Effect of Culture Time on the Basal Expression Levels of Drug Transporters in Sandwich-Cultured Primary Rat Hepatocytes

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Abbreviations: ADE, adverse drug effects; Bcrp, breast cancer resistant protein; Bsep, bile salt export pump; Cyp, Cytochrome P450; DPP IV, dipeptidyl peptidase IV; ECM, extracellular matrix; E3S, estrone-3-sulfate; E17BG, estradiol 17β-D-glucuronide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MPP+, 1-methyl-4-phenylpyridinium; Mdr, multidrug resistance protein; Mrp, multidrug resistance-related protein; NME, new molecular entity; Ntcp, Na$^+$-taurocholate cotransporting polypeptide; (OTF1) POU domain, class 2, transcription factor 1; Oatp, organic anion transporting

polypeptide; Oat2, organic anion transporter 2; Oct1, organic cation transporter 1; PAH,

p-aminohippuric acid; PB, probenecid; P-gp, P-glycoprotein; qRT-PCR, real-time

quantitative reverse transcription polymerase chain reaction; Slc, solute carrier organic

transporter; TEA, tetraethylammonium; ZO-1, zonula occludens protein 1; 18S rRNA,

18S ribosomal RNA.

Abstract

Sandwich-cultured (SC) rat hepatocytes are employed in drug discovery for pharmacological and toxicological assessment of drug candidates, yet their utility as a functional model for drug transporters has not been fully characterized. To evaluate the system as an in vitro model for drug transport, expression changes of hepatic transporters relative to whole liver and freshly-isolated hepatocytes (day-0) were examined by RT-qPCR for 4 consecutive days of culture. No significant differences in transporters expression levels were observed between freshly-isolated hepatocytes and whole liver. Two distinct mRNA profiles were detected over time showing: (1) more than 5-fold decline in levels of uptake transporters such as Ntcp, Oat2, Oatp1a1, 1a4, and 1b2; and (2) greater than 5-fold increase of efflux transporters Pgp, Bcrp, Mrp1, 2, and 3 and 4. Additionally, protein levels and functional activities for selected transporters were also determined. Protein levels for Mrp2, Bcrp, Pgp, Ntcp, and Oatp1a4 corresponded to changes in mRNA. Functional activities of Oatps and Oct1 exhibited 3- and 4-fold decrease on day-2 and day-4, respectively, relative to day-0, while more than 10-fold reduction in Oat2 activity was observed. These results indicate that cell culture conditions used herein did not provide an optimal environment for expression of all hepatic transporters. Significant time-dependent alterations in basal gene expression patterns of transporters were detected as compared to liver or freshly-isolated hepatocytes. Further work and new strategies are required to improve the validity of this model as an in vitro tool for in vivo drug transport or biliary clearance prediction.

Introduction

Drug transporters are membrane proteins that play important roles in the absorption, distribution and elimination of a wide range of drugs, nutrients, and metabolites. They are distributed and expressed in many tissues including intestine, liver, kidney, and brain (Kusuhara and Sugiyama, 2002). In particular, hepatic drug transporters contribute significantly to the hepatic exposure and biliary excretion of various endogenous and exogenous compounds. Uptake (OATPs, OCT1, and OAT2) and efflux transporters (MRP3, 4, and 5) expressed at sinusoidal (basolateral) membranes mediate the translocation of drugs or endogenous metabolites from blood into liver and from liver into blood respectively. Transporters located at canalicular (apical) membranes (Pgp, BCRP, MRP2) serve to pump small molecules from liver into the bile (Muller and Jansen, 1997; Suzuki and Sugiyama, 1999; Sai, 2005).

The exact roles of many drug transporters are still under active investigation; however, several in vitro, preclinical and clinical studies have demonstrated that interactions with transporters contribute to the toxicity and therapeutic efficacy of small molecules. Similar to the liabilities observed in drug metabolizing enzymes, transporter-mediated drug–drug interactions (DDI) have been reported in clinic (Giacomini et al., 2010). Working alone or in concert with drug-metabolizing enzymes, these interactions are known to significantly impact the exposure, efficacy and safety profiles of drugs (Greiner et al., 1999; Lau et al., 2007; Wu and Benet, 2005). Therefore it has become critically important to characterize a drug candidate as a substrate, inhibitor and/or an inducer of transporters during the selection of new chemical entities in drug discovery.

A variety of well established in vitro approaches have been used in lead identification and optimization to study drug transporter activities, such as cells over-

expressing individual or multiple transporters, and primary cultured hepatocytes (Sahi J et al., 2005). Primary hepatocytes cultured in a sandwich configuration (between two layers of ECM) represent a relevant in vitro experimental model for assessment of hepatic metabolism and transport, drug-drug interactions, and hepatotoxicity (Annaert and Brouwer, 2005; Shitara et al., 2005). In contrast to transfected cells which express only one or two specific transporters and often lack drug metabolizing enzymes, hepatocytes contain all the complement of transporters and metabolizing enzymes, and retain regulation mechanisms as well. They are extensively used as an experimental model to investigate metabolism, transport and hepatotoxicity of investigational drugs (Ansede et al., 2010; Liu et al., 1999). Furthermore, they are also employed to explore the dynamic interplay between drug metabolism enzymes and transporters (Wu et al., 2005; Hewitt et al., 2007; Lau et al., 2002; Shitara et al., 2005), and regulation of hepatic transporters by nuclear receptor ligands (Jigorel et al., 2006). Additionally they are utilized to predict in vivo hepatobiliary disposition of drug candidates (Annaert et al., 2005; Hewitt et al., 2007; Hoffmaster et al., 2005; Liu et al., 1999).

While the expression of hepatic membrane transporters has been investigated in primary cultured hepatocytes to some extent (Hoffmaster et al., 2005; Zhang et al., 2005c; Jorgensen et al., 2007), a comprehensive understanding of transporter expression relative to culture time is limited. Distribution and elimination of drugs are often mediated by multiple transporters; hence the coordinated expression and activities of transporters at the sinusoidal and canaliculi side of hepatocytes could significantly influence drug disposition, DDI risk and toxicity. Thus, in order to further characterize this system and gain more insights into its utility and limitations, in the present communication, the gene expression profiles and functionality of major hepatic drug transporters (uptake or efflux) were studied in sandwich-cultured rat hepatocytes, a

widely used in vitro model. Further, since gene expression is a dynamic and highly regulated process and may change in response to the functional requirements of the hepatocytes, the expression of less important xenobiotic transporter (Mrp1, Mrp6) were also evaluated. The temporal changes in overall transporters gene expression profiles were examined over a period of 4-days of culture time. To investigate whether changes in mRNA levels corresponded to the changes in protein expressions, western blot analysis was performed for selected transporters. Additionally, functional activities of uptake transporters (Oatps, Octs, and Oats) at different culture time points were also assessed over time for comparison. With respect to functional activity of canalicular transporters they have been well characterized elsewhere (Annaert and Brouwer, 2005; Liu et al., 1999).

Materials and Experimental Procedures

Chemicals and Reagents

[^{3}H]Estradiol 17-β-d-glucuronide (E17BG) (45.2 Ci/mmol), *p*-[glycyl-^{14}C]-aminohippuric acid ([^{14}C]PAH) (55 mCi/mmol), and [^{3}H]1-methyl-4-phenylpyridinium acetate ([^{3}H]MPP+) (83 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Cell culture reagents and products were purchased from Mediatech (Manassas, VA). Williams' E medium (WEM) was purchased from Sigma-Aldrich (St. Louis, MO). Standard Hanks' balanced salt solution (HBSS) and insulin were obtained from Life Science Technologies (Carlsbad, CA). Matrigel™ and fibronectin were purchased from BD Biosciences (Bedford, MA). Supplement of insulin, transferrin and selenium (ITS, 500X) was from Lonza Inc. (Allendale, NJ). The reagents for total protein measurement were from Pierce (Rockford, IL). Primary antibodies, Mrp2 (M2III-6) and Bcrp (BXP-53) were purchased from Enzo-Life Sciences (Plymouth Meeting, PA);

Human MDR1 (D-11) that cross-reacts with rodent Mdr1a/b was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); Oatp1a1 (Slco1a1), and Oatp1a4 (Slco1a4) were from Millipore (Billerica, MA); POU domain, class 2, transcription factor 1 (POU class 2 homeobox 1), Bsep, Ntcp, and Mrp1 were from Abcam (Cambridge, MA); DPP IV-CD26-ADA binding protein was from Thermo Scientific (Rockford, IL); GAPDH was obtained from Sigma-Aldrich (St. Louis, MO), and ZO-1 was from Life Science Technologies (Carlsbad, CA). Secondary antibodies and western blot reagents were from LI-COR Biosciences (Lincoln, NE). RT-PCR enzymes and reagents were from Life Science Technologies (Carlsbad, CA). RNA extraction and purification kits were from Qiagen (Carlsbad, CA). Fluorescein isothiocyanate (FITC), Alexa 488, and 4',6'-diamidino-2-phenylindole (DAPI) were from Life Science Technologies (Carlsbad, CA).

Animals

Hepatocytes were isolated from male Sprague-Dawley rats weighing approximately 250 to 300 g obtained from Charles River Laboratories Inc. (Raleigh, NC). Animals were provided free access to food and water prior to surgery. All animal procedures were compliant with the guidelines of the Institutional Animal Care and Use Committee (Amgen Inc.).

Isolation and In Vitro Culture of Primary Rat Hepatocytes

Freshly-isolated rat hepatocytes were prepared according to a published method (Moldéus et al., 1978) based on collagenase digestion and separation of liver parenchymal cells. The isolated cells were resuspended in WEM containing L-glutamine (4 mM) and then centrifuged at 50xg (2-min, 4°C) to remove collagenase and cell debris. Three additional washes provided platable cells with viability (as assessed by trypan

blue exclusion testing) ranging from 87-95%. The hepatocytes culture condition was adapted from published method with minor modifications (Turncliff et al., 2006). Briefly, hepatocytes were resuspended in plating medium, Dulbecco's modified Eagle medium (DMEM), containing insulin (1 μg/ml), fibronectin (5 μg/ml), dexamethasone (0.1 μM), 50 U/ml penicillin, 50 mg/ml streptomycin and 10% fetal bovine serum (FBS), and seeded at a density of 400,000 cells/well in 24-well BD BioCoat™ collagen coated plates (BD Biosciences, Bedford, MA). Cells were incubated at 37ºC in 5% CO_2, 95% air and allowed to attach for 2-4 hr. After attachment, plating medium was discarded along with unattached hepatocytes, and the cells were washed once with 1XPBS. Ice cold hepatocytes plating media (DMEM,0.5 ml) supplemented with 50 U/ml penicillin, 50 mg/ml streptomycin, 1Xglutamine, insulin-transferrin-selenium (1%), dexamethasone (0.1 mM), 5% FBS containing 0.25 mg/mL Matrigel™ was then added to each well. Following an overnight incubation with Matrigel™ overlay, medium was replaced by culture media (WEM, without phenol red) supplemented with 50 U/ml penicillin, 50 mg/ml streptomycin, 1Xglutamine, insulin-transferrin-selenium (1%), dexamethasone (0.1 mM). Medium was replaced daily and cultured hepatocytes were maintained at 37°C in 5% CO_2, 95% air.

RNA Isolation and RT-qPCR

Total RNA was isolated from rat livers and cultured hepatocytes using Qiagen RNA mini-kits (Valencia, CA). On-column digestion was performed to remove all traces of residual DNA. The purified RNA was quantified by spectrophotometry on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), and the quality was evaluated by visualization of the isolated RNA on a capillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). For each of the test and

reference samples, 1 μg of total RNA was reverse transcribed according to the manufacturer's directions, using random hexamer primers with the SuperScript® III First-Strand Synthesis System for RT-PCR kit (Life Sciences; Carlsbad, CA). The resulting cDNA was treated with RNase H to remove residual RNA.

Customized TaqMan® Arrays employing 384-Well Micro Fluidic Cards (Applied Biosystems, Foster City, CA) were used to determine the relative expression and profiling of the selected transporters. The probe and primer sets for 20 transporter genes, 3 metabolism enzyme genes, and 18S rRNA were selected from predesigned gene expression assays. The selected PCR primers were gene specific and were designed to span an exon-exon junction. Two μl of single-stranded cDNA of each sample (equivalent to 100 ng of total RNA) were mixed with 48 μl of nuclease-free water and 50 μl of TaqMan® universal PCR master mix. The mixture was then transferred into a loading port on the card. The card was centrifuged, sealed, and PCR amplification was then performed using an Applied Biosystems Prism 7900HT sequence detection system under the following thermal cycler conditions: 10-min at 95°C (activation), 40 cycles of denaturation at 95°C for 15-sec, and annealing and extension at 60°C for 1-min.

TaqMan Data Analysis and Statistical Procedures

RQ Manager 1.2 software (Applied Biosystems, Foster City, CA) was used to determine the amplification curves based on the comparative threshold cycle (Ct) method (Bookout and Mangelsdorf, 2003). Relative quantitation (RQ) of the transcription levels was analyzed by RealTime StatMiner™ software package (Integromics; Granada, Spain) for automated data analysis. Gene expression values were normalized to 18S rRNA.

Statistical comparison of groups was performed using the parametric t-test functions of the Limma package (RealTime StatMiner™ software, Integromics Inc Philadelphia, PA) (Smyth, 2004). Benjamini-Hochberg method was used to calculate the false discovery rate (FDR) and to pick out genes whose expression levels are significantly different between two groups (Benjamini et al., 1995). For each gene the FDR was estimated and used to determine the corresponding adjusted (adj) *p* value. Only genes exhibiting expression changes at a significant level (adj *p*<0.05, fold change of 2) were considered to be differentially expressed. All samples were run in duplicate from three separate experiments.

Western Blot Analysis

Plated hepatocytes were washed twice with ice-cold PBS and incubated with ice-cold Complete Lysis-M Buffer containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) for 10-min. Cells were scraped off the plates and transferred into a centrifuge tube. The suspension was gently mixed on an orbital shaker at 4°C for 15-min to lyse the cells, and then centrifuged at 14,000xg at 4°C for 15-min. The supernatant was transferred to a fresh centrifuge tube and diluted at least 1:10 before determining the protein concentration using the BCA method (Pierce, Rockford, IL) (Smith et al., 1985). Samples were divided into aliquots and stored frozen at -80°C until subsequent analysis.

Protein samples were heat-denatured and 20 μg was loaded for separation on SDS NuPage® Bis-Tris gels (Life Technologies, Carlsbad, CA). After transfer, the membranes were blocked with LI-COR Odyssey® blocking buffer for 45-min and treated with primary antibodies (supplemental table 1), followed by anti-mouse or anti-rabbit conjugated secondary antibodies according to the manufacturer's instructions (IRDye

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800CW or IRDye 680LT; LI-COR, Lincoln, NE). The protein bands were visualized using LI-COR Odyssey® Imager, near-infrared (NIR) fluorescence detection imaging system. A monoclonal antibody against GAPDH (1:5000) (Sigma-Aldrich Inc, St. Louis, MO) served as a loading control. The protein levels of the bile canalicular protein, dipeptidyl peptidase IV (DPP IV), was used to assess the functional polarization, while POU domain, class 2, transcription factor 1 (OTF1) was used as a control for transcriptional activities. All immunoblots were repeated at least three times.

In Vitro Uptake Experiments

Activity of sinusoidal drug transporters was determined by cellular accumulation of radiolabeled probe substrates (Zamek-Gliszczynski et al., 2003). Briefly, after attachment (2-4hr) plated cells were washed with the assay buffer (HBSS containing 10 mM (N'-2-Hydroxyethylpiperazine-N'-2 ethanesulphonic acid; HEPES) and allowed to equilibrate for 20-min at 37°C with or without inhibitor. Dosing solutions containing the probe substrates [^{14}C]PAH (1 μM) for Oat2, [^{3}H]E17BG (1 μM) for Oatps, and [^{3}H]MPP+ (1 μM) for Oct1 in the presence or absence of the respective inhibitors probenecid (PB) (50 μM), estrone-3-sulfate (E3S) (100 μM), and tetraethylammonium (TEA) (250 μM) were added at 37°C or 4°C for 3-min. To terminate the active uptake process (3-min), cells were washed with ice-cold assay buffer. Cells were lysed and substrate accumulation was determined by liquid scintillation counting analysis. Passive diffusion was determined by carrying out parallel experiments at 4°C. Protein content of the hepatocytes in each well was measured with the BCA method. For analysis of differences between the groups, unpaired t-test with the Bonferroni correction for multiple comparisons was used following a two-way analysis of variance (ANOVA). Differences were considered significant at the $p < 0.05$ level.

Light Microscopy

Light microscopy was used to confirm the integrity of the canalicular networks. Photographic images were taken with a Fisher Scientific Micromaster Digital Inverted Microscope at 200xmagnification (Fisher Scientific; Wilmington, DE).

Immunofluorescence

Immunolocalization of selected transporter proteins on day-4 post-culture time was investigated by confocal microscopy. Hepatocytes plated onto glass chamber slides in sandwich configuration were fixed with ice-cold acetone for 10-min. After three 0.5 ml washes with PBS, cells were incubated with 1% ultra pure bovine serum albumin (BSA) for 60-min. Blocking buffer was removed and cells were incubated with the following diluted specific primary antibodies for rat Mrp2, Bcrp, Oatp1, Pgp or ZO-1. Samples were washed three times with 1 ml of PBS containing 0.05% Tween-20 (PBST), and then incubated with the fluorophores conjugated secondary antibodies (Alexa Fluor 488 or FITC). Following three washes with 1 ml of PBST/SDS (containing 0.01% SDS), the cells were counterstained with DAPI to label the nucleus. Cells were then mounted onto glass coverslips, examined using a Leica SP2 confocal microscope (Leica Microsystems, Bannockburn, IL) and differential interference contrast (DIC) images were collected. After sequential excitation, blue and green fluorescent images of the same cell were merged for colocalization. Background fluorescence was determined by applying the secondary conjugated antibody alone, and by replacement of the primary antibody with non-immune serum.

Results

Morphological Assessment of Cultured Rat Hepatocytes

Figure1 illustrates the morphological changes in cultured hepatocytes occurring over 4-days of cell culture. Microscopic observations showed that hepatocytes displayed mostly spherical and singular shapes at early stages after seeding. After incubation for 24-h, hepatocytes assumed the characteristic cuboidal shape with visible intercellular boundaries. Reaggregation of cell clusters and reestablishment of cell-to-cell contact was also apparent 24-h post seeding. Repolarization and formation of an extensive bile canalicular network was apparent at 72-h post-seeding time (Fig. 1).

Fluorescence Immunocytochemistry Using Confocal Microscopy in Cultured Rat Hepatocytes

Cellular distribution of selected transporter proteins (Pgp, Bcrp, Oatp1a1, and Mrp2) in day-4 cultures of hepatocytes was assessed by indirect immunofluorescence (Fig. 2). A characteristic fluorescent pattern (green) restricted to canalicular domain was observed in the hepatocytes immunostained for Bcrp. Immunofluorescence signals of Mrp2 and Pgp indicated expression and localization of these proteins primarily on the canalicular domain of adjacent cells and areas near cell junctions. Oatp1a1 immunoreactivity was demonstrated with a well-demarcated surface staining (green).

Samples stained with the labeled secondary antibody alone as negative control did not exhibit nonspecific staining suggesting minimum contribution of the background to the observed signal in hepatocytes (Fig. 2). Nuclei were stained with the blue fluorescent stain, DAPI. ZO-1, a marker protein for tight junctions (Stevenson et al., 1989), showed a visible fine network on the membrane areas of cell-cell contact

demonstrating defined membrane boundaries reflecting normal development of tight junctions and epithelial polarization.

Time Dependent Changes of Basal Transporters Expression Levels in Primary Rat Hepatocytes

Examination of the hepatic transporters gene expression levels in primary rat hepatocytes cultured in sandwich configuration showed no significant changes between whole liver and freshly-isolated hepatocytes on day-0, except for Mrp1 (~18-fold difference), indicating that the isolation procedure had minimal impact on expression levels (Figs. 3 and 4). Most of the transcripts remained relatively constant as well on day-1 compared to day-0. No significant change in transporters' relative mRNA levels was detected on day-1, except for Mdr1 where a significant increase was evident (Fig. 3).

Large variations in expression levels between day-0 and day-2 to day-4 were present. A clear difference in gene expression pattern between the uptake and efflux transporters was observed. Gene expression levels of most uptake transporters decreased during culture (indicated in green, Fig. 4), whereas most efflux transporters increased over time (as shown in red, Fig. 4). For example transcripts for uptake transporters including Oatp1a4, Oatp1b2, and Ntcp were significantly reduced (more than 10-fold) over time relative to day-0. The opposite pattern was observed for the efflux transporters such as Mrp1, Mrp2, Mrp3, Mdr1a, b and Bcrp with an increase of more than 10-fold in expression levels (Fig. 3). The mRNA levels for Oct1 and mdr2 remained relatively unchanged or less significant (Oct1, day-4). For Mrp5 and Oat1 however, the mRNA expression levels were very low and the real changes in gene expression could not be determined.

To assess the impact of culture time on phase I enzyme mRNA expression levels, selected metabolic enzymes were also included in this analysis. Cyp4a1 relative expression levels continuously declined, while Cyp3a11 remained relatively constant on day-0 to day-3 with about a 2-fold decrease by day-4. Cyp1a1 transcripts levels were not detectable in freshly-isolated hepatocytes and were detected at very low levels in rat liver. However, an increase in the Cyp1a1 mRNA levels was observed at later culture time points (day-2, day-3, and day-4). Taken together, these results indicate that the expression profiles of cultured rat hepatocytes are different from rat liver or freshly-isolated rat hepatocytes.

Time Dependent Changes of Transporter Protein Levels

To determine whether the changes in mRNA levels correspond to protein expression levels, western blot analysis of uptake transporters Oatp1a1, Oatp1a4, Ntcp and efflux transporters Bcrp, Mdr1, Mrp1, Mrp2, Bsep, and Mdr2 was performed. Protein levels for the canalicular marker DPP IV and the transcription factor OTF1 were assessed as measurement of apical functional polarization and normal transcriptional activities. Consistent with changes in mRNA levels, efflux transporters Mdr1, Mrp2 and Bcrp showed a time-dependent increase in protein amount after an initial decrease on day-1 (Fig. 5). Protein levels of Mrp1 remained relatively constant (Fig. 5). In concert with the mRNA transcription levels, a time-dependent decline in the protein levels of Oatp1a4 and Ntcp levels were also observed. After an initial decline on day 1, the protein levels of the uptake transporter Oatp1a1 improved on day-4 of culture despite down regulation at mRNA level. A minor decrease in the protein levels of Bsep was only observed on day-3 which appeared to recover on day-4 of culture (Fig. 5).

Sinusoidal Membrane Transporter Activities

The influence of culture time on selected uptake transporter activities was dramatic (Fig. 6). Uptake of E17BG (Oatp substrate) and MPP+ (Oct substrate) decreased approximately 4-fold. Uptake of PAH, a marker for Oat2 activity was decreased by about 8-fold. The decline in activities generally paralleled the observed decrease in the mRNA levels except for Oct1 where mRNA levels remained relatively unchanged.

Discussion

Primary sandwich cultured hepatocytes are the closest model to the in vivo situation and are recognized to maintain some of liver-specific functions (Wu et al., 2005; Hewitt et al., 2007; Lau et al., 2002; Shitara et al., 2005). Nevertheless, isolation of hepatocytes results in loss of cell polarity and architecture. While culturing hepatocytes in sandwich configuration restores cell polarization and excretory function (Hoffmaster et al., 2005; LeCluyse et al., 1994), temporal transcriptional changes in gene and protein expression have been reported (Boess et al., 2003; Li et al., 2009; Richert et al., 2006; Swift et al., 2010). The current study was performed to determine an optimal culture time point where the expression of sinusoidal or canalicular membrane transporters are comparable to that of freshly-isolated hepatocytes. Hence, basal expression levels of hepatic drug transporters was examined over a period of four days post culture by RT-qPCR. Protein expression of selected transporters with commercially available antibodies was also determined for comparison. Finally, activities of major hepatic uptake transporters were assessed to investigate the impact of expression change on their functions.

The culture conditions in the present study were adapted from published methods optimized in other laboratories (Turncliff et al., 2006; Chandra et al., 2001) except that fibronectin was included in initial culture stages to aid hepatocyte attachment and differentiation (Pankov et al., 2002). Phase contrast microscopic analysis confirmed that culture conditions used herein were optimal for hepatocyte viability, morphology and canalicular network formation. Cultured hepatocytes demonstrated intact bile canaliculi reconstruction following day-2 (Fig. 1), indicative of propagation and polarized distribution of apical transporters. Immunocytochemistry demonstrated correct regional localization and distribution of selected transporters at either basolateral (Oatp1a1) or apical (Pgp, Mrp2 and Bcrp) domains of the hepatocytes (Fig. 2). Visualization of the tight junction protein ZO-1 showed an organized network on the membrane areas of cell-cell contact further validating restoration of bile canaliculi and bipolar configuration (Fig. 2) (Stevenson et al., 1989).

Gene expression data confirmed that transporters expression levels were not influenced by the hepatocytes isolation procedures (Luttringer et al., 2002). In contrast, pronounced differences in transporters gene expression profiles were observed at different culture stages. Opposite time dependent expression profiles were found for uptake and efflux transporters. In general, mRNA levels of most uptake transporters were suppressed (Fig. 3), while a sharp increase in most of the efflux transporters was observed, relative to freshly isolated hepatocytes or liver. In particular, expression levels of Mdr1a, Mdr1b, Mrp1, Mrp2, Mrp3, Mrp4 and Bcrp improved, Mdr2 did not change, while Bsep and Mrp6 declined.

Transporters expression data at the mRNA levels are limited in sandwich cultured hepatocytes. Borlak et al., (2004) reported a substantial reduction in basolateral transporters levels (Oatp1a1, Oatp1a4 and Ntcp) and an increase in Mdr1b levels.

These observations are in accordance with the current results. For Mrp2, Bsep, Mdr2, and Mdr1a the report indicated no significant changes although high variability in expression levels was noted. Luttringer et al., (2002) examined changes in transporters (uptake or efflux) expression levels using microarray analysis. While these changes were to some extent in accordance with the present observations, the magnitude of the changes was less significant in some cases (Mdr1a, Bsep, Mrp1, Mrp2, Oatp1a4, and Oatp1a1). It is well documented that qRT-PCR is more sensitive than microarray. Microarray results may underestimate actual changes in gene expression and account for the observed discrepancy (Morey et al., 2006). Furthermore, down regulation of uptake transporters (Oatp1a1, Oatp1a4) and upregulation of efflux transporters (Mdr1a/b) were observed in both conventional and sandwich cultures (Jørgensen et al., 2007). Therefore, the results of this study confirm previous reports and provide a direct observation of global hepatic transporters transcriptional changes as a function of time in sandwich cultured rat hepatocytes.

With respect to protein expression, a decrease on day-1 of culture was detected for Mrp2, Bcrp and Pgp (Fig. 5) which was followed by a time dependent increase that paralleled mRNA expressions. Protein levels decline on day-1 could be caused by the rapid protein degradation or loss of cell polarity and architecture shortly after hepatocytes isolation. Bsep and Mrp1 protein levels remained relatively constant over different culture stages. As Mrp1 protein expression is considerably low and barely detectable in hepatocytes, the observed levels of Mrp1 protein suggest an apparent increase in hepatic Mrp1 protein. Alternatively, it may be also caused by cross-reactivity of the Mrp1 antibody to other proteins leading to an increase in the background signal. Similar to the mRNA expression, protein levels of uptake transporters Oatp1a4 and Ntcp declined over time while Oatp1a1 levels were relatively well maintained after an initial

decline on day-1 (Fig. 5). The fast recovery of Oatp1a1 protein levels may be related to regulated control mechanisms that stabilizes the protein in response to the functional requirements of the hepatocytes. The protein expression results (uptake and efflux) were consistent with previously published data (Hoffmaster et al., 2005; Swift et al., 2010). A comparable or higher protein levels were attained for Mrp2, Mrp1, Bcrp, Pgp and Bsep in cultured hepatocytes on day-4 which indicated that hepatocytes were able to synthesize and maintain certain proteins under the current culture conditions.

The uptake activity measured herein declined in parallel to the mRNA (Oatp, Oct, Oat) and protein levels (Oatp). Uptake across the sinusoidal membrane is an important first step in hepatic exposure and hepatobiliary excretion of small molecules. In some cases, as in highly polar drugs (e.g., pravastatin), the hepatocellular uptake is carrier-mediated and rate-limiting step for hepatic elimination (Treiber et al., 2004; Kotani et al., 2011). The impaired hepatic uptake could therefore limit the influx and hepatocellular exposure of such molecules and result in decreased metabolism or in vitro biliary clearance. Indeed, Kotani et al. recently showed that the hepatic clearances of taurocholate, digoxin, pravastatin and rosuvastatin predicted from uptake clearances in 4-day sandwich cultured rat hepatocytes markedly underestimated in vivo hepatic clearance due to down regulation of Oatps. These observations should be considered when utilizing sandwich cultured rat hepatocytes to evaluate therapeutic agents for hepatic uptake or in vitro biliary clearance. Likewise, changes in the expression levels of efflux transporters (Mrp2, Bcrp, Pgp, Mrp3, and Bsep) may lead to inaccurate estimation of in vitro biliary clearance of actively transported compounds, thus limiting the predictability of this model for in vivo biliary clearance.

Many elements could contribute to the dynamic changes observed in mRNA, protein levels, and activity. After isolation, hepatocytes lose many of their liver-specific

functions and are placed in a setting that is dramatically different from the native liver architecture; consequently the transcriptional changes could be related to loss of native physiology and architecture of hepatocytes in culture and subsequent adaptive changes to reduce stress and enhance cell survival rate. Conversely, it could be the result of protective mechanisms to reduce toxic waste materials including nutrient remnants generated during ordinary cellular metabolism and maintain homeostasis. Therefore, a down-regulation in the expression of absorptive mechanisms would limit the uptake of potentially toxic substances, while increase or maintenance of the expression levels of Pgp, Bcrp, Bsep, Mrp2, Mdr2 at the canalicular membrane could facilitate the secretion of potentially hepatotoxic endogenous compounds (Nies et al., 2009; Zollner et al., 2003).

Related human hepatocytes studies are scarce. In general, a similar trend in the expression (protein and gene) of uptake and efflux transporters has been described in human hepatocytes (Jigorel et al., 2005; Hoffmaster et al., 2005). Although an overall better maintenance of some uptake transporters (NTCP) as compared to rat hepatocytes has been reported (Jigorel et al., 2005), a comprehensive evaluation of all major hepatocellular transporters as described in the present studies may be necessary to determine the time profile expression of human hepatic transporters in cultured system.

In summary, this report presented a comprehensive survey of culture time related changes in relative gene or protein expression levels of transporters in sandwich hepatocytes. Our temporal analysis defined 18 hepatic transporter genes displaying dynamic expression changes across the time course studied. These data show concordance when compared with previous reports (Borlak and Klutcka 2004; Jigorel et al., 2005; Li et al., 2009; Richert et al., 2006; Swift et al., 2010). The findings from the present study indicate that under the current culture conditions an optimum environment

for the simultaneous expression of all of the transporters was not attained. Further

optimization of culture conditions is needed to attain basolateral transporters levels

comparable to liver.

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Authorship Contributions

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Performed tissue collection, and hepatocyte isolation: Craig Uyeda and Mark P. Grillo

Performed data analysis: Eskouhie H. Tchaparian

Wrote the manuscript: Eskouhie H. Tchaparian

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Footnotes

This work was presented as a poster at the FIP PSWC/AAPS Annual Meeting and Exposition on November 14, 2010; Ernest N., Morial Convention Center, New Orleans, LA, Abstract: M1222

Legends for figures:

Fig.1: Time course morphology images of primary rat hepatocytes. Light microscopic images show pattern of time-dependent differentiation and polarization of cultured hepatocytes as well as bile canalicular formation. The bile canalicular network is visible as bright dots or belts between adjacent hepatocytes.

Fig. 2: Immunofluorescence localization of ZO-1 (tight junction marker), Bcrp, Pgp, Mrp2 and Oatp1a1. Low-magnification confocal microscopic views show the localization of apical Oatp1a1, ZO-1 and basolateral Bcrp of cultured rat hepatocyte on day-4. To compare the expression level of Oatp1a1, Bcrp and ZO-1 in the rat hepatocytes, the labeling conditions and settings of the confocal microscope were identical for all observations. A microscopic image with non-immune serum (negative control) is also shown as an example.

Fig. 3: Time Dependent Changes in Membrane Transporters Expression in Cultured Rat Hepatocytes. Gene expression changes of sinusoidal and canalicular membrane transporters or Cyp450s in rat primary hepatocytes cultures on day-1 to day-4, and total liver as compared to day-0 (freshly-isolated hepatocytes) is shown in Figure 3 (a, b, c). Total RNA was isolated from freshly-isolated rat hepatocytes (day-0), whole liver and 1, 2, 3, and 4-day old cultured primary hepatocytes as described in materials and methods. The values of columns are expressed as the log 10 of mean of fold change. Statistical comparison of groups was performed using the parametric t-test functions. Benjamini-Hochberg method was used to determine the adjusted p values and statistical significance of each point (*adj $p < 0.05$, as described in materials and methods).

Fig. 4: Hierarchical cluster analysis of differentially expressed transporters genes in primary rat hepatocytes cultures. Data were represented as expression values relative to the freshly-isolated hepatocytes (day-0) at p < 0.05. Each column represents a selected culture time-point (day-1, -2, -3, -4) and whole liver (column 1), shades of red and green indicate up- or down-regulation of genes according to the color scheme shown. Black indicates no change. Genes represented by rows were clustered according to their similarities in pattern of expression.

Fig. 5: Western blotting analysis of the expression of transporters proteins in cultured rat hepatocytes. Western immunoblots were performed using either cultured hepatocytes lysate samples or total liver homogenate (20 mg protein/lane) as indicated in the materials and methods. GAPDH was used as the loading control, DPP IV as the canalicular marker, and OTF1 as transcriptional control for housekeeping genes.

Fig. 6: Time dependent changes in sinusoidal membrane transporters activities are shown in Figure 6a (Oatps), 6b (Oat2), 6c (Oct1). Primary rat hepatocytes were incubated with radiolabeled transporters probe substrates: $[^{3}H]$E17BG (1 μM), $[^{14}C]$PAH (1 μM), and $[^{3}H]$MPP+ (1 μM) which are transported by Oatps, Oat2, and Oct1, respectively. The incubations were performed as described in materials and methods in the presence or absence of E3S (100 μM), PB (50 μM), and TEA (250 μM) which are known inhibitors for Oatps, Oat2, and Oct1 respectively. Additionally, 4°C incubations were evaluated to determine passive diffusion of the substrate. Cells were lysed in lysis buffer and cellular accumulation of the radiolabeled compounds was determined by scintillation counting. Data were normalized relative to the protein values and expressed as pmol/mg/min. Data represented are the mean values of 3 separate experiments performed in triplicate. *p<0.05, **p<0.01, and ***p<0.001.
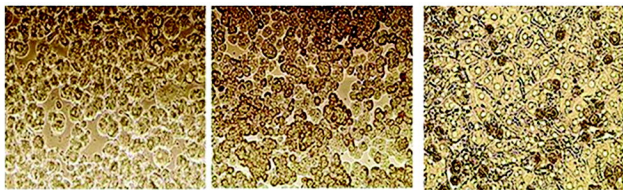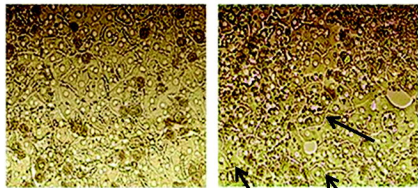
Figure 1

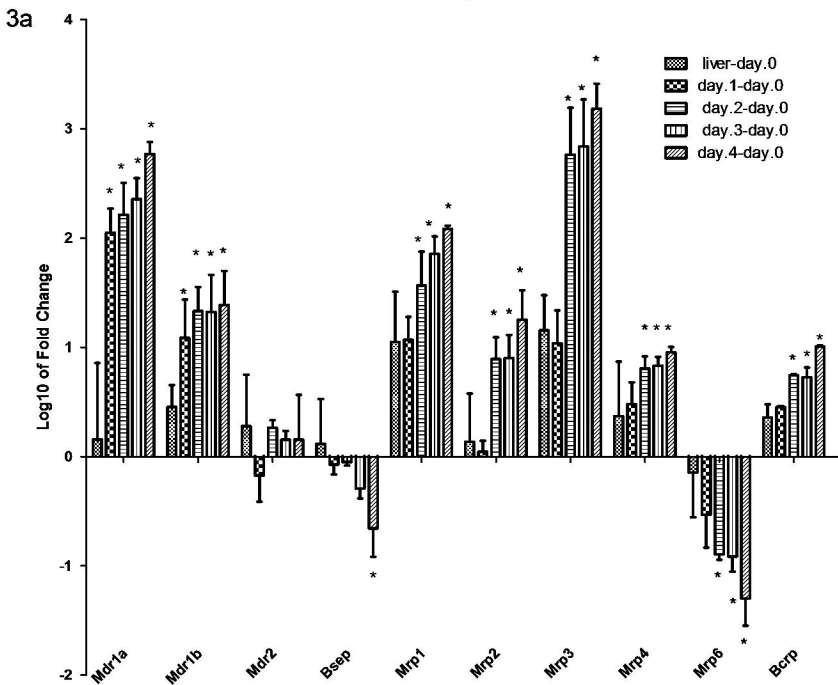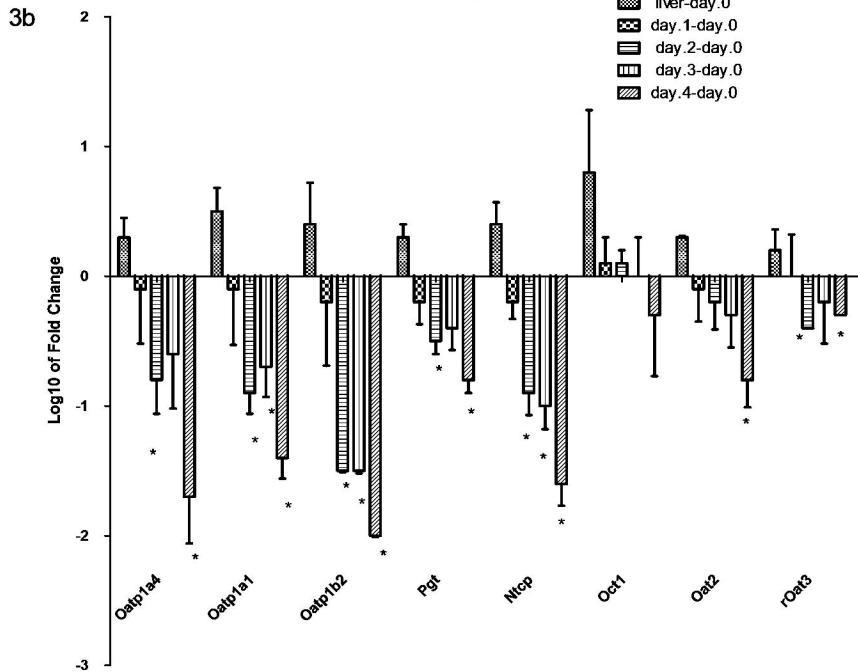

Days in Culture

0 (4hr) 1 2

3 4

Bile canaliculi

Figure 2

Figure 3

3b

Slc Transporters

Legend: liver-day.0, day.1-day.0, day.2-day.0, day.3-day.0, day.4-day.0

Y-axis: Log10 of Fold Change

X-axis categories: Oatp1a4, Oatp1a1, Oatp1b2, Pgt, Ntcp, Oct1, Oat2, rOat3

3c

CYP450s

Legend:
- liver-day.0
- day.1-day.0
- day.2-day.0
- day.3-day.0
- day.4-day.0

Y-axis: Log10 of Fold Change

Bar group labels: Cyp3a23/3a1, Cyp4a1
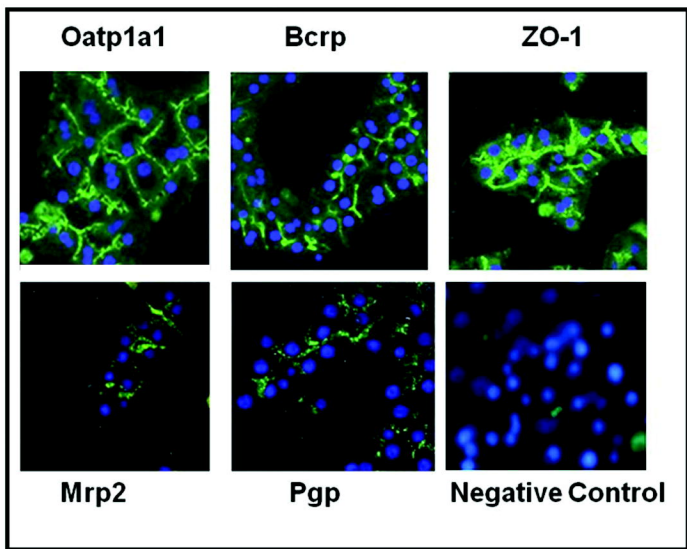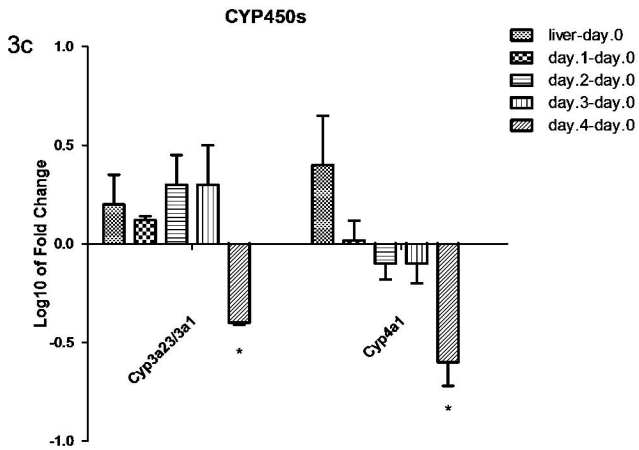
Figure 4

Figure 5

Figure 6

6a

6b

6c