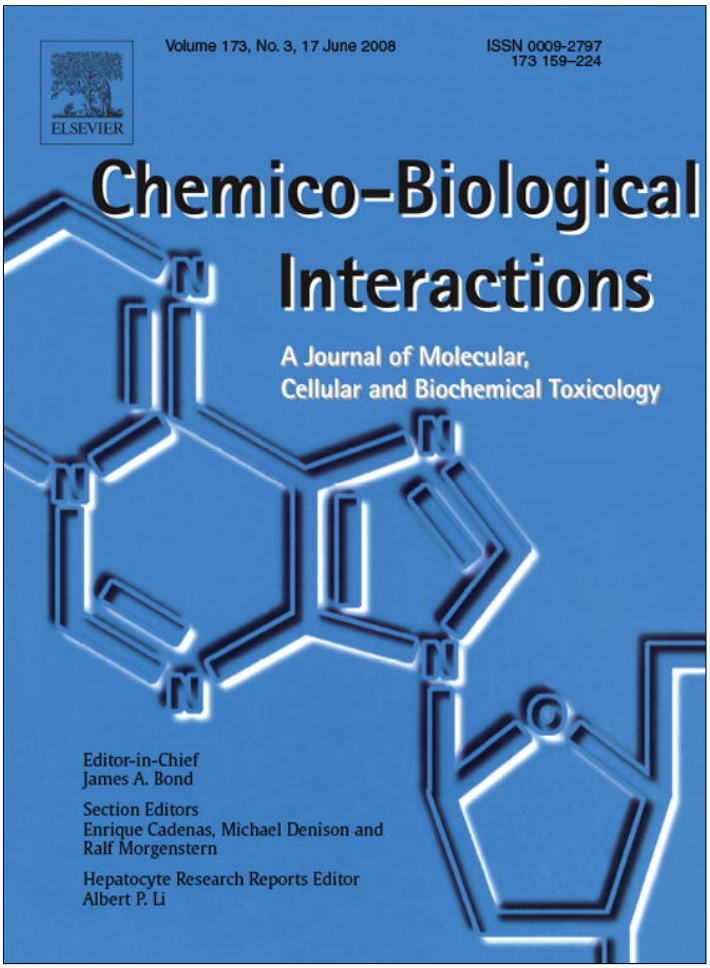

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Preservation of hepatic phenotype in lentiviral-transduced primary human hepatocytes

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ABSTRACT

Lentiviral vectors effectively transduce both dividing and non-dividing cells and stably integrate into the genome of the host cell. In this study, we evaluated the usefulness of a lentiviral system for genetic modulation of primary human hepatocyte cultures. Infection with GFP-expressing lentivectors shows that Huh7 and HepG2 cell lines, as well as primary cultures of human hepatocytes, are efficiently transduced by lentiviral vectors. Real-time RT-PCR analyses demonstrate that infection with lentivectors does not alter hepatic hallmarks such as the expression of the nuclear receptors CAR, PXR, RXRα, or HNF4α, or expression of the secretory protein, albumin. Additionally, infected hepatocytes retain the capacity for CYP3A4 induction in response to treatment with phenobarbital, a uniquely sensitive indicator of hepatic differentiation status. Lentivectors may be used for both over-expression and knockdown analyses in primary hepatocytes, as demonstrated in this study by >200-fold CAR over-expression and knockdown of CAR to less than 40% of endogenous levels, with corresponding effects on CYP2B6 expression. In summary, lentiviral vectors provide a novel methodology by which primary human hepatocytes may be stably genetically manipulated, with minimal effects on the differentiated hepatic phenotype. These approaches offer considerable advantage over current methodologies, providing a valuable alternative for use in pharmacological and toxicological investigations involving primary human hepatocyte models and potentially for cell-based therapeutics to treat hepatic dysfunction *in vivo*.

1. Introduction

Healthy hepatic function is integral to the disposition and metabolism of a diverse array of endogenous and exogenous substances. However, the liver is the target of many congenital and acquired diseases, some of which are amenable to gene therapy. Additionally, cultures of primary human hepatocytes serve as vital models for drug metabolism research and as predictors of toxicological outcomes that may result following exposure to xenobiotic agents. The ability to modulate gene expression within the fully differentiated hepatocyte is imperative for the realization of the full potential of hepatic gene therapy, and for manipulating the hepatocyte model in research investigations.

Development of an ideal methodology for the genetic modulation of primary human hepatocyte cultures has proven challenging. Primary human hepatocytes are refractory to most common transfection techniques. While adenoviral vectors transduce hepatocytes effectively, the vectors are non-replicating and remain episomal, and thus gene expression is transient [1,2]. Retroviral vectors integrate into the host genome, but most require a round of cell division for the integration event to occur [3–5]. Such viral vectors are therefore ineffectual for stable genetic

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modulation in quiescent primary hepatocyte cultures [6]. Baculovirus vectors are reasonably efficient for hepatocyte transduction, however, previous studies in our laboratory have demonstrated that the baculoviral infection event adversely affects the differentiated hepatic phenotype [7,8], with infection reducing hepatic albumin levels and ablating the phenobarbital induction response; both sensitive indicators of hepatocyte differentiation status [9].

Lentiviral vectors are unique within the retroviral vector family because lentivirus is capable of infecting both dividing and non-dividing cells and stably integrates into the host genome, thereby facilitating long-term transgene expression [10–12]. Several studies have shown that lentiviral vectors effectively transduce the widely used hepatoma cell lines, Huh7 and HepG2 [13–18]. More recently, researchers demonstrated that lentivectors afford high efficiency transduction of primary cultures of human hepatocytes [14,19–21]. While these results are promising, it is imperative to evaluate whether the infection event alters the differentiated hepatic phenotype, or otherwise adversely affects hepatocytes in primary culture.

In this investigation, we verify that lentiviral vectors efficiently transduce Huh7 and HepG2 hepatoma-derived cell lines, and demonstrate that transgene expression is preserved through cell division. We further confirm that primary human hepatocyte cultures are effectively transduced by lentivectors and that hepatocytes retain transgene expression for the duration of the culture period. Importantly, the results of our studies demonstrate that lentiviral infection does not alter mRNA expression levels of albumin or of key nuclear receptors in primary human hepatocytes, including constitutive androstane receptor (CAR), retinoid X receptor alpha (RXRα), pregnane X receptor (PXR), or hepatocyte nuclear factor four alpha (HNF4α). Additionally, we show that lentiviral-infected hepatocytes retain the capacity for cytochrome P450 3A4 (CYP3A4) induction in response to treatment with phenobarbital, a response consistent with a highly differentiated hepatic phenotype [22]. Further, we demonstrate that a lentiviral infection strategy in hepatocytes is effective for achieving both over-expression and knockdown of select genes with concomitant modulation of specific target gene responses, and identify a novel siRNA sequence capable of knocking down CAR to ~40% of endogenous levels in human hepatocytes.

2. Materials and methods

2.1. Materials

Cell lines were purchased from ATCC (Manassas, VA). Dulbecco's Modified Eagle's Media (DMEM) + GlutaMAX, Minimum Essential Media (MEM) + Earle's Salts + L-glutamine, William's E Media, non-essential amino acids, sodium bicarbonate, sodium pyruvate, HEPES, penicillin, streptomycin, GlutaMAX, and fetal bovine serum (FBS) were acquired from Gibco/Invitrogen (Grand Island, NY). Dexamethasone, insulin, selenium, transferrin, and linoleic acid/albumin were obtained from Sigma (St. Louis, MO). 6-(4-Chlorophenyl)imidazo[2,1–b] [1,3] thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) was purchased from BioMol (Plymouth Meeting, PA). Phenobarbital (PB) was purchased from Sigma (St. Louis, MO). All oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). pTracer-CMV2 was purchased from Invitrogen (Carlsbad, CA). pCDH1-MCS1-EF1-copGFP cDNA lentivector, pSIH1-H1-copGFP shRNA lentivector, and pPACKH1 packaging plasmid mix were purchased from System Biosciences (Mountain View, CA). QIAquick Gel Extraction Kit and QIAfilter Plasmid Maxi Kit were obtained from Qiagen (Valencia, CA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). PEG-8000 was acquired from Sigma (St. Louis, MO). Nikon inverted fluorescence microscope was purchased from Nikon USA (Melville, NY), and digital camera and SpotRT software were purchased from Diagnostic Instruments (Sterling Heights, MI). TRIzol Reagent was obtained from Invitrogen (Carlsbad, CA). High Capacity cDNA Archive Kit, Assays-on-Demand Gene Expression Products, and ABI 7300 Real-time PCR System were purchased from Applied Biosystems (Foster City, CA).

2.2. Cell culture and treatment

293T/17 transformed human embryonic kidney cells were maintained in DMEM + GlutaMAX supplemented with 0.1 mM non-essential amino acids, 0.75 g/l sodium bicarbonate, 1 mM sodium pyruvate, 10 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. HepG2 and Huh7 human hepatoma-derived cell lines were maintained in MEM + Earle's Salts + L-glutamine supplemented with 0.1 mM non-essential amino acids, 0.75 g/l sodium bicarbonate, 1 mM sodium pyruvate, 10 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. Primary human hepatocyte cultures were obtained through the Liver Tissue Procurement and Distribution System under NIH Contract #N01-DK-9-2310 (refer to Table 1 for hepatocyte donor information). Hepatocytes were isolated by a three-step collagenase perfusion technique and plated on rat-tail collagen as previously described [23]. Hepatocytes were maintained in William's E Media supplemented with 10 mM HEPES, 2 mM GlutaMAX, 100 units/ml penicillin, 100 μg/ml streptomycin, 25 nM dexamethasone, 10 nM insulin, 5 ng/ml selenium, 5 μg/ml transferrin, and 1% linoleic acid/albumin.

2.3. Lentiviral cDNA expression vector construction

Human CAR (NM_005122) was initially PCR amplified from human liver cDNA using the primer sequences: F

Table 1
Human hepatocyte donor information

Donor	Age	Gender	Ethnicity	Cause of death
HH-A	54	M	C	N/A (resection)
HH-B	45	M	C	Head trauma
HH-C	54	M	C	N/A (resection)
HH-D	55	M	C	Cerebrovascular accident
HH-E	74	F	C	N/A (resection)
HH-F	49	M	C	N/A (resection)
HH-G	69	F	C	N/A (resection)
HH-H	44	M	C	N/A (resection)
HH-I	57	F	C	N/A (resection)

5′-GATCGAATTCGTCATGGCCAGTAGGGAAGATGAG-3′, R 5′-GATCGATATCTCAGCTGCAGATCTCCTGGAGCCAG-3′ (restriction sites underlined). The amplicon was then digested with EcoRI and EcoRV and cloned by ligation into pTracer-CMV2. The hCAR fragment was subsequently sub-cloned into pCDH1-MCS1-EF1-copGFP (pCDH1) cDNA lentivector. Briefly, hCAR was digested from the original vector using EcoRI and EcoRV, electrophoresed through a 0.6% agarose gel, purified using QIAquick Gel Extraction Kit, and cloned by ligation into EcoRI and SwaI sites of the pCDH1 vector. Plasmids were purified using QIAfilter Plasmid Maxi Kit.

2.4. siRNA design and lentiviral siRNA expression vector construction

Small interfering RNA (siRNA) target sequences within the hCAR mRNA were identified using GenScript Corporation's "siRNA Target Finder" (https://www.genscript.com/ssl-bin/app/rnai) and confirmed by searching a number of other publically accessible siRNA target sequence identifiers. Template sequences encoding a short hairpin RNA (a stem-loop structure consisting of both the sense and anti-sense strands of the targeted sequence separated by a loop sequence) were generated and cloned into pSIH1-H1-copGFP (pSIH1) shRNA lentivector. Briefly, two complementary oligonucleotides (Table 2, restriction sites underlined) were annealled, digested, electrophoresed through a 3% agarose gel, purified using QIAquick Gel Extraction Kit, and ligated into BamH1 and EcoRI sites of the pSIH1 vector. Plasmids were purified using QIAfilter Plasmid Maxi Kit.

2.5. Lentiviral production, titering and target cell infection

Lentivirus production and subsequent target cell infections were performed essentially according to manufacturer's instructions, except that Lipofectamine 2000 was utilized for transfection of the 293T/17 packaging cells with pPACKH1 packaging plasmids and either pCDH1 or pSIH1 expression vectors. Psuedoviral supernatant harvested from packaging cells was either used to infect target cells directly, or was concentrated prior to infection by 10% PEG-8000 precipitation. Viral titer was estimated by infecting 100,000 293T/17 cells with 10-fold dilutions of virus immediately after plating. 48 h post-infection the percentage of GFP+ cells in three to five random fields was determined by counting cells at 200× magnification and used to calculate the number of transducing units per milliliter of viral supernatant (TU/ml). GFP expression in infected cells was observed using a Nikon inverted fluorescence microscope, and images were captured using SpotRT software with a digital camera.

2.6. Real-time RT-PCR

Primary human hepatocyte RNA was isolated using TRIzol Reagent and converted to cDNA using High Capacity cDNA Archive Kit, both according to manufacturers' instructions. Real-time RT-PCR was performed using Assays-on-Demand Gene Expression Products according to manufacturer's protocol. Briefly, 100 ng of cDNA template, 25 μl 2× Taqman Universal Master Mix, and 2.5 μl 20× Target Assay Mix were combined into 50 μl reactions. Each reaction was divided in half and run on an ABI 7300 Real-time PCR System. Real-time RT-PCR data were analyzed using the $\Delta\Delta C_T$ Method as previously described [24]. Briefly, C_T values for each half-reaction were averaged and this value used in subsequent calculations. Target gene expression levels for each sample were normalized to those of the corresponding 18S endogenous control by calculating the ΔC_T [$\Delta C_T = C_{T\text{target}} - C_{T18S}$]. Target gene expression levels of samples infected with gene- or siRNA-expressing virus were further normalized to those infected with empty virus by computing the $\Delta\Delta C_T$ [$\Delta\Delta C_T = \Delta C_{T\text{gene/siRNA-exp.virus}} - \Delta C_{T\text{emptyvirus}}$]. The relative fold change of target gene expression levels of samples infected with gene- or siRNA-expressing virus relative to those infected with empty virus was calculated by raising 2 to the $-\Delta\Delta C_T$ power [$2^{-\Delta\Delta CT}$].

3. Results

3.1. Lentiviral vectors effectively transduce Huh7 and HepG2 cells, as well as primary human hepatocyte cultures

In order to evaluate the usefulness of a lentiviral system for genetic modulation of hepatic models, we first tested the ability of the lentiviral vectors to transduce two commonly used hepatic model cell lines, Huh7 and HepG2. Indeed, when infected with lentiviral vectors engineered to express green fluorescent protein (GFP) at a multiplicity of infection (MOI) of ~5 TU/cell, >95% of Huh7 cells, and >75% of HepG2 cells were estimated to be GFP_+ at 5 days post-infection (Fig. 1A and B, upper panels). These results are consistent with those of previous studies which have reported that Huh7 cells are more efficiently transduced by lentivectors than HepG2 cells [14,16]. To confirm retention of transgene expression throughout cell division, infected Huh7 and HepG2 cells were maintained for over 3 months, throughout 20 passages, in the absence of any selective agent. Results indicated that GFP expression is retained in close to 100% of the cells in the initial GFP_+ Huh7 and HepG2 populations, through cell proliferation and without selective pressures, although there is a decrease in mean fluorescent intensity of the GFP_+ cells over time, most markedly in the HepG2 cells (Fig. 1A and B, middle and lower panels).

Primary cultures of human hepatocytes are the current gold standard in hepatic models. Thus, subsequent experiments assessed the effectiveness of a lentiviral system for genetic manipulation of difficult-to-transfect primary human hepatocyte cultures (refer to Table 1 for hepatocyte donor information). Primary human hepatocytes were infected with GFP-expressing lentiviral vectors at a MOI of ~5 TU/cell. Infected hepatocytes displayed high levels of GFP expression (>75% GFP_+ cells) at 2 days post-infection (Fig. 2A), and expression of GFP was retained for the duration of the hepatocyte culture time (14 days) (Fig. 2B). Microscopic examination revealed that neither the hepatic cell lines nor the primary human hepatocytes

Table 2
Sequences of oligonucleotides used to generate pSIH1 lentivectors expressing siRNAs targeted to CAR (restriction sites underlined)

Template I.D.	Strand 1	Strand 2
CAR 210	5′-GGTACCGGATCCGCCACAGGCTACCACTTTAATCTTCCTGT-CAGAATTAAAGTGGTAGCCTGTGGCTTTTTGAATTCGAATTC-GGTACC-3′	5′-GGTACCGAATTCGAATTCAAAAAGCCACAGGCTACCACTTT-AATTCTGACAGGAAGATTAAAGTGGTAGCCTGTGGCGGATCC-GGTACC-3′
CAR 379	5′-ACCGGATCCCTGGCATGAGGAAAGACATGACTTCCTGTCAG-ATCATGTCTTTCCTCATGCCAGTTTTTGAATTCGGT-3′	5′-ACCGAATTCAAAAACTGGCATGAGGAAAGACATGATCTGAC-AGGAAGTCATGTCTTTCCTCATGCCAGGGATCCGGT-3′
CAR 572	5′-ACCGGATCCAGCTCATCTGTTCATCCATCACTTCCTGTCAG-ATGATGGATGAACAGATGAGCTTTTTTGAATTCGGT-3′	5′-ACCGAATTCAAAAAAGCTCATCTGTTCATCCATCATCTGAC-AGGAAGTGATGGATGAACAGATGAGCTGGATCCGGT-3′
CAR 755	5′-ACCGGATCCGGAAATCTGTCACATCGTACTCTTCCTGTCAG-AAGTACGATGTGATGACATATTTCCTTTTTGAATTCGGT-3′	5′-ACCGAATTCAAAAAGGAAATCTGTCACATCGTACTTCTGAC-AGGAAGAGTACGATGTGACAGATTTCCGGATCCGGT-3′

exhibited morphological abnormalities upon lentiviral infection.

3.2. Lentiviral infection does not affect select markers of the differentiated hepatic phenotype

For primary human hepatocyte culture models to most accurately reflect *in vivo* responses, it is vital that the cultures maintain a highly differentiated status, i.e. that they retain hepatic signature hallmarks such as expression of specific nuclear receptors and hepatic secretory proteins, as well as metabolic enzyme induction capacity. As a genetic manipulation tool, the lentivirus system would be most valuable for use in a primary human hepatocyte model if the infection event does not alter these hepatic characteristics, or otherwise adversely affect the cell. To examine these parameters, we next evaluated whether lentiviral infection would alter an array of hepatic markers, including expression of the nuclear receptors CAR, RXRα, PXR, and HNF4α albumin expression, or induction of the CYP3A4 gene following phenobarbital treatment. Primary human hepatocyte cultures were transduced with lentivectors at a MOI of ∼10 TU/cell, a level sufficient to infect >80% of the cells as demonstrated by GFP marker gene expression (data not shown). Under these conditions, we observed no morphological changes in lenti-infected hepatocytes. Additionally, real-time RT-PCR analyses demonstrated that infection with lentiviral vectors did not alter hepatic mRNA expression of CAR, RXRα, PXR, or HNF4α (Fig. 3A). Albumin mRNA expression remained similarly unchanged by viral infection (Fig. 3B). Although variable between donors, as documented previously [25], induction of CYP3A4 upon phenobarbital treatment was preserved in lentivirally infected hepatocytes of individual donors (Fig. 3C).

3.3. Effectiveness of the lentiviral system for both over-expression and knockdown of CAR

In our studies, lentiviral vectors efficiently transduced primary human hepatocyte cultures with little effect on the expression of the assessed hepatic hallmarks and hepatic-

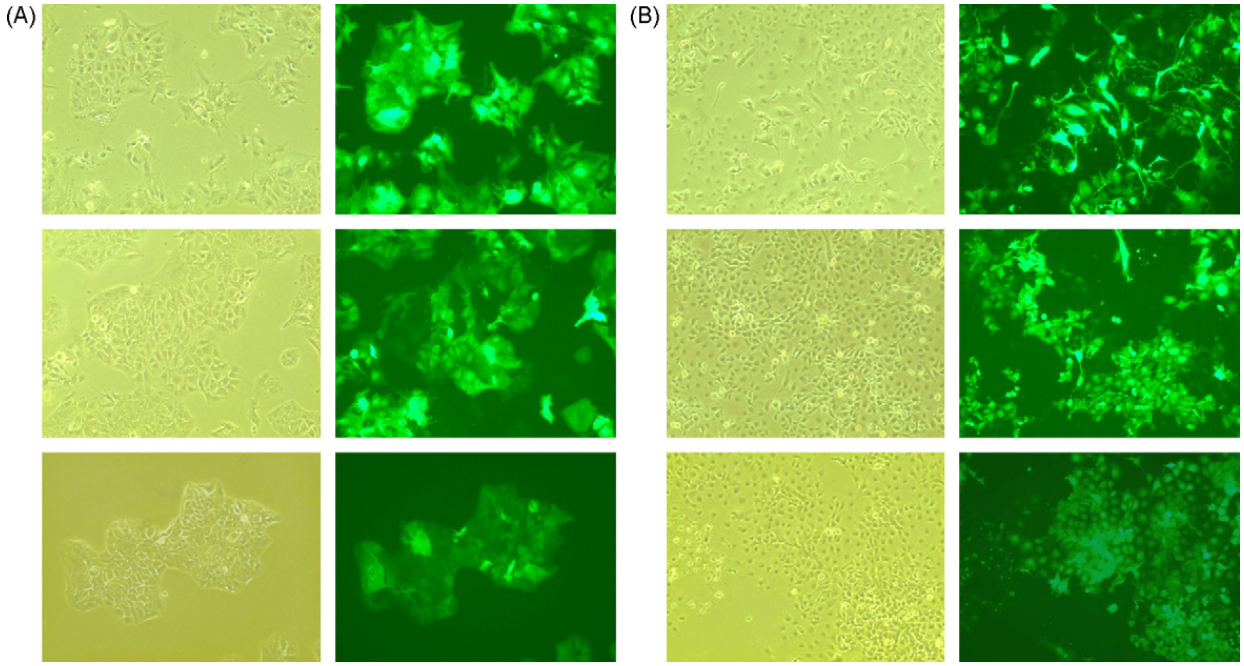

Fig. 1. GFP expression in Huh7 and HepG2 cells infected with GFP-expressing lentiviral vectors. Huh7 and HepG2 cells were infected while in suspension with pSIH1 empty lentivectors at a MOI of ∼5 transducing units/cell (TU/cell) and maintained through multiple passages in the absence of selective pressures. Cells were imaged at 100× magnification in both phase-contrast and fluorescence microscopy. (A) Upper panel, Huh7 cells at 5 days post-infection; middle panel, Huh7 cells after 5 passages; lower panel, Huh7 cells after 20 passages. (B) Upper panel, HepG2 cells at 5 days post-infection; middle panel, HepG2 cells after 5 passages; lower panel, HepG2 cells after 20 passages.

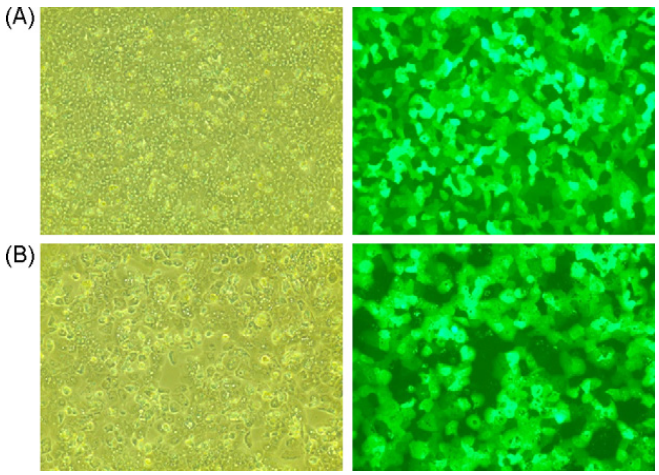

Fig. 2. GFP expression in primary human hepatocyte cultures infected with GFP-expressing lentiviral vectors. Hepatocyte cultures (HH-C) were infected with pSIH1 empty lentivectors at a MOI of ~5 TU/cell. Cells were imaged at 100× magnification in both phase-contrast and fluorescence microscopy. (A) Hepatocyte cultures at 2 days post-infection. (B) Hepatocyte cultures at ~2 weeks post-infection.

specific responses. We next evaluated the usefulness of the lentiviral genetic modulation system for over-expression and knockdown analyses in primary human hepatocyte cultures.

For CAR over-expression assessment, hepatocytes were infected with lentivirus expressing CAR. Real-time RT-PCR results showed that CAR mRNA expression levels were increased >200-fold in cells infected with CAR-expressing lentivirus (Fig. 4A). Whether such over-expression was functionally relevant was assessed by measuring mRNA expression levels of the CAR target gene, CYP2B6 (Fig. 4B). Consistent with previous findings, human hepatocytes exhibited inter-individual variability in the level of CYP2B6 induction in response to treatment with the human CAR agonist, CITCO 2- to 6-fold) [25,26]. Hepatocytes infected with CAR-expressing lentivirus exhibited a highly variable increase in basal CYP2B6 expression levels (2-to 26-fold). CYP2B6 induction in hepatocytes infected with CAR-expressing lentivirus was slightly enhanced upon CITCO treatment compared to that of hepatocytes infected with empty lentivirus (empty virus induction: HH-B 3-fold, HH-D 2-fold, HH-G 6-fold, and CAR-expressing virus induction: HH-B 3-fold, HH-D 2.5-fold, and HH-G 9-fold). These results indicate that some portion of the over-expressed CAR pool is engaged in cycling between the cytosolic and nuclear compartments and, due to its constitutive activity, that fraction that equilibrates to the nucleus participates in transactivation of endogenous gene targets similarly as would the resident activated receptor.

For CAR knockdown analysis, primary human hepatocytes were infected with lentivirus expressing four unique siRNAs targeted to CAR at specific locations along the mRNA sequence. Real-time RT-PCR analyses indicated that the siRNA targeted to CAR at position 755 exerted the most effective knockdown (Fig. 5A). CAR mRNA expression was

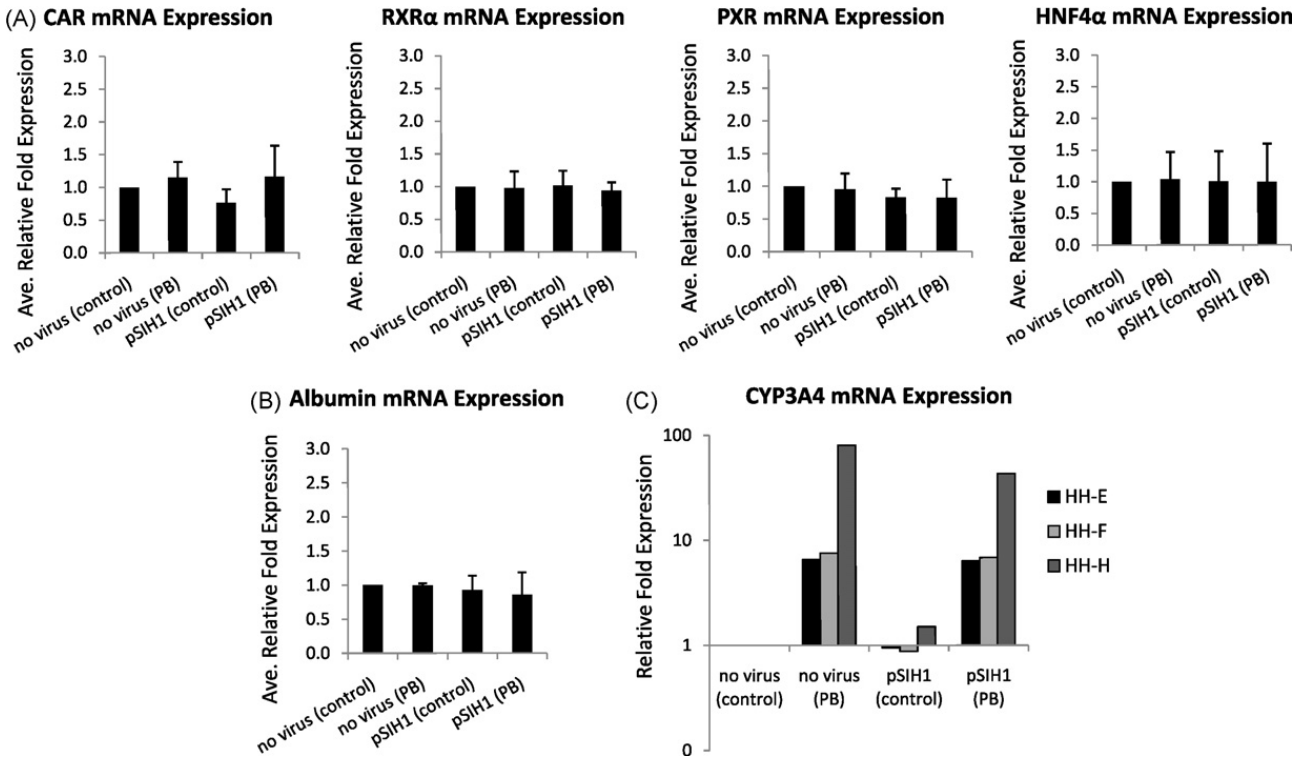

Fig. 3. mRNA expression levels of hepatic hallmarks in cultures of primary human hepatocytes infected with lentiviral vectors. Hepatocyte cultures from three individual donors (HH-E, HH-F, and HH-H) were infected with pSIH1 empty lentivectors at a MOI of ~10 TU/cell. Hepatocytes were treated with 500 μM PB for 24 h. RNA was extracted 3–4 days post-infection, converted to cDNA, subjected to real-time RT-PCR, and the data analyzed using the $\Delta\Delta C_T$ method to determine relative mRNA expression levels. (A) mRNA expression levels of the hepatic nuclear receptors CAR, RXRα, PXR and HNF4α; values are mean ±S.D. of the three hepatocyte donors. (B) Albumin mRNA expression levels; values are mean ±S.D. of the three hepatocyte donors. (C) mRNA expression levels of CYP3A4 hepatic enzyme; values are individual expression levels for each of the three donors.

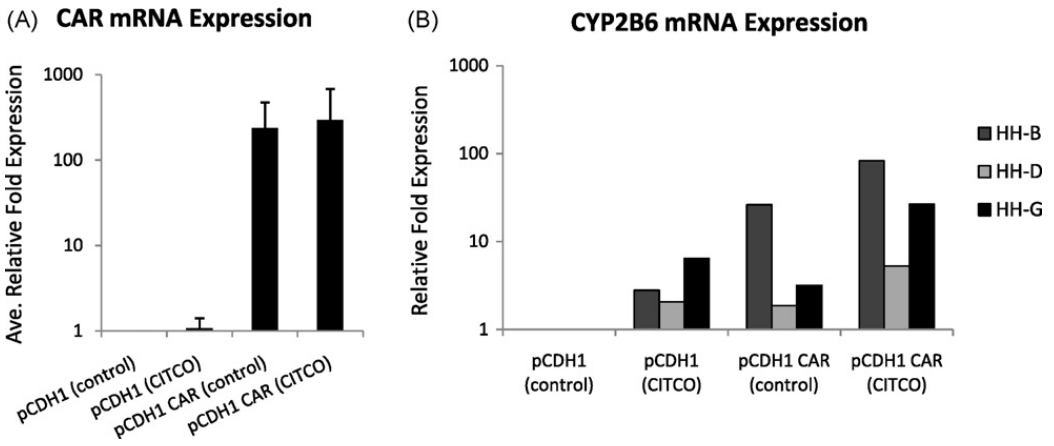

Fig. 4. CAR and CYP2B6 mRNA expression in primary human hepatocyte cultures infected with CAR-expressing lentiviral vectors. Hepatocyte cultures from three individual donors (HH-B, HH-D, and HH-G) were infected with lentivectors expressing CAR or pCDH1 empty lentivectors. Hepatocytes were treated with 100 nM CITCO for 24–48 h. RNA was extracted 2–5 days post-infection, converted to cDNA, subjected to real-time RT-PCR, and the data analyzed using the $\Delta\Delta C_T$ method to determine relative mRNA expression levels. (A) CAR mRNA expression levels; values are mean ±S.D. of the three hepatocyte donors. (B) CYP2B6 mRNA expression levels; values are individual expression levels for each of the three donors.

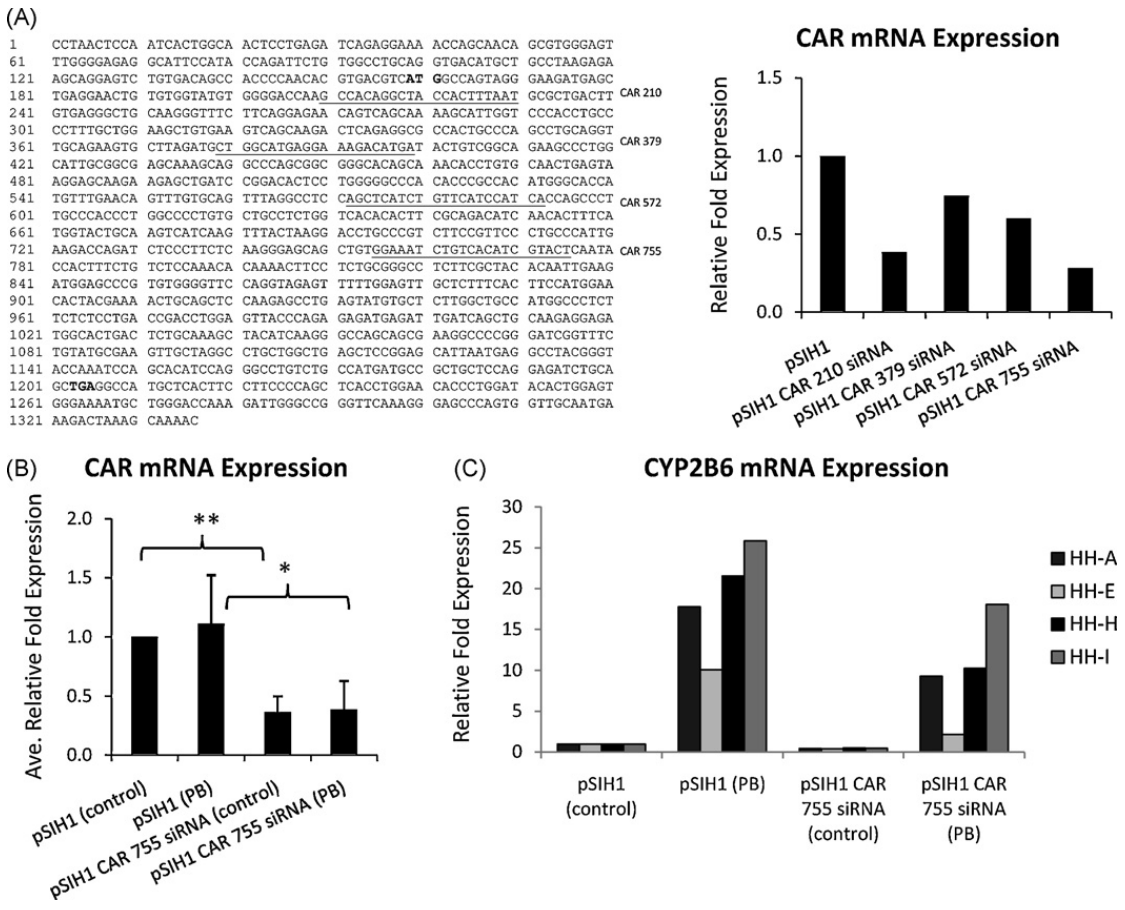

Fig. 5. CAR and CYP2B6 mRNA expression in Hepatocyte cultures infected with lentiviral vectors expressing siRNA against CAR. Hepatocyte cultures from four individual donors (HH-A, HH-E, HH-H, and HH-I) were infected with pSIH1 lentivectors expressing siRNA against CAR or pSIH1 empty lentivectors. RNA was extracted 4–7 days post-infection, converted to cDNA, subjected to real-time RT-PCR, and the data analyzed using the $\Delta\Delta C_T$ method to determine relative mRNA expression levels. (A) Left panel, Schematic showing target locations of the CAR siRNAs assessed for CAR knockdown capability. Underlined sequence indicates siRNA target location; bolded sequence denotes translation initiation and termination codons. Right panel, graph illustrating the effect of the siRNA lentivectors on CAR expression. (B) CAR mRNA expression levels from hepatocytes infected with pSIH1 empty lentivectors or pSIH1 CAR 755 siRNA lentivectors (MOI of ~3 TU/cell) and treated with 500 μM PB for 24–48 h; values are mean ±S.D. of the four hepatocyte donors ($^{*}p < .05$, $^{**}p < .01$ by Student's *t*-test, one-tailed, two sample unequal variance). (C) CYP2B6 mRNA expression levels from hepatocytes infected with pSIH1 empty lentivectors or pSIH1 CAR 755 siRNA lentivectors (MOI of ~3 TU/cell) and treated with 500 μM PB for 24–48 h; values are individual expression levels from each of the four donors.

reduced to ~40% of endogenous cellular levels in hepatocytes infected with lentivirus expressing the siRNA against CAR at position 755 at a MOI of ~3 TU/cell (virus sufficient to infect >60% of the cells) (Fig. 5B). Functional significance of the CAR knockdown was confirmed by assessing mRNA expression levels of CYP2B6 in the presence and absence of phenobarbital. Results demonstrated that CAR knockdown led to a corresponding ~50% decrease in both basal and phenobarbital-induced mRNA expression levels of CYP2B6 (Fig. 5C).

4. Discussion

Previous investigations demonstrated that lentiviral vectors efficiently transduce the hepatic model cell lines Huh7 and HepG2 [13–18], as well as cultures of primary human hepatocytes [14,19–21], with Nguyen and colleagues reporting a MOI of 5 TU/cell as sufficient for transduction of ~75% of the primary hepatocyte culture [20]; all findings with which our results are consistent. Although these investigations demonstrated that lentivectors transduce primary human hepatocyte cultures with high efficiency, they did not evaluate whether the infection event alters hepatic signature hallmarks, such as expression of nuclear receptors or secreted proteins, or metabolic enzyme induction.

To determine whether lentiviral infection may adversely affect the differentiated hepatic character, we measured expression levels of a number of hepatic-enriched nuclear receptors subsequent to lentiviral infection. The nuclear receptors CAR, PXR, RXRα, and HNF4α were selected for evaluation as they regulate expression of a myriad of hepatic genes encoding, generally, drug metabolizing enzymes (such as cytochromes P450, sulfotransferases, UDP-glucuronosyltransferases, and glutathione *S*-transferases), drug transporters (such as multidrug-resistance proteins, multidrug resistance-associated proteins, and organic anion-transporting proteins), as well as other nuclear receptors [27,28]. Our data demonstrate that the levels of these hepatic nuclear receptors remain constant despite lentiviral transduction at a MOI of ~10 TU/cell (virus sufficient to infect >80% of hepatocytes), therefore indicating that hepatic gene regulation by these transcription factors is not impaired by the infection event. This conclusion is further confirmed by the retention of albumin expression levels in lentivector-infected hepatocyte cultures. In contrast, albumin expression was ablated by baculoviral infection of primary hepatocytes [9]. Importantly, phenobarbital-mediated induction of CYP3A4, a sensitive indicator of hepatocyte differentiation status [22], was fully preserved in lentivirally transduced cells.

Results from this study show that lentivectors may be employed for both over-expression and knockdown investigations in primary human hepatocyte cultures (as demonstrated here by CAR modulation), with the functional significance of these manipulations apparent by measure of effects on downstream target gene expression. Since the lentivectors integrate into the genome of the host cell, duration of transgene expression is theoretically limited only by the length of time that hepatocytes can be maintained in culture. Our results demonstrate that lentivirus-infected Huh7 and HepG2 cell lines maintain robust GFP transgene expression for over 3 months in culture, spanning 20 passages (duration of experiment). Likewise, transgene expression is retained for at least 14 days post-infection in primary cultures of human hepatocytes. Further, we identified a novel siRNA sequence capable of knocking down CAR to ~40% of endogenous levels, with specific knockdown of CAR mRNA expression remaining evident at 7 days post-infection. More lengthy time-course studies to confirm preservation of transgene and siRNA expression are underway.

Using a lentivector expressing a siRNA targeted to CAR at position 755, we were able to demonstrate knockdown of CAR to ~40% of endogenous levels in human hepatocytes. The data in Fig. 5 represent the CAR mRNA expression levels from the entire hepatocyte culture, of which ~60% of the cells were infected. It is possible that the ~40% of uninfected hepatocytes may account for the remaining CAR expression, suggesting that this siRNA may approach close to 100% knockdown efficiency in infected cells. Experiments are being conducted in our laboratory to enrich for the infected population by sorting the cells by FACS. Recently, Chen et al. identified a siRNA capable of knocking down CAR to ~30% of endogenous levels [29]. However, the knockdown experiment was performed in Caco-2 cells transiently transfected with both a CAR expression vector and the siRNA against CAR. Thus, to our knowledge, our report is the first to demonstrate direct knockdown of endogenous CAR.

This study demonstrates that lentiviral vectors may be utilized successfully to infect Huh7 cells, HepG2 cells, and, importantly, cultures of primary human hepatocytes. Data presented herein show that lentiviral infection does not alter hepatic markers including expression of albumin and an array of hepatic nuclear receptors. Further, infected hepatocytes retain the capacity for CYP3A4 induction by phenobarbital treatment, a response that is specific to highly differentiated hepatocytes. Both over-expression and knockdown analyses may be successfully undertaken using a lentiviral system in human hepatocytes. Lentiviral vectors thus offer a powerful methodology for achieving stable genetic modulation of primary human hepatocytes. These strategies offer a promising approach for both pharmacological and toxicological investigations within the primary hepatocyte model, as well as ultimately for use in *ex vivo* gene therapy to ameliorate hepatic disease.

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References

[1] Y. Ilan, H. Saito, N.R. Thummala, N.R. Chowdhury, Adenovirus-mediated gene therapy of liver diseases, Semin. Liver Dis. 19 (1999) 49–59.
[2] Q. Li, M.A. Kay, M. Finegold, L.D. Stratford-Perricaudet, S.L. Woo, Assessment of recombinant adenoviral vectors for hepatic gene therapy, Hum. Gene Ther. 4 (1993) 403–409.

[3] N. Ferry, O. Duplessis, D. Houssin, O. Danos, J.M. Heard, Retroviral-mediated gene transfer into hepatocytes in vivo, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 8377–8381.
[4] D.G. Miller, M.A. Adam, A.D. Miller, Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection, Mol. Cell. Biol. 10 (1990) 4239–4242.
[5] M. Yamashita, M. Emerman, Retroviral infection of non-dividing cells: old and new perspectives, Virology 344 (2006) 88–93.
[6] J.A. Wolff, J.K. Yee, H.F. Skelly, J.C. Moores, J.G. Respess, T. Friedmann, H. Leffert, Expression of retrovirally transduced genes in primary cultures of adult rat hepatocytes, Proc. Natl. Acad. Sci. U.S.A. 84 (1987) 3344–3348.
[7] C. Hofmann, V. Sandig, G. Jennings, M. Rudolph, P. Schlag, M. Strauss, Efficient gene transfer into human hepatocytes by baculovirus vectors, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 10099–10103.
[8] V. Sandig, C. Hofmann, S. Steinert, G. Jennings, P. Schlag, M. Strauss, Gene transfer into hepatocytes and human liver tissue by baculovirus vectors, Hum. Gene Ther. 7 (1996) 1937–1945.
[9] N.B. Beck, J.S. Sidhu, C.J. Omiecinski, Baculovirus vectors repress phenobarbital-mediated gene induction and stimulate cytokine expression in primary cultures of rat hepatocytes, Gene Ther. 7 (2000) 1274–1283.
[10] M.I. Bukrinsky, S. Haggerty, M.P. Dempsey, N. Sharova, A. Adzhubel, L. Spitz, P. Lewis, D. Goldfarb, M. Emerman, M. Stevenson, A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells, Nature 365 (1993) 666–669.
[11] P. Lewis, M. Hensel, M. Emerman, Human immunodeficiency virus infection of cells arrested in the cell cycle, Embo J. 11 (1992) 3053–3058.
[12] L. Naldini, U. Blomer, P. Gallay, D. Ory, R. Mulligan, F.H. Gage, I.M. Verma, D. Trono, In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector, Science 272 (1996) 263–267.
[13] Y.Z. Cao, A.E. Friedman-Kien, Y.X. Huang, X.L. Li, M. Mirabile, T. Moudgil, D. Zucker-Franklin, D.D. Ho, CD4-independent, productive human immunodeficiency virus type 1 infection of hepatoma cell lines in vitro, J. Virol. 64 (1990) 2553–2559.
[14] C.M. Kowolik, J.K. Yee, Preferential transduction of human hepatocytes with lentiviral vectors pseudotyped by Sendai virus F protein, Mol. Ther. 5 (2002) 762–769.
[15] W. Liao, G. Ning, Knockdown of apolipoprotein B, an atherogenic apolipoprotein, in HepG2 cells by lentivirus-mediated siRNA, Biochem. Biophys. Res. Commun. 344 (2006) 478–483.
[16] K.L. Nash, B. Jamil, A.J. Maguire, G.J. Alexander, A.M. Lever, Hepatocyte-specific gene expression from integrated lentiviral vectors, J. Gene Med. 6 (2004) 974–983.
[17] J. Seppen, M. Rijnberg, M.P. Cooreman, R.P. Oude Elferink, Lentiviral vectors for efficient transduction of isolated primary quiescent hepatocytes, J. Hepatol. 36 (2002) 459–465.
[18] O. Sirin, F. Park, Regulating gene expression using self-inactivating lentiviral vectors containing the mifepristone-inducible system, Gene 323 (2003) 67–77.
[19] C. Giannini, S. Morosan, J.G. Tralhao, J.E. Guidotti, S. Battaglia, K. Mollier, L. Hannoun, D. Kremsdorf, H. Gilgenkrantz, P. Charneau, A highly efficient, stable, and rapid approach for ex vivo human liver gene therapy via a FLAP lentiviral vector, Hepatology 38 (2003) 114–122.
[20] T.H. Nguyen, J. Oberholzer, J. Birraux, P. Majno, P. Morel, D. Trono, Highly efficient lentiviral vector-mediated transduction of nondividing, fully reimplantable primary hepatocytes, Mol. Ther. 6 (2002) 199–209.
[21] C. Selden, N. Mellor, M. Rees, J. Laurson, M. Kirwan, D. Escors, M. Collins, H. Hodgson, Growth factors improve gene expression after lentiviral transduction in human adult and fetal hepatocytes, J. Gene Med. 9 (2007) 67–76.
[22] J.S. Sidhu, F. Liu, C.J. Omiecinski, Phenobarbital responsiveness as a uniquely sensitive indicator of hepatocyte differentiation status: requirement of dexamethasone and extracellular matrix in establishing the functional integrity of cultured primary rat hepatocytes, Exp. Cell Res. 292 (2004) 252–264.
[23] S.C. Strom, L.A. Pisarov, K. Dorko, M.T. Thompson, J.D. Schuetz, E.G. Schuetz, Use of human hepatocytes to study P450 gene induction, Methods Enzymol. 272 (1996) 388–401.
[24] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408.
[25] A. Moreau, P. Maurel, M.J. Vilarem, J.M. Pascussi, Constitutive androstane receptor-vitamin D receptor crosstalk: consequence on CYP24 gene expression, Biochem. Biophys. Res. Commun. 360 (2007) 76–82.
[26] J.M. Maglich, D.J. Parks, L.B. Moore, J.L. Collins, B. Goodwin, A.N. Billin, C.A. Stoltz, S.A. Kliewer, M.H. Lambert, T.M. Willson, J.T. Moore, Identification of a novel human constitutive androstane receptor (CAR) agonist and its use in the identification of CAR target genes, J. Biol. Chem. 278 (2003) 17277–17283.
[27] J.J. Eloranta, P.J. Meier, G.A. Kullak-Ublick, Coordinate transcriptional regulation of transport and metabolism, Methods Enzymol. 400 (2005) 511–530.
[28] R.G. Tirona, R.B. Kim, Nuclear receptors and drug disposition gene regulation, J. Pharm. Sci. 94 (2005) 1169–1186.
[29] X. Chen, J. Zhang, S.M. Baker, G. Chen, Human constitutive androstane receptor mediated methotrexate induction of human dehydroepiandrosterone sulfotransferase (hSULT2A1), Toxicology 231 (2007) 224–233.