct1 activity. By contrast, intracellular accumulation of the Oat2 substrate PAH was only slightly inhibited by the Oat2 inhibitor probenecid (by a 1.3-fold factor) (Fig. 7A), suggesting that Oat2 activity was rather low in 4 h-cultured hepatocytes. Ntcp, Oatp and Oct1 activity in primary hepatocytes were next found to be markedly down-regulated with time in culture, whatever the culture conditions (Fig. 7B); 3 days-old hepatocytes cultured in a sandwich/matrigel configuration displayed however higher Ntcp and Oct1 activities than their counterparts maintained in monolayer/plastic or monolayer/collagen conditions, but these differences did not reach a significant level (Fig. 7B).

Secretion of drugs into bile canaliculi-like structures was studied using Ca^{2+} withdrawal-mediated disruption of canaliculi, as previously described (Liu et al., 1999a). Four days-old sandwich-cultured hepatocytes pre-incubated in Ca^{2+}-free conditions exhibited decreased retention of taurocholate, in contrast to counterparts maintained in monolayer/collagen conditions (Fig. 8A). These data confirmed that sandwich-cultured hepatocytes display functional bile canaliculi, in agreement with Fig. 1B, thus permitting the calculation of the BEI for taurocholate (Fig. 8A). Decreased accumulation of estradiol-17β-glucuronide (BEI=16 %), rhodamine 123 (BEI=17%) or CF (BEI=22 %) were also found in sandwich-cultured hepatocytes pre-incubated in Ca^{2+}-free medium (Fig. 8B). Sandwich-cultured hepatocytes with intact bile canaliculi were finally found to exhibit increased total accumulation of CF in the presence of the Mrp inhibitor probenecid, likely reflecting a probenecid-inhibited sinusoidal efflux of the fluorescent dye, with a SEI value of 29 % (Fig. 8C).

3.5. Regulation of drug transporter mRNA expression by prototypical activators of drug sensing receptors.

To determine whether primary mouse hepatocytes may express signaling ways involved in transporter regulation, cultured hepatocytes were exposed to prototypical ligands of drug sensing receptors and mRNA expressions of various transporters were analyzed (Fig. 9). The PXR activator dexamethasone (Moore et al., 2000) induced expression of Oatp1a4, Mrp2 and Mrp4; TCDD, a potent agonist of AhR (Hankinson, 1995), increased expression of Mrp4. Phenobarbital, which activates the CAR signaling cascade (Masahiko and Honkakoski, 2000), enhanced expression of Ntcp, Oatp1a4, Bsep and Mrp2. These inductions were observed in hepatocytes cultured in monolayer/plastic, monolayer/collagen or sandwich/matrigel configurations (Fig. 9). However the level of induction was somewhat different according to the culture conditions. Indeed, Oatp1a4 was rather less induced in dexamethasone- or phenobarbital-treated hepatocytes maintained in sandwich configuration, than in monolayer-cultured counterparts, whereas Mrp4 induction by dexamethasone reached a significant level only in hepatocytes cultured in sandwich/matrigel configuration (Fig. 9).

Discussion

The present study demonstrated that expression of drug transporters in primary mouse hepatocytes is markedly influenced by time in culture, whatever the culture conditions used, i.e., monolayer/plastic, monolayer/collagen or sandwich/matrigel configurations. Indeed, mRNA expression of SLC transporters, especially those of Ntcp, Oatp1b2, Oct1, Oat2 and Mate1, rapidly and remarkably fall during primary culture. This is associated with a concomitant loss of Ntcp protein expression and a reduction of Ntcp, Oatp and Oct1 transport activities. Expression of ABC transporters is also markedly impaired, but mostly in an

opposite manner. Indeed, various ABC transporters, such as Mdr1a, Mdr1b, Mrp1, Mrp3, Mrp4 and Bcrp, display a marked increase of mRNA expression (and protein expression for Mrp4) with time in culture, whatever the culture conditions used. By contrast, the bile salt canalicular transporter Bsep is down-regulated at both mRNA and protein level with time in culture. Only Mrp2 mRNA expression appears to be not significantly changed during primary culture of mouse hepatocytes. When considering transporter expression profiles, those found in 4 days-old hepatocytes cultured in monolayer/plastic, monolayer/collagen or sandwich/matrigel configurations fail to correlate with that of freshly isolated hepatocytes, but correlate each over. The use of sandwich versus monolayer configurations does not therefore prevent major changes in transporter expression occurring in primary mouse hepatocytes. Similarly, the use of sandwich cultures did not counteract reduction of sinusoidal SLC transporter expression in primary rat hepatocytes (Borlak and Klutcka, 2004; Kotani et al., 2011; Tchaparian et al., 2011), whereas, by contrast, SLC transporter expression is rather better preserved in primary human hepatocytes (Jigorel et al., 2005).

If the culture conditions do not influence overall transporter expression profile in primary mouse hepatocytes, some differences between monolayer and sandwich configurations have however to be underlined. First, 3 days-old sandwich-cultured hepatocytes display rather limited mRNA up-regulation of the ABC transporters Mdr1b, Mrp1, Mrp4, Mrp5 and Bcrp when compared to their counterparts cultured in monolayer configurations, indicating that the sandwich configuration may transiently limit culture-associated over-expression of some ABC transporters. Secondly and probably most importantly, sandwich-cultured mouse hepatocytes exhibit functional bile canaliculi, which, by contrast, were not present in mouse hepatocytes cultured in monolayer configurations. Canalicular secretion of the bile salt taurocholate has been consequently detected only in sandwich-cultured mouse hepatocytes. BEI calculated for taurocholate in our sandwich-

cultured mouse hepatocytes was 36%, which is very similar to the value previously reported in sandwich-cultured mouse hepatocytes by Swift and Brouwer (Swift and Brouwer, 2010). For17-β-estradiol glucuronide, CF and rhodamine 123, BEI values calculated from our data are around 15-20 % and therefore lower than that found for taurocholate. Similarly, BEI values reported for CF (28 %) and rhodamine 123 (8 %) in sandwich/matrigel-cultured rat hepatocytes are lower than that calculated for taurocholate (60 %) (Turncliff et al., 2006). Interestingly, polarization of sandwich-cultured mouse hepatocytes allowed us to detect probenecid-inhibited sinusoidal efflux of CF, likely reflecting the activity of sinusoidal Mrps, and especially Mrp3, known to handle CF (Zamek-Gliszczynski et al., 2003) .

The precise cellular and molecular mechanisms involved in transporter expression changes occurring in primary mouse hepatocytes remain to be determined. A link with the de-differentiation process, which commonly occurs in primary rodent hepatocytes (Olsavsky Goyak et al., 2010), has most likely to be considered. This *in vitro* down-regulation of liver-specific functions is thought to result from the disruption of the normal tissue architecture, as well as from adaptation to *in vitro* environment (Elaut et al., 2006) and the subsequent loss of liver-enriched transcription factors such as C/EBPα and hepatocyte nuclear factors (HNFs) (Olsavsky Goyak et al., 2010), that directly or indirectly control drug transporter expression (Geier et al., 2008; Li and Klaassen, 2004). In addition, hepatocyte isolation process and primary culture are associated with production of oxygen reactive-species, which may contribute to transporter regulation (Ziemann et al., 1999). Interestingly, contact between hepatocytes and extracellular matrix proteins as well as the maintenance of a tridimensional morphology have been postulated to play a key-role in the maintenance of a well-differentiated state for hepatocytes (Schuetz et al., 1988) and thus likely contribute to the polarized status of mouse sandwich-cultured hepatocytes. Such cells nevertheless exhibit major changes in transporter expression levels, indicating that the use of a sandwich/matrigel

it is not sufficient to keep a full differentiated status. The global profile of transporter expression changes in primary mouse hepatocytes, i.e. down-regulation of sinusoidal SLC transporters and up-regulation of most of ABC transporters, may be in fact closed to a cholestatic expression pattern, as already hypothesized for cultured rat hepatocytes (Rippin et al., 2001). It may reflect an adaptative protective response for hepatocytes, resulting in decreased accumulation of drugs and chemical toxins through reduced uptake and increased efflux.

Primary mouse hepatocytes were found to retain major signaling ways regulating drug transporter expression in response to chemical inducers, whatever the culture conditions used. Indeed, Mrp4, a well-known target for AhR (Xu et al., 2010), was up-regulated in TCDD-treated primary mouse hepatocytes. Similarly, expression of the PXR targets Mrp2 and Oatp1a4 (Cheng et al., 2005; Kast et al., 2002) were induced in cultured mouse hepatocytes exposed to the PXR agonist dexamethasone, whereas phenobarbital treatment increased expression of Mrp2 and Mrp4, knowing that these ABC transporters have been previously shown to be regulated by CAR (Assem et al., 2004; Kast et al., 2002; Maher et al., 2005). Phenobarbital also induced expression of the SLC transporters Oatp1a4 and Ntcp and of the ABC transporter Bsep in primary mouse hepatocytes. These transporters have however not been previously reported to constitute targets for CAR (Wagner et al., 2005), thus suggesting the implication of CAR-independent effects of phenobarbital, possibly related to induction of HNF-4α by the barbiturate (Bell and Michalopoulos, 2006).

Data from our present study indicate that primary mouse hepatocytes can be used for drug transporter studies, with however the necessity to respect adequate culture conditions for that. Short time-cultured hepatocytes on plastic dishes, i.e. 4-h old primary hepatocytes, are likely to be retained for investigating whether a xenobiotic can be handled, or can inhibit a sinusoidal hepatic influx drug transporter, owing to the high transporter activities displayed by

these cells. Only Oat2-mediated PAH uptake was low in such cells, thus supporting the need to test additional Oat2 substrates or culture conditions to better characterize Oat2 transport in cultured mouse hepatocytes. For the study of canalicular and sinusoidal efflux transporter activities, the use of sandwich-cultured hepatocytes is the unique choice since polarity was retained only for this culture configuration; the marked altered profile of drug transporter expression in sandwich-cultured mouse hepatocytes has however to be kept in mind when interpreting experimental data and further studies are likely required to precise the exact relevance of *in vitro* data generated with primary mouse hepatocytes to *in vivo* situations. Finally, primary mouse hepatocytes can also be used for transporter regulation studies, whatever the culture conditions.

In summary, functional expression and regulation of drug transporters have been characterized in primary mouse hepatocytes, demonstrating the feasibility of using such cells for investigating interactions of xenobiotics with hepatic transporter activity or regulation. Such studies could be especially interesting to perform with primary hepatocytes from wild-type and transporter- or drug sensing receptor-deficient knockout mice, in order to investigate the implication of the deleted gene in term of drug transporter activity or regulation.

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Legends to figures

Fig. 1. Schematic representation of drug transporter expression in mouse hepatocyte.

Fig 2. Cell morphology and formation of functional canalicular networks in primary mouse hepatocytes.

Primary mouse hepatocytes were cultured in monolayer/plastic, monolayer/collagen and sandwich/matrigel configurations for 3 and 4 days. Hepatocyte morphology was then examined by light microscopy (A) whereas functional bile canaliculi were detected through CF labeling (B), as described in Materials and Methods. Bile canaliculi are indicated by arrows. Bar = 25 μM.

Fig. 3. Expression of SLC transporter mRNAs in primary mouse hepatocytes.

Primary mouse hepatocytes were cultured in monolayer/plastic, monolayer/collagen or sandwich/matrigel configurations for various lengths of time after seeding (from 4 h to 4 days). SLC transporter mRNA expressions were then determined by RT-qPCR, as described in Materials and Methods. Data are expressed for each transporter as percentage of expression found in freshly isolated mouse hepatocytes (FIH), arbitrarily set at the value of 100 %; they are the means $\pm$ SEM of values from at least three independent assays. *, $p<0.05$ when compared to FIH; #, $p<0.05$ when compared to 4 h-old cultured hepatocytes. D, day.

Fig. 4. Expression of ABC transporter mRNAs in primary mouse hepatocytes.

Primary mouse hepatocytes were cultured in monolayer/plastic, monolayer/collagen or sandwich/matrigel configurations for various lengths of time after seeding (from 4 h to 4 days). ABC transporter mRNA expressions were then determined by RT-qPCR, as described

in Materials and Methods. Data are expressed for each transporter as percentage of expression found in freshly isolated mouse hepatocytes (FIH), arbitrarily set at the value of 100 %; they are the means ± SEM of values from at least three independent assays. *, $p<0.05$ when compared to FIH; #, $p<0.05$ when compared to 4 h-old cultured hepatocytes. D, day.

Fig. 5. Correlation between drug transporter mRNA expression profiles in freshly isolated mouse hepatocytes and primary mouse hepatocytes.

Drug transporters were ranked according to their level of mRNA expression in freshly isolated mouse hepatocytes (FIH), 4 h-old cultured hepatocytes and 4 days-old hepatocytes cultured in monolayer/plastic, monolayer/collagen or sandwich/matrigel configurations. Correlations between transporter expression profiles were next analyzed using the Spearman's rank correlation method. Spearman's rank coefficients (ρ), p values and equations for the regression lines are provided at bottom right of each correlation graph.

Fig. 6. Ntcp, Bsep and Mrp4 protein expression in primary mouse hepatocytes.

Total protein extracts were prepared from freshly isolated mouse hepatocytes (FIH) and from primary mouse hepatocytes cultured in monolayer/plastic, monolayer/collagen or sandwich/matrigel configurations for various lengths of time after seeding (4 h, 1 or 3 days). Transporter protein expression was then analyzed by Western-blotting. Data shown are representative of two independent experiments.

Fig. 7. Sinusoidal drug influx activities in primary mouse hepatocytes.

(A) Primary mouse hepatocytes were cultured in monolayer/plastic configuration for 4 h after seeding. Cells were then incubated with the Ntcp substrate taurocholate (43.4 nM), the Oct1 substrate TEA (40 μM), the Oatp substrate E3S (3.4 nM) or the Oat2 substrate PAH (200 nM)

for 5 min, in the presence or absence of sodium (for taurocholate), 50 μM verapamil (for TEA), 2 mM probenecid (Prob) (for estrone-3-sulfate) or 10 mM probenecid (for PAH). Intracellular accumulations of substrates were then determined by scintillation counting. Data are the means ± SEM of at least three independent experiments. *; $p<0.05$ when compared to uptake in the presence of sodium (for taurocholate) or in the absence of inhibitors (for TEA, E3S and PAH). (B) Primary mouse hepatocytes were cultured in monolayer/plastic, monolayer/collagen or sandwich/matrigel configurations for various lengths of time after seeding (from 4 h to 4 days). Sodium-dependent accumulation of taurocholate (Ntcp activity), verapamil-inhibitable accumulation of TEA (Oct1 activity) and probenecid-inhibitable uptake of E3S (Oatp activity) were then determined as described above. Data are the means ± SEM of three independent assays. *, $p<0.05$ when compared to 4 h-old cultured hepatocytes. D, day.

Fig. 8. Canalicular (A, B) and sinusoidal (C) drug efflux activities in primary mouse hepatocytes.

(A) Primary mouse hepatocytes were cultured in monolayer/collagen or sandwich/matrigel configurations for 4 days after seeding. Cells were then pre-incubated in the presence or absence of Ca^{2+} for 10 min, and next incubated with taurocholate for 10 min. Intracellular accumulations of taurocholate in cells + bile canaliculi (condition with Ca^{2+}) or in cells only (condition without Ca^{2+}) were next determined by scintillation counting. Data are the means ± SEM of five independent experiments. *, $p<0.05$ when compared to cells pre-incubated in the presence of Ca^{2+}. BEI for taurocholate, calculated as described in Materials and Methods, is indicated on the top of the graph. (B) 4 days-old sandwich-cultured mouse hepatocytes were pre-incubated in the presence or absence of Ca^{2+} for 10 min and intracellular accumulations of estradiol-17β glucuronide, rhodamine 123 and CF in cells + bile canaliculi (condition with

Ca^{2+}) or in cells only (condition without Ca^{2+}) were then determined as described in Materials and Methods. Data are the means ± SEM of at least three independent experiments. *, $p<0.05$ when compared to cells pre-incubated in the presence of Ca^{2+}. BEI calculated as described in Materials and Methods are indicated on the top of the graphs. (C) 4 days-old sandwich-cultured mouse hepatocytes were loaded with CF diacetate for 10 min at 37°C in the presence of Ca^{2+}. Hepatocytes were next incubated in CF diacetate-free medium for 20 min in the absence or presence of 2 mM probenecid (Prob). CF retention into cells + bile canaliculi was then determined by spectrofluorimetry. The data are the means ± SEM of five independent experiments. *, $p<0.05$ when compared to cells not exposed to probenecid. SEI for CF, calculated as described in Materials and Methods, is indicated on the top of the graph. FAU, fluorescence arbitrary unit.

Fig. 9. Regulation of drug transporter expression by drug-sensing receptor agonists in primary mouse hepatocytes.

Primary mouse hepatocytes, cultured in monolayer/plastic, monolayer/collagen or sandwich/matrigel configurations, were either untreated (Unt) or exposed to 10 µM dexamethasone (Dex), 10 nM TCDD or 3.2 mM phenobarbital (Pheno) for three days. Drug transporter mRNA expressions were then determined by RT-qPCR, as described in Materials and Methods. Data are expressed as induction factors, i.e., the ratio transporter mRNA level in xenobiotic-treated hepatocytes versus that found in untreated counterpart for each culture condition. Data are the means ± SEM of five independent experiments. *, $p<0.05$ when compared to control untreated cells.

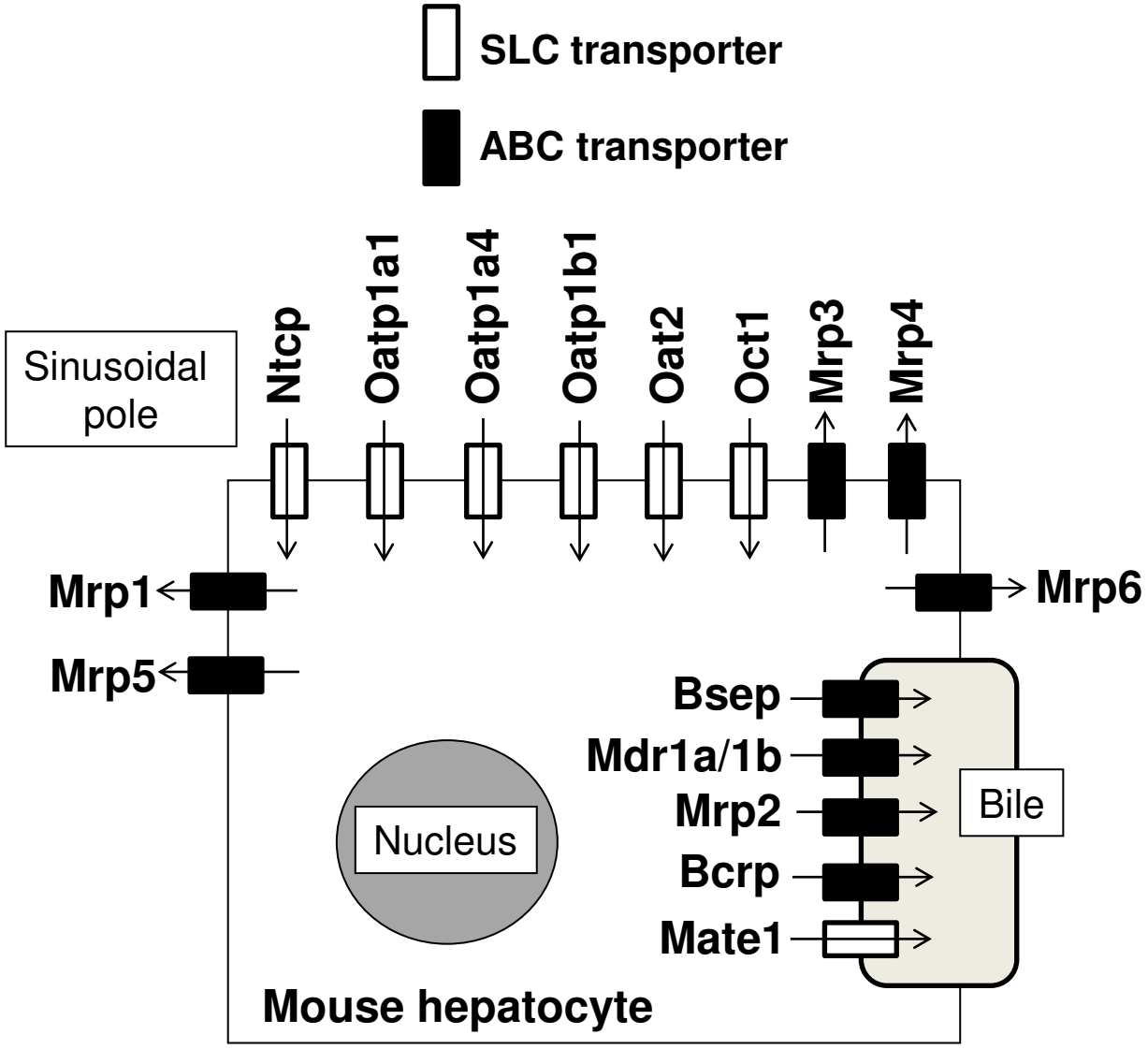

SLC transporter
ABC transporter
Sinusoidal pole
Ntcp
Oatp1a1
Oatp1a4
Oatp1b1
Oat2
Oct1
Mrp3
Mrp4
Mrp1
Mrp5
Mrp6
Bsep
Mdr1a/1b
Mrp2
Bcrp
Mate1
Bile
Nucleus
Mouse hepatocyte

Figure 1

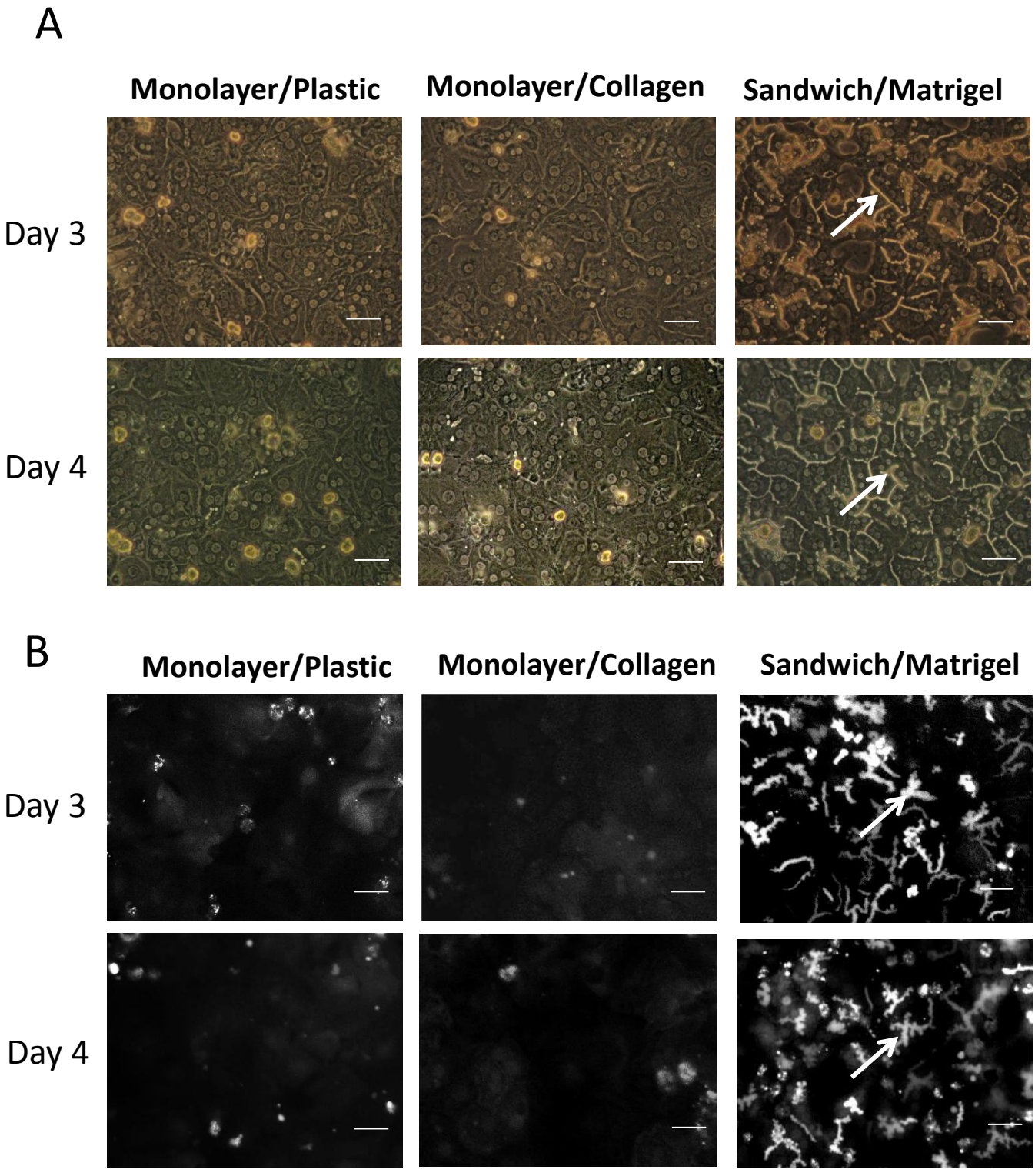

A
Monolayer/Plastic
Monolayer/Collagen
Sandwich/Matrigel
Day 3
Day 4
B
Monolayer/Plastic
Monolayer/Collagen
Sandwich/Matrigel
Day 3
Day 4

Figure 2

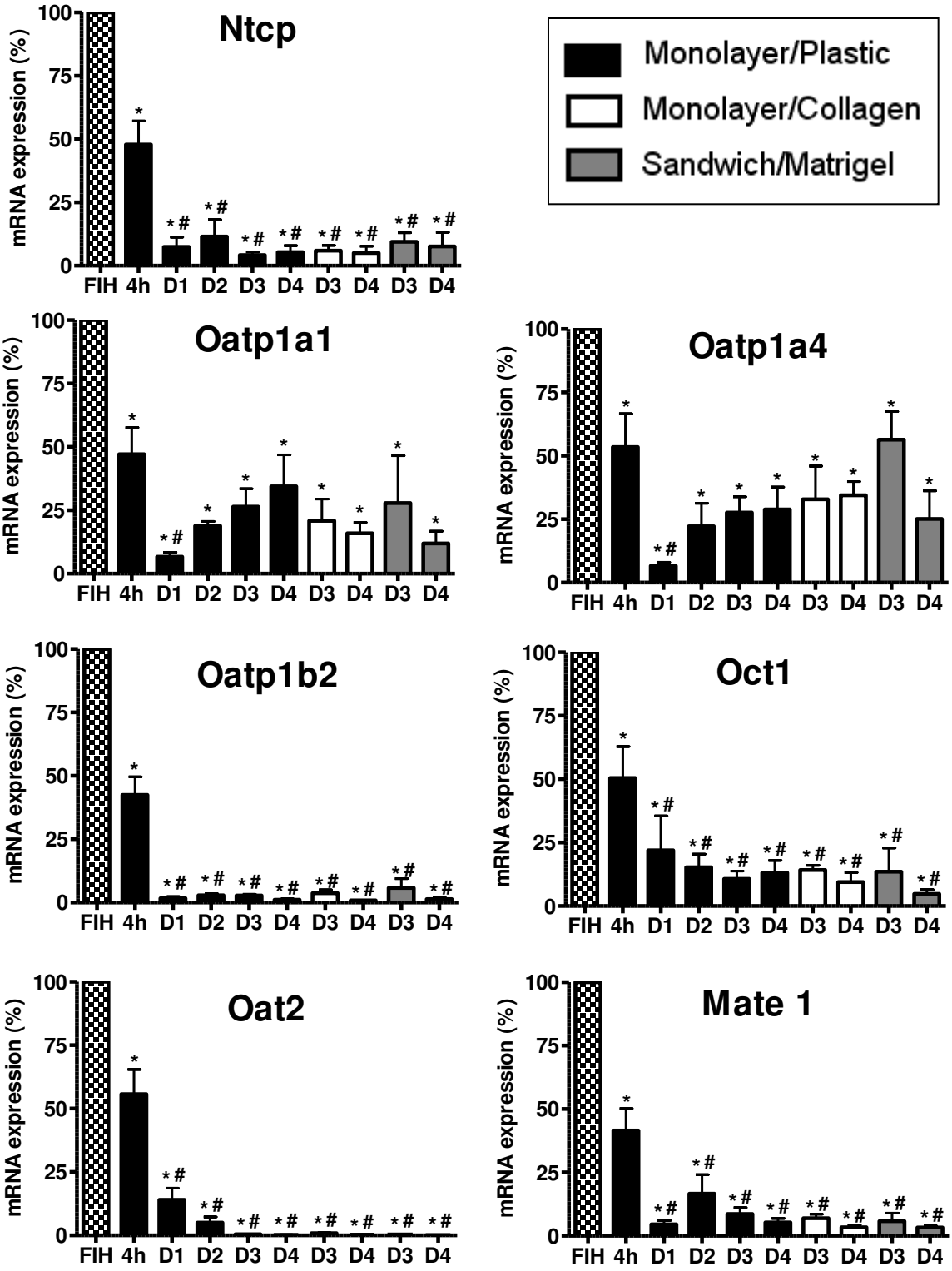
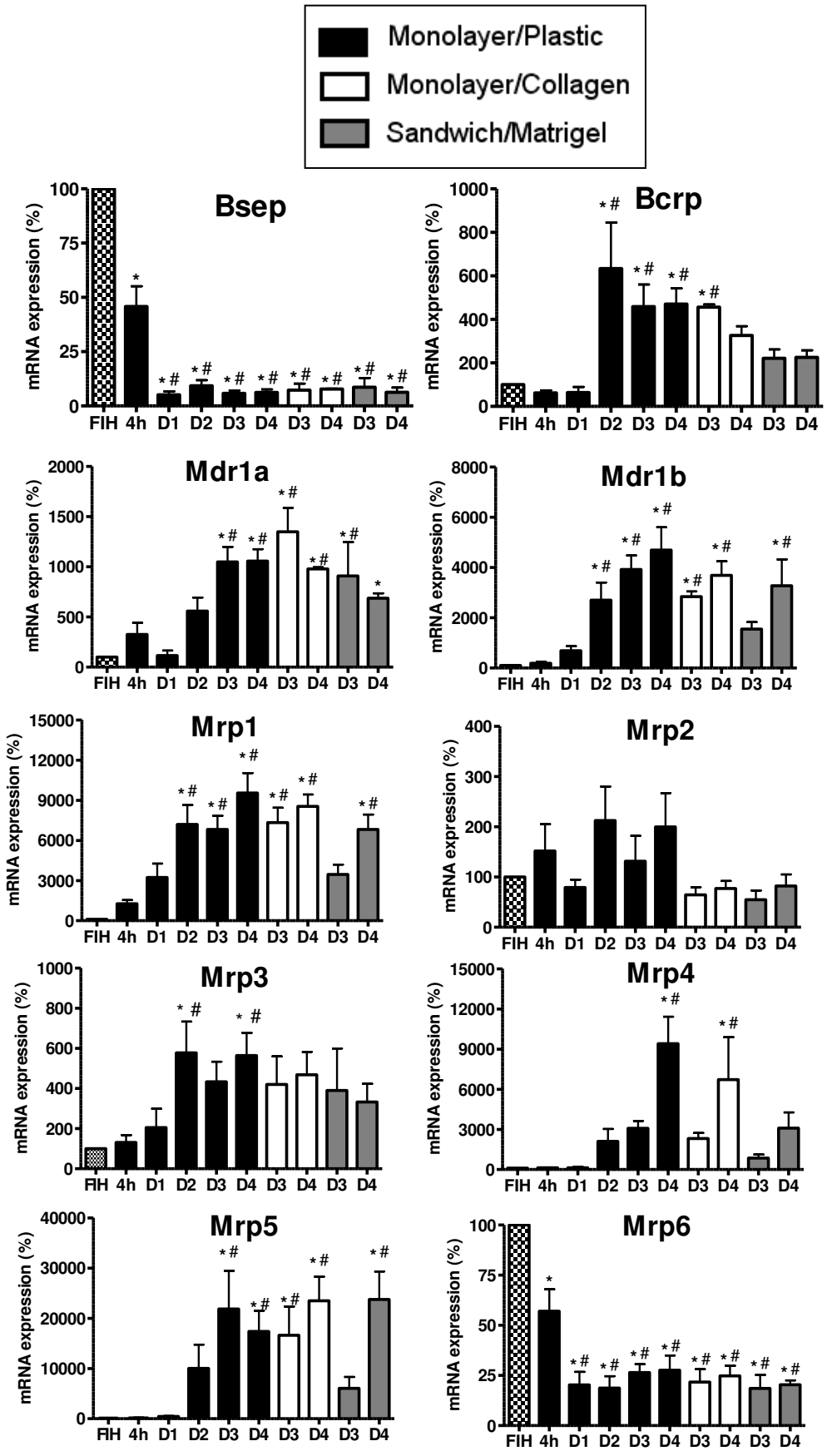

Ntcp
Oatp1a1
Oatp1a4
Oatp1b2
Oct1
Oat2
Mate 1
mRNA expression (%)
100
75
50
25
0
FIH 4h D1 D2 D3 D4 D3 D4 D3 D4
Monolayer/Plastic
Monolayer/Collagen
Sandwich/Matrigel

Figure 3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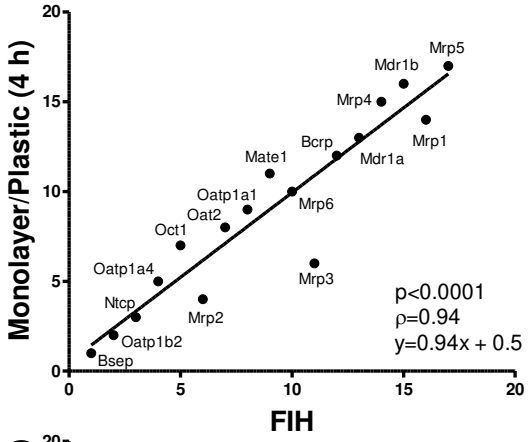
Monolayer/Collagen
Sandwich/Matrigel
Bsep
Bcrp
Mdr1a
Mdr1b
Mrp1
Mrp2
Mrp3
Mrp4
Mrp5
Mrp6
mRNA expression (%)
FIH 4h D1 D2 D3 D4 D3 D4 D3 D4

Figure 4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y=0.94x + 0.5
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Mdr1b
Mrp4
Mrp1
Bcrp
Mdr1a
Mate1
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Mrp6
Oat2
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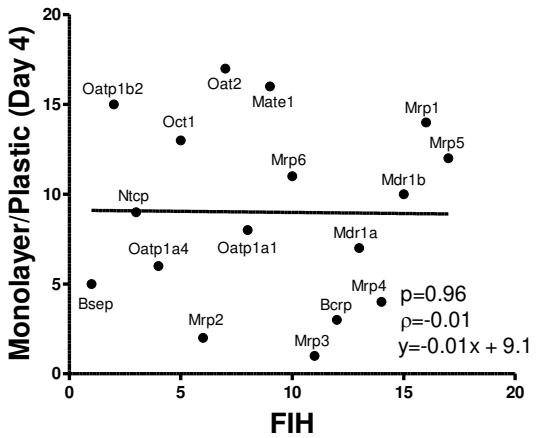
Bsep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Oct1
Mrp5
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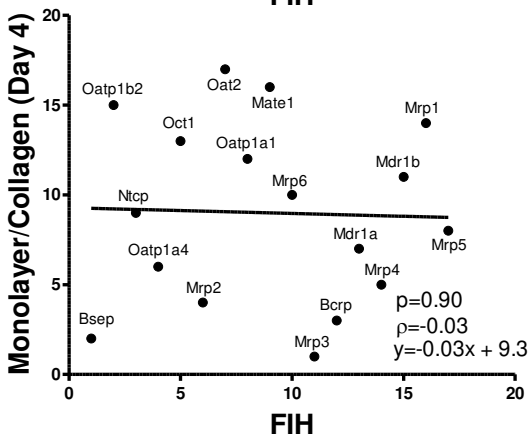
Mrp3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Mrp1
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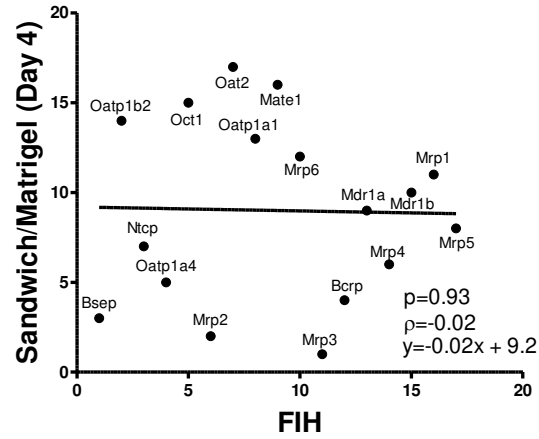
Bsep
Mrp3


Sandwich/Matrigel (Day 4)
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Oct1
Oatp1a1
Mrp6
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Mdr1b
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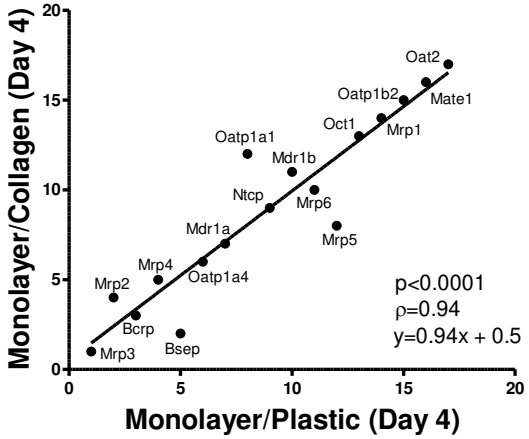
Bcrp
Bsep
Mrp2
Mrp3


Monolayer/Collagen (Day 4)
Monolayer/Plastic (Day 4)
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y=0.94x + 0.5
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Oatp1b2
Mate1
Oct1
Mrp1
Oatp1a1
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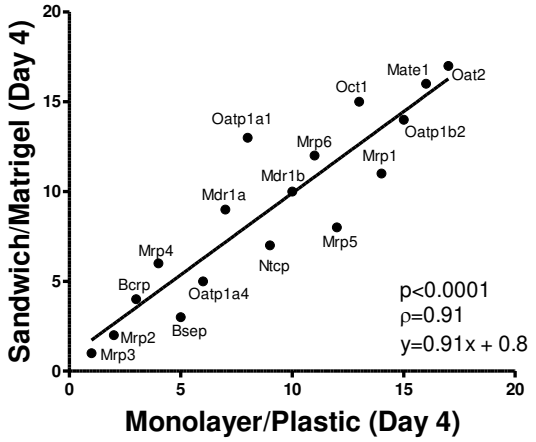
Sandwich/Matrigel (Day 4)
Monolayer/Plastic (Day 4)
p<0.0001
ρ=0.91
y=0.91x + 0.8
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Oct1
Oat2
Oatp1a1
Oatp1b2
Mrp6
Mrp1
Mdr1b
Mdr1a
Mrp5
Mrp4
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Oatp1a4
Bsep
Mrp2
Mrp3

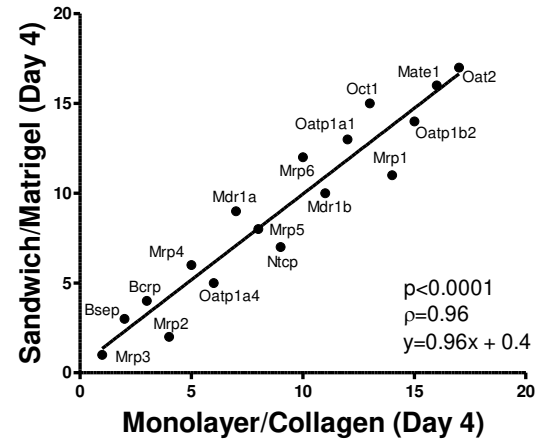
Sandwich/Matrigel (Day 4)
Monolayer/Collagen (Day 4)
p<0.0001
ρ=0.96
y=0.96x + 0.4
Mate1
Oct1
Oat2
Oatp1a1
Oatp1b2
Mrp6
Mrp1
Mdr1a
Mdr1b
Mrp5
Mrp4
Ntcp
Bcrp
Oatp1a4
Bsep
Mrp2
Mrp3

Figure 5

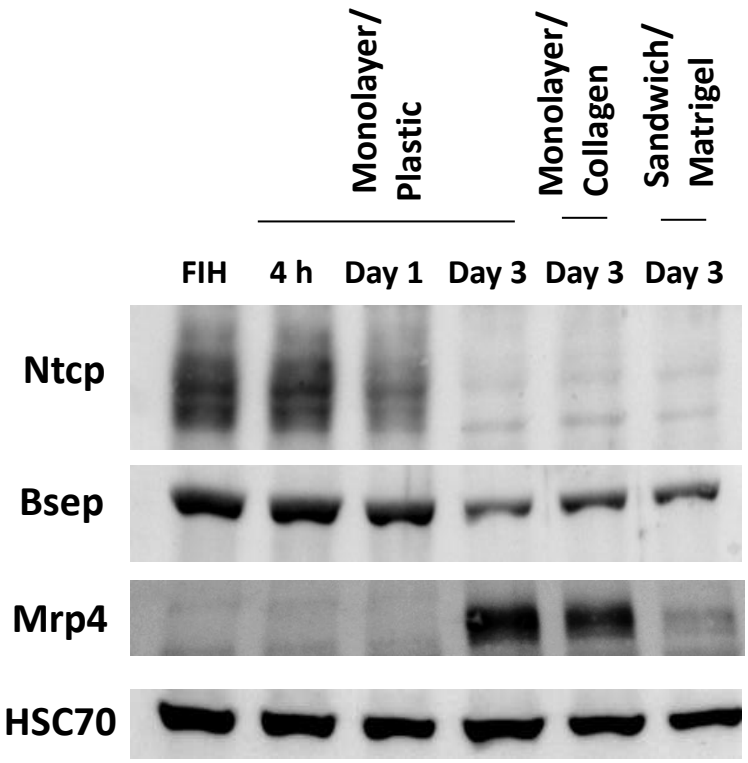
Monolayer/ Plastic
Monolayer/ Collagen
Sandwich/ Matrigel
FIH
4 h
Day 1
Day 3
Day 3
Day 3
Ntcp
Bsep
Mrp4
HSC70

Figure 6

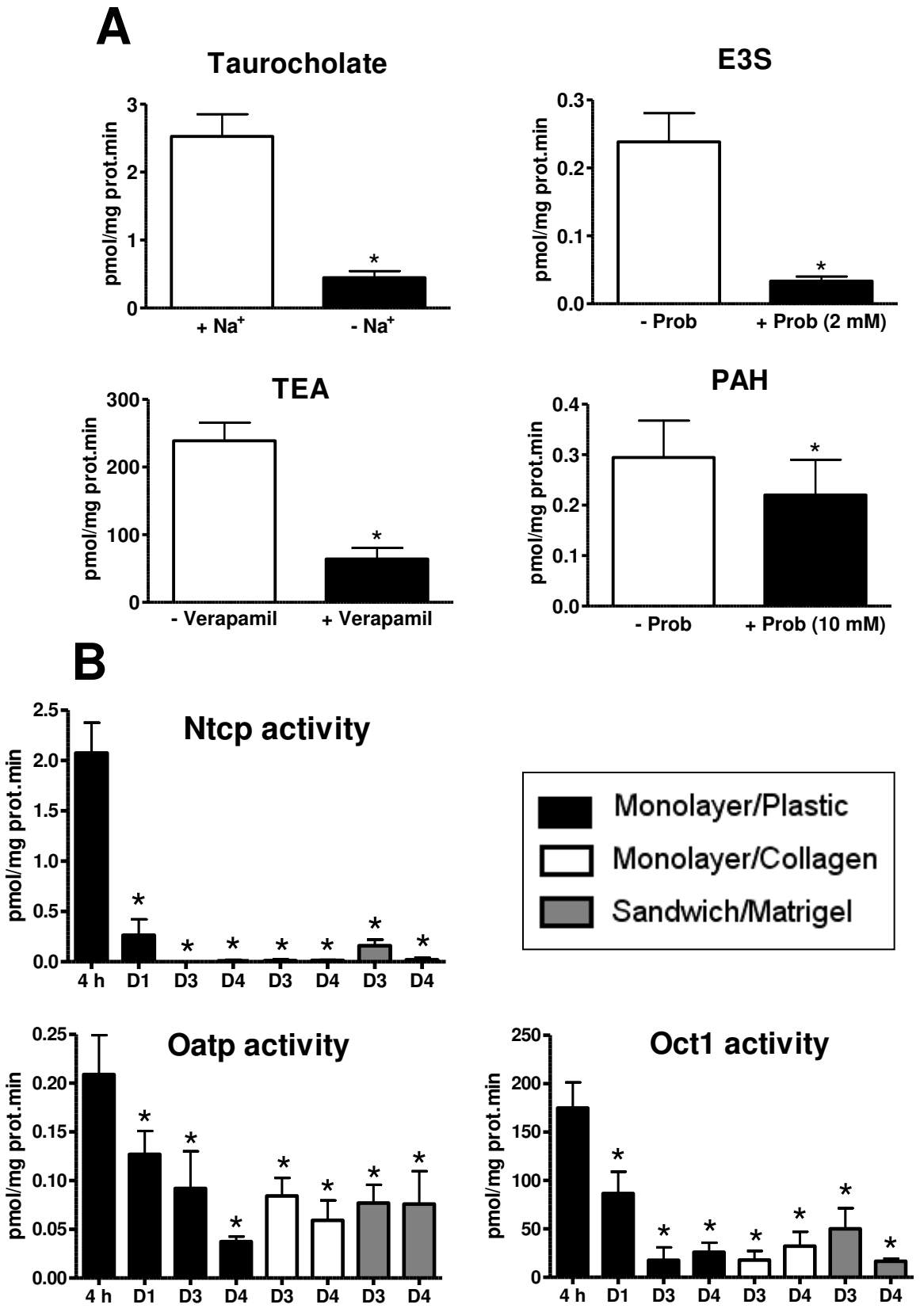
A
Taurocholate
pmol/mg prot.min
+ Na+
- Na+
E3S
pmol/mg prot.min
- Prob
+ Prob (2 mM)
TEA
pmol/mg prot.min
- Verapamil
+ Verapamil
PAH
pmol/mg prot.min
- Prob
+ Prob (10 mM)
B
Ntcp activity
pmol/mg prot.min
4 h
D1
D3
D4
D3
D4
D3
D4
Monolayer/Plastic
Monolayer/Collagen
Sandwich/Matrigel
Oatp activity
pmol/mg prot.min
Oct1 activity
pmol/mg prot.min

Figure 7

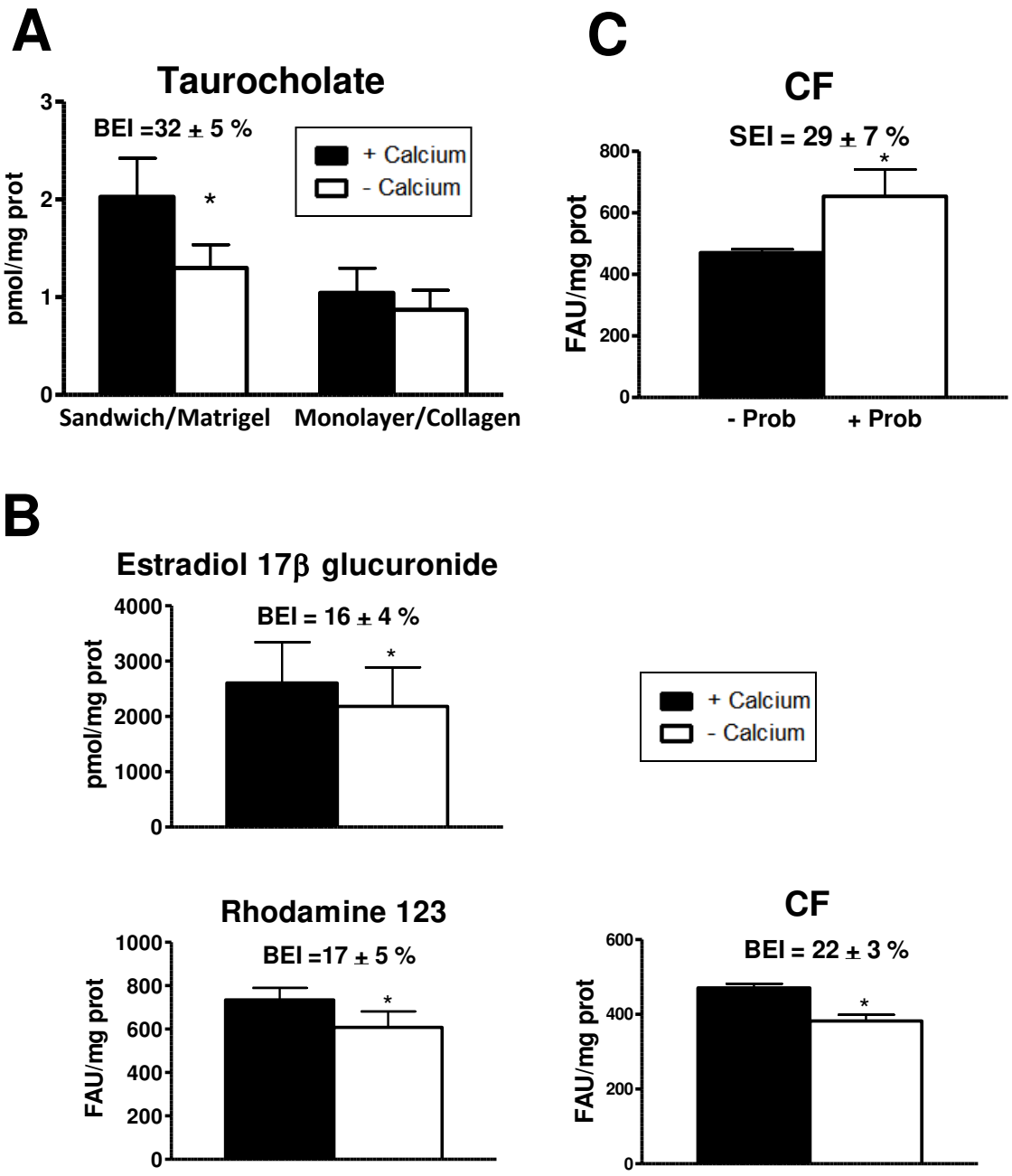
A
Taurocholate
BEI =32 ± 5 %
+ Calcium
- Calcium
pmol/mg prot
3
2
1
0
*
Sandwich/Matrigel
Monolayer/Collagen
C
CF
SEI = 29 ± 7 %
FAU/mg prot
800
600
400
200
0
*
- Prob
+ Prob
B
Estradiol 17β glucuronide
BEI = 16 ± 4 %
pmol/mg prot
4000
3000
2000
1000
0
*
+ Calcium
- Calcium
Rhodamine 123
BEI =17 ± 5 %
FAU/mg prot
1000
800
600
400
200
0
*
CF
BEI = 22 ± 3 %
FAU/mg prot
600
400
200
0
*

Figure 8

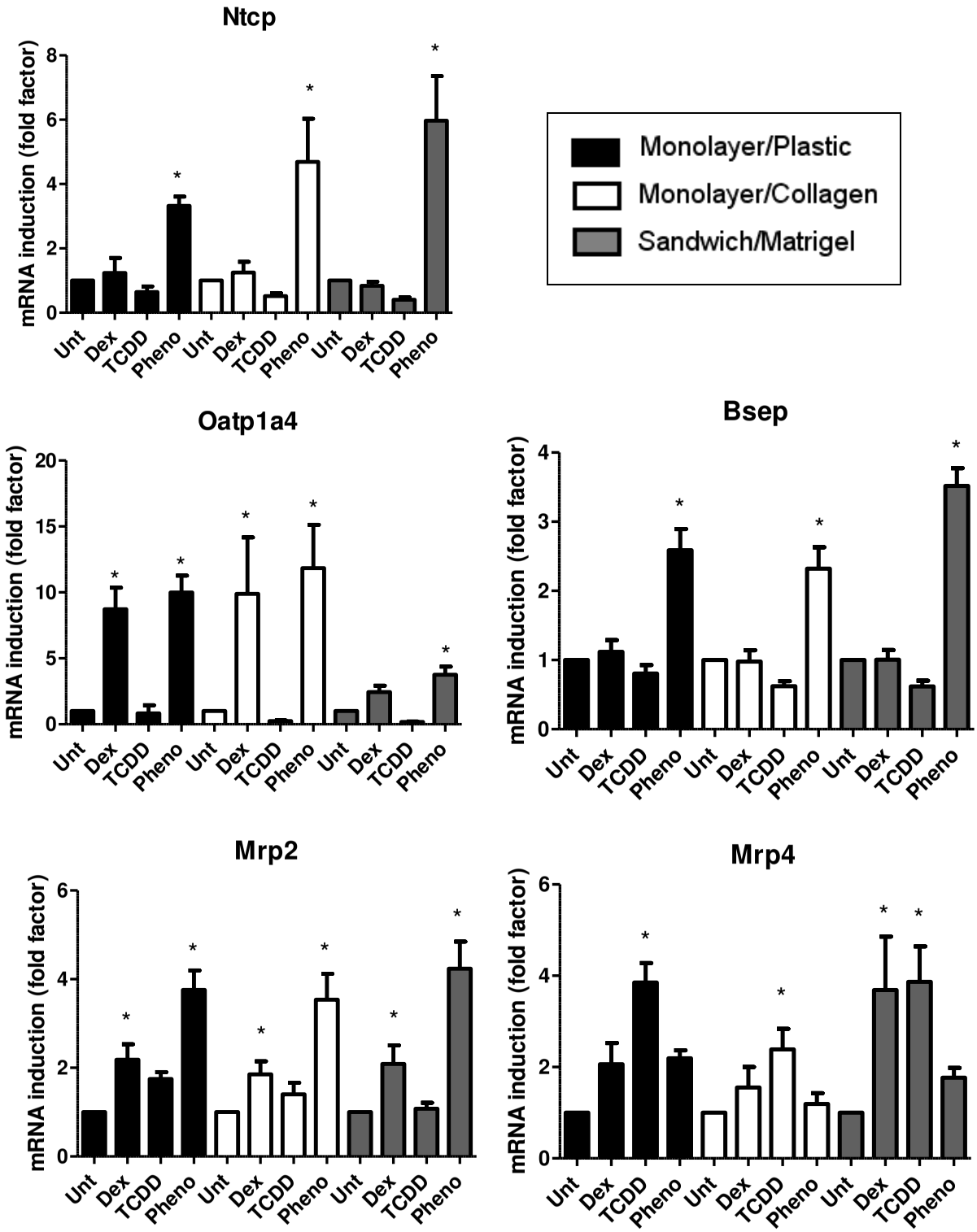
Ntcp
mRNA induction (fold factor)
Monolayer/Plastic
Monolayer/Collagen
Sandwich/Matrigel
Oatp1a4
Bsep
Mrp2
Mrp4
Unt
Dex
TCDD
Pheno

Figure 9

Supplementary Table 1. Primers used for qPCR assays

Gene	Sense	Antisense
Ntcp	GCTGGTCAGATGAAAGACCT	GGAGTTCAGCAAGATCAAGG
Oatp1a1	TAGCTTGCCTCCAGTATGCCTT	ACAGGCCAAATGCTATGTATGC
Oatp1a4	CAAGCTTTCTCCCTGCACTCTT	TCCTTCGCAGTGAGCTTCATT
Oatp 1b2	AGCAATGATCGGACCAATCCT	AACCCAACGAGCATCCTGA
Oat2	GATCACTGCTCTGGTGGTGA	GAGTGCAGTGAATCCCATTCC
Oct1	TGGCTGGGTGTACGACACTC	TCCTGTAGCCAGAGCCGACA
Mate1	GAATGGGTGGGCCAAGTATG	CATTGACCTGTCGTGCTGGAT
Mdr1a	ATGACAGATAGCTTTGCAAGTGTA	GGCAAACATGGCTCTTTTATCG
Mdr1b	AAGCCAGTATTCTGCCAAGCAT	CTCCAGACTGCTGTTGCTGATG
Bsep	CCTGTCAGCATTTTTGTCTG	TCATCGCCTTCCTCTTTAAC
Bcrp	TGGCTGTCCTGGCTTCAGTAC	CCAAGAATTCATTATACTGCAA
Mrp1	CGATCAAGAGTGGCGAAGG	AGGTGATGCCATTCAGTGTG
Mrp2	GCTGGGAGAAATGGAGAATGTC	ACTGCTGAGGGACGTAGGCTA
Mrp3	TCCCACTTTTCGGAGACAGTAAC	CTGAGGACCTTGAAGTCTTGGA
Mrp4	TGCTCCTCGTCGTAAGTGTG	TGGAGGGAGGACGATAAAT
Mrp5	GCTCCCTTTTTCCTGTTCACCTGTGC	ATGGGGAGGAGCCTTGTTCTTGATTC
Mrp6	GGATTGACAGCAGAAGAGG	GCAGAGGAAGAGGAACAGG
18S	CGCCGCTAGAGGTGAAATTC	TTGGCAAATGCTTTCGCTC