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The human constitutive androstane receptor promotes the differentiation and maturation of hepatic-like cells

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ABSTRACT

Expression of the constitutive androstane receptor (CAR, NR1I3) is enriched in the mature mammalian liver and increasingly recognized for its prominent role in regulating a myriad of processes including biotransformation, chemical transport, energy metabolism and lipid homeostasis. Previously, we demonstrated that CAR levels were markedly enhanced during the differentiation of hepatic-like cells derived from hESCs, prompting the hypothesis that CAR contributes a key functional role in directing human hepatogenesis. Here we demonstrate that over-expression of CAR in human embryonic stem cells (ESCs), transduced by a lentiviral vector, accelerates the maturation of hepatic-like cells, with CAR over-expressing cells exhibiting a 2.5-fold increase in albumin secretion by day 20 in culture differentiation, and significantly enhanced levels of mRNA expression of several liver-selective markers, including hepatic transcription factors, plasma proteins, biotransformation enzymes, and metabolic enzymes. CAR over-expressing cells also exhibited enhanced CITCO-inducible CYP3A7 enzymatic activity. Knockdown of CAR via siRNA attenuated the differentiation-dependent expression programs. In contrast, expression levels of the pregnane X receptor (PXR), a nuclear receptor most similar to CAR in primary sequence, were negligible in human fetal liver tissues or in the differentiating hESCs, and stable over-expression of PXR in hepatic-induced hESCs failed to enhance expression of hepatic phenotype markers. Together, these results define a novel role for human CAR in hepatic lineage commitment.

Introduction

Differentiation of hESCs among hepatic lineage is typically divided into four stages: endoderm induction (days 1–3), hepatic specification (days 4–8), hepatoblast expansion (days 9–13) and hepatic maturation (days 14–20) (Snykers et al., 2009). These processes are controlled by a myriad of specific molecular and genetic signals that are the subject of active investigation. Recent studies have focused on defining the roles of key transcriptional factors and other signaling pathways that drive the complex process of hepatic differentiation. For example, transduction of hepatocyte nuclear factor 4α (HNF4α) and co-transduction of forkhead box A2 (FoxA2) and HNF1α appear to facilitate hepatic differentiation from human embryonic stem cells (ESCs) and from human induced pluripotent stem cells (hiPSCs) (Takayama et al., 2012). Impressively, Huang et al. demonstrated that transduction of GATA4, HNF1α and FoxA3, together with the inactivation of p19^{ARf} induces the development of mouse tail-tip fibroblasts into functional hepatocyte-like cells (Huang et al., 2011). Other researchers have reported on a variety of matrices and culture supplements to promote hepatic differentiation, including the use of media containing fibroblast growth factor (Agarwal et al., 2008; Baharvand et al., 2006; Basma et al., 2009; Cai et al., 2007; Chiao et al., 2008; Lavon et al., 2004; Schwartz et al., 2005; Shiraki et al., 2008; Soto-Gutierrez et al., 2006), bone morphogenetic protein (Cai et al., 2007), hepatocyte growth factor (Agarwal et al., 2008; Baharvand et al., 2006; Basma et al., 2009; Chen et al., 2006; Hay et al., 2008b; Ishii et al., 2008; Moore and Moghe, 2009; Schwartz et al., 2005; Shiraki et al., 2008; Soto-Gutierrez et al., 2006), dexamethasone (Agarwal et al., 2008; Baharvand et al., 2006; Basma et al., 2009; Moore and Moghe, 2009; Shirahashi et al., 2004; Shiraki et al., 2008; Soto-Gutierrez et al., 2006), insulin (Baharvand et al., 2006; Shirahashi et al., 2004), oncostatin M (Agarwal et al., 2008; Baharvand et al., 2006; Hay et al., 2008b; Moore and Moghe, 2009; Shiraki et al., 2008), activin A (Agarwal et al., 2008; Basma et al., 2009; Cai et al., 2007; Chen et al., 2006; D'Amour et al., 2005; Hay et al., 2008a, 2008b; Ishii et al., 2008; Moore and Moghe, 2009; Shiraki et al., 2008), wnt3a (Hay et al., 2008a; Moore and Moghe, 2009), and sodium butyrate (Hay et al., 2008b; Rambhatla et al., 2003). The review by Snykers et al. offers

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a comprehensive synopsis of the progress in driving hepatocyte differentiation from hESCs (Snykers et al., 2009). While promising, the differentiation methodologies defined remain insufficient to derive fully functional hepatocytes from hESCs.

Previously, we defined a hepatic differentiation approach involving the culture of hESCs for 20 days on a collagen matrix in the presence of a defined hepatocyte culture medium (Zamule et al., 2011). The resulting hepatic-like cell population exhibited decreased expression of 'stemness' markers as well as enhanced expression of a variety of hepatic transcription factors, nuclear receptors, liver-generated plasma proteins, protease inhibitors, metabolic enzymes, and biotransformation enzymes. Further, these hESC-derived hepatic-like cells developed the capacity to transport anionic compounds and store glycogen. Notably, expression of the constitutive androstane receptor was markedly increased in the hepatic-like cells.

CAR is a member of the nuclear receptor (NR) superfamily and is expression is highly enriched in the liver (Swales and Negishi, 2004). Subsequent to their activation, both CAR and the pregnane X receptor (PXR) function as transcriptional regulators of genes participating in hepatic biotransformation and drug transport (Chang and Waxman, 2006), affecting the dispositional fate of many drugs (Wei et al., 2000), chemical carcinogens (Xie et al., 2003) as well as endogenous substances such as steroids (Xie et al., 2003), heme and bilirubin (Xie et al., 2003), thyroid hormone (Maglich et al., 2004), and cholesterol/bile acids (Guo et al., 2003; Stedman et al., 2005). CAR's function has also emerged as an important regulator of lipid and energy metabolism (Wada et al., 2009) and as a modulator of genes involved in a diverse array of physiological processes that include cell growth and differentiation (Page et al., 2007; Liu et al., 2009; Baskin-Bey et al., 2006; Blanco-Bose et al., 2008).

Evolving evidence supports the concept that the NRs function as critical mediators in both the maintenance of 'stemness' as well as in stem cell differentiation (Jeong and Mangelsdorf, 2009). However, limited data exist regarding CAR's expression and its potential role in the hepatic specification process. A recent report comparing expression levels of members of the NR superfamily during embryoid body differentiation indicated that hESCs express increasing levels of CAR during the first six days of development (Xie et al., 2009). These findings were corroborated in a 30-day differentiation protocol reporting increased expression levels of CAR in hepatic-like cells derived from hESCs (Ek et al., 2007). In the developing liver, CAR mRNA expression correlates with that of its transcriptional regulator, HNF4α (Pascussi et al., 2007), a NR that plays an integral role in hepatic differentiation (Li et al., 2000). Interestingly, despite inter-individual and developmental stage variability, in a panel of human fetal liver samples CAR mRNA expression levels in fetal liver averaged approximately 40% that of postnatal liver levels and were approximately 4-fold greater than that of HNF4α (Vyhlidal et al., 2006).

Given these observations, and considering our previous results, we hypothesized that CAR contributes an important biological role directing the differentiation of hESCs along the hepatic lineage. The data presented here confirm that CAR's expression is markedly enhanced during the process of human hepatic differentiation. More importantly, in differentiating hESCs, CAR over-expression and conversely, its reduction, contributed major modulatory effects on a battery of biochemical, molecular and functional hepatic markers impacting a variety of developmental programs. In contrast to the contributions of CAR in human hepatic specification, PXR was expressed only marginally in hESCs or in human fetal liver and its forced over-expression did not significantly impact the level of any hepatic-specification marker tested. The results of the current investigation support a critical role for CAR as a key regulator in the complex scheme of human hepatic differentiation.

Materials and methods

Chemicals

Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (St. Louis, MO). Rifampicin (RIF) was purchased from VWR Biosciences. 6-(4-chlorophenyl): imidazo[2,1-*b*]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA).

Human fetal tissues and primary hepatocyte cultures

Human fetal liver tissues were obtained from Dr. Thomas Shepard from the Central Laboratory of Human Embryology at the University of Washington, Seattle, WA. Cultures of primary human hepatocytes were obtained from Dr. Stephen Strom from the University of Pittsburgh through the Liver Tissue Procurement and Distribution System (NIH Contract # N01-DK-7-0004/HHSN267200700004C). Primary human hepatocytes were isolated by a three-step collagenase perfusion technique and plated on rat-tail collagen as described previously (Strom et al., 1996). Hepatocytes were maintained in William's E Media (Gibco; Grand Island, NY) supplemented with 10 mM HEPES (Gibco), 2 mM GlutaMAX (Gibco), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco), 25 nM dexamethasone (Sigma; St. Louis, MO), 10 nM insulin (Sigma), 5 ng/ml selenium (Sigma), 5 μg/ml transferrin (Sigma), and 1% linoleic acid/albumin (Sigma) (Olsavsky et al., 2007).

Human embryonic stem cell culture

The WA09 (H9) and WA01 (H1) human embryonic stem cell lines were acquired from the National Stem Cell Bank through WiCell Research Institute (Madison, WI). The cells were maintained on irradiated human foreskin fibroblast (hFF) feeder layer cells (ATCC; Manassas, VA) in hESC media consisting of Dulbecco's Modified Eagle Media F-12 (Gibco) supplemented with 20% knock-out serum replacement (Gibco), 1 mM GlutaMAX (Gibco), 0.1 mM non-essential amino acids (Gibco), 25 ng/ml basic fibroblast growth factor (National Cancer Institute; Bethesda, MD), and 0.1 mM β-mercaptoethanol (Sigma). Media was changed daily and differentiated colonies were removed from the culture by manual dissociation 2–3 times per week, depending on the culture density. Cells were passaged weekly by manual dissociation and plated on fresh hFF feeder layers. The data presented were generated from the H9 cell line; although analogous results as tested were also obtained with the H1 cells (data not shown).

Hepatic differentiation of human embryonic stem cells and treatments

Human embryonic stem cells (hESCs) were first differentiated into hepatic-like cells using hepatocyte media. Hepatocyte-like morphology and hepatocyte-specific functions including inodcyanine green uptake and glycogen storage capacity were presented in a previous report (Zamule et al., 2011). The data shown in Figs. 5 and 6 C–E were generated based on this protocol. To further optimize the stem cell differentiation strategy, various growth factors were sequentially added, according to the methods of Cai et al. (2007). Briefly, culture plates were coated with a ice cold 2.4% (v/v) of rat tail type I collagen (Sigma) in MCDI solution (*N*-Cyclohexyl-*N*′-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate, Sigma), incubated at 37 °C for 4 h and washed with PBS. Initially, hESCs were plated on collagen-coated plates in hESC media. After 1 day, knock-out serum replacement was reduced to 2% and supplemented with 100 ng/ml Activin A (Peprotech, NJ).

After 3 days of Activin A treatment, the differentiated cells were cultured in hepatocyte media (see above) containing 100 ng/ml basic fibroblast growth factor and 20 ng/ml bone morphogenic protein2 (BMP2, Peprotech, NJ) for 5 days. The differentiated cells were then incubated with hepatocyte media supplemented with 20 ng/ml hepatocyte growth factor (HGF, Peprotech, NJ) for 5 days. Finally, hepatocyte maturation was further stimulated by culture in hepatocyte media with 10 ng/ml oncostatin M (OSM, Peprotech, NJ).

Alkaline phosphatase staining

An alkaline phosphatase detection kit (Millipore) was used for alkaline phosphatase staining, conducted essentially according to the manufacturer's instructions. Briefly, cells were fixed with 4% formaldehyde in PBS, rinsed with TBST (20 mM Tris–HCl, 0.15 M NaCl, 0.05% Tween-20), stained for 15 min with a 2:1:1 ratio of Fast Red Violet:Naphthol:water, rinsed with TBST, and covered with PBS. Cells were visualized using a Nikon TE-2000 inverted fluorescent microscope (Nikon USA; Melville, NY) and images were captured using a digital camera and SpotRT software (Diagnostic Instruments; Sterling Heights, MI).

Lentiviral cDNA and siRNA expression vector construction

Lentivectors were obtained from System Biosciences (Mountainview, CA). The pCDH1-MCS1-EF1-copGFP cDNA lentivector expressing human CAR (NM_005122) and the pSIH1-H1-copGFP shRNA lentivector expressing a small interfering RNA targeted to CAR were generated as described previously (Zamule et al., 2008). The pCDH1-MCS1-EF1-copGFP cDNA lentivector expressing human PXR (NM_003889) was engineered as follows. Briefly, PXR was PCR-amplified from human liver cDNA using the primer sequences: Forward, 5′-GATCGAATTCGACATGGAGGTGAGACCCAAAGAAAG-3′; Reverse, 5′- GATCGATATC TAGAAGGCACAGTCGAGG-3′ (restriction sites underlined). The amplicon and vector were then digested with EcoRI/EcoRV and EcoRI/SwaI, respectively, electrophoresed through a 0.6% agarose gel, purified using QIAquick Gel Extraction Kit (Qiagen; Valencia, CA), and cloned by ligation. Plasmids were purified using QIAfilter Plasmid Maxi Kit (Qiagen) and sequence verified.

Lentiviral production and target cell infection

Production of lentiviral particles and target cell transductions were performed according to the manufacturer's instructions (System Biosciences) with minor adaptations. Briefly, human embryonic kidney (HEK) 293T/17 transformed virus packaging cells (ATCC), cultured as previously described (Zamule et al., 2008), were transfected with lentiviral expression plasmids and pPACKH1 packaging plasmid mix (System Biosciences) using Lipofectamine 2000 (Invitrogen; Carlsbad, CA). Pseudoviral supernatants were collected from packaging cell cultures at 72 h post-transfection, filtered, and used for direct target cell infections in the presence of 6 μg/ml polybrene. Target cell media was replaced the following day. Transduction efficiency was assessed using green fluorescent protein as a marker for gene expression and monitored with a Nikon inverted fluorescence microscope. Images were captured using a digital camera and SpotRT software.

RNA isolation from cultured cells and human fetal liver tissues, cDNA archiving, and Taqman or SYBR green real-time PCR

RNA was isolated from cultured cells and human fetal liver tissues, using TRIzol Reagent (Invitrogen) and converted to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems; Foster City, CA), both according to manufacturers' protocols. Real-time RT-PCR was carried out using Taqman®Gene Expression Assays (Applied Biosystems), according to the manufacturer's instructions. Briefly, 50 ng of cDNA template, 15 μl 2 × Taqman Universal Master Mix, and 1.5 μl 20 × Target Assay Mix were combined into 30 μl reactions. Otherwise, real-time RT-PCR was performed with PerfeCTa SYBR Green SuperMix, UNG, ROX (Quanta BioSciences, Gaithersburg, MD). Fifty ng of cDNA template, 15 μl 2 × SYBR green Master Mix, 0.1 μM final concentrations of forward and reverse primers were added into 30 μl reactions. The reactions were divided in half to generate technical replicates and run on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Data were analyzed using the $\Delta\Delta C_T$ method as previously described (Livak and Schmittgen, 2001; Zamule et al., 2008). Standard curves were generated by amplifying a serial dilution of plasmid DNA containing CAR or PXR. A strong linear relationship between C_T values (y) and log of cDNA copy numbers (x) was observed between 30 and 3×10^6 copies ($y = -3.455 \times \log10(x) + 37.169$, $r^2 = 0.999$, 94.73% efficiency, for CAR; $y = -3.429 \times \log10(x) + 35.762$, $r^2 = 0.999$, 95.72% efficiency, for PXR). All experiments were performed in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiment (MIQE) guidelines (Bustin et al., 2009). SYBR Green Primers and Taqman® probes are summarized in Supplemental Table 1.

Albumin secretion ELISA assay

Conditioned media from the differentiated hESCs was collected at day 20 and stored at −80 °C until assayed. The concentration of human albumin secreted into the cell culture medium was determined using a human albumin ELISA quantitation kit (Bethyl Laboratory, Montgomery, TX, USA), according to the manufacturer's instructions. Briefly, the plate was prepared by incubating with the human albumin coating antibody for 1 h, washed 5 times, incubated with blocking solution containing 1% BSA for 30 min, and then washed 5 times. Then, 100 μl of each standard, control, or samples were loaded to each well and incubated for 1 h, followed by 5 washes. The plate was incubated with HRP-conjugated human albumin detection antibody for 1 h, washed 5 times, and immersed in tetramethylbenzidine (TMB) substrate solution for 15 min in the dark. Color development was stopped by addition of 0.18 M H_2SO_4. The plate was read at 450 nm using a Packard Spectra Count (Meriden, CT) reader. The concentration of human albumin was normalized to the number of total cells determined from each well.

CYP activity assays

CYP3A4/7 and CYP2C9 activity were measured using the P450-Glo™ CYP assay kit (Promega, WI). Intracellular CYP enzymes convert the luminogenic substrate to the luciferin product, which is detected in a subsequent reaction with the Luciferin Detection Reagent. The amount of luminescence produced is directly proportional to CYP activity. Briefly, hepatic-like cells were incubated with the fresh culture medium containing CYP3A4/7 or CYP2C9 pGlo substrates. After incubation for 3–4 h at 37 °C, 50 μl of the medium from each well was transferred to a 96-well opaque white luminometer plate and 50 μl of luciferin detection reagent was added to initiate the luminescent reaction. The plate was incubated at room temperature for 20 min and luminescence was read using a Tecan Infinite m200 Pro luminometer (Switzerland). Net signals were calculated by subtracting background luminescence values from DMSO and NR activators-treated values.

Statistical analyses

Data were generated from at least two independent trials, and presented as mean ± SEM. A Student's *t*-test (one-tailed; two-sample, unequal variance) was used for two-group comparisons.

One-way ANOVA with Tukey's analysis was used to compare the means of three or more groups. A two-way ANOVA with Bonferroni analysis was used to determine how a response was influenced by two factors. Statistical significance was set as $p < 0.05$.

Results

Hepatic differentiation of hESCs results in increased CAR expression

Previously we demonstrated that culturing hESCs on a collagen substrate within a highly-defined culture media enabled the differentiation of hepatic-like cells that exhibited enhanced expression of selective markers including transcription factors, nuclear receptors, plasma proteins, metabolic and biotransformation enzymes, as well as augmented hepatic functional indices such as transport of anionic compounds and glycogen storage, coincident with attenuated expression of pluripotency markers and 'stemness' function (Zamule et al., 2011). In the current report, the differentiation culture conditions were further optimized (see Materials and methods) such that enhanced levels of CAR mRNA were now detected in the developing hepatoblasts after 3 days of culture, and these levels were further enhanced throughout the extended study period, especially at the stages equivalent to hepatic specification (day 8) and hepatoblast expansion (day 13) (Fig. 1A).

CAR expression in human fetal liver tissue

To determine the extent to which CAR is expressed in human fetal liver, we quantified CAR mRNA levels in tissues acquired from nine subjects encompassing a range of gestational stages. CAR hepatic mRNA levels were relatively highly expressed late in the first trimester and early in the second trimester, although at levels less than those measured in adult liver (Fig. 1B). These findings are consistent with those previously reported by Vyhildal et al. (Vyhlidal et al., 2006). Microarray expression data from a panel of human tissue generated using Affy U95 (publicly available through the UCSC Genome Browser website http://genome.ucsc.edu/ (Karolchik et al., 2008; Kent et al., 2002)), further confirms that CAR mRNA is relatively robustly and selectively expressed in fetal liver.

Lentivirus efficiently transduces hESCs and does not alter select markers of 'stemness'

To enable experimental analyses of CAR function in the stem cell differentiation model, a stable genetic modulation system utilizing lentivirus was deployed. Initially with this approach, hESC colonies were transduced with lentivirus containing an expression construct for green fluorescent protein (GFP). Although inter- and intra-colony variability in transduction efficiency was noted, infected hESC colonies generally exhibited high levels of GFP expression (Supplementary Fig. 1A, upper panels). The most homogenously GFP-positive colonies were selected for subsequent propagation. GFP expression was retained in infected hESCs for over two months in culture (throughout six passages) in the absence of selective pressures (Supplementary Fig. 1A, lower panels). Although available data suggest that lentiviral infection does not affect hESC pluripotency (Gropp et al., 2003; Xiong et al., 2005), we examined this issue by quantifying the relative mRNA expression levels of selected markers implicated in self-renewal and pluripotency in lentivirus-infected hESCs. Infected hESC colonies exhibited no morphological abnormalities or signs of toxicity. Further, when compared to uninfected control cells, lentiviral infection posed no detectable alteration of mRNA expression levels for the 'stemness' markers, octamer-binding transcription factor 4 (OCT4), nanog homeobox (NANOG), SRY-box containing gene 2 (SOX2), alkaline phosphatase, or the tyrosine-protein kinase C-KIT (Supplementary Fig. 1B). Confirmation that lentivirus infection did not alter alkaline phosphatase expression was attained at the functional level using an alkaline phosphatase activity staining technique (Supplementary Fig. 1C).

CAR expression enhances hepatic differentiation of hESCs

To determine the impact of CAR expression on hepatic differentiation capacity, the lentivirus system was used to stably express exogenous CAR in hESCs which were then subjected to the hepatic differentiation protocol. These cells exhibited significantly increased expression of CAR mRNA compared with cells infected with empty virus (Fig. 2A), without any accompanying noted difference in morphology or viability changes that could be observed. Stage-specific differentiation markers were examined to allow assessment of the relative extent of hepatic cellular differentiation and maturation. Transduced CAR-overexpressing cells exhibited enhanced increased mRNA expression of the hepatic transcription factors, CCAAT/enhancer binding protein alpha (C/EBPα), HNF1α, and HNF4α (Fig. 2), representing primary members of the combinatorial regulatory network that governs transcription of most genes during and after hepatic specification (Kyrmizi et al., 2006). mRNA levels of α-fetoprotein (AFP), a hepatic endoderm cells marker, were significantly enhanced in CAR infected cells on day 8, and progressively elevated until day 20 of culture differentiation. mRNA expression of albumin, a hepatic progenitor cell marker, and AAT were initially detected at day 13 in CAR over-expressing cells, reaching higher levels by

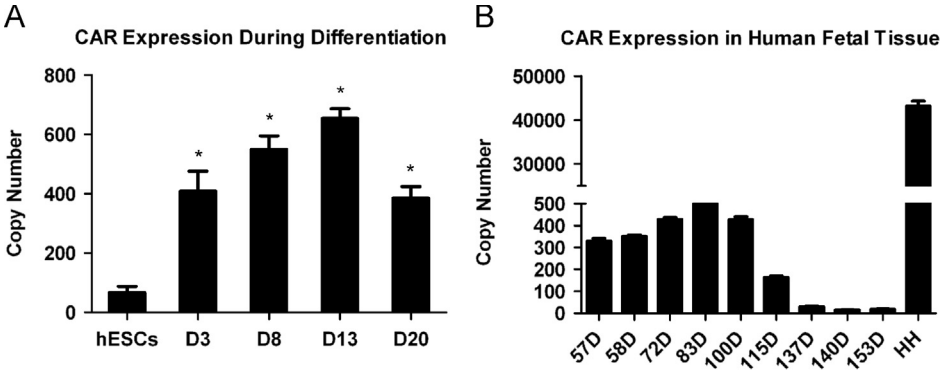

Fig. 1. CAR mRNA is expressed during hepatic differentiation. Real-time PCR was performed using cDNA prepared from (A) HESCs or differentiated stem cells at indicated days, and (B) human fetal liver tissue or pooled samples from eight primary human hepatocyte donors (HH). Copy number was calculated based on the standard curve generated by amplifying a dilution series of a standard plasmid DNA containing CAR. *$p < 0.05$.

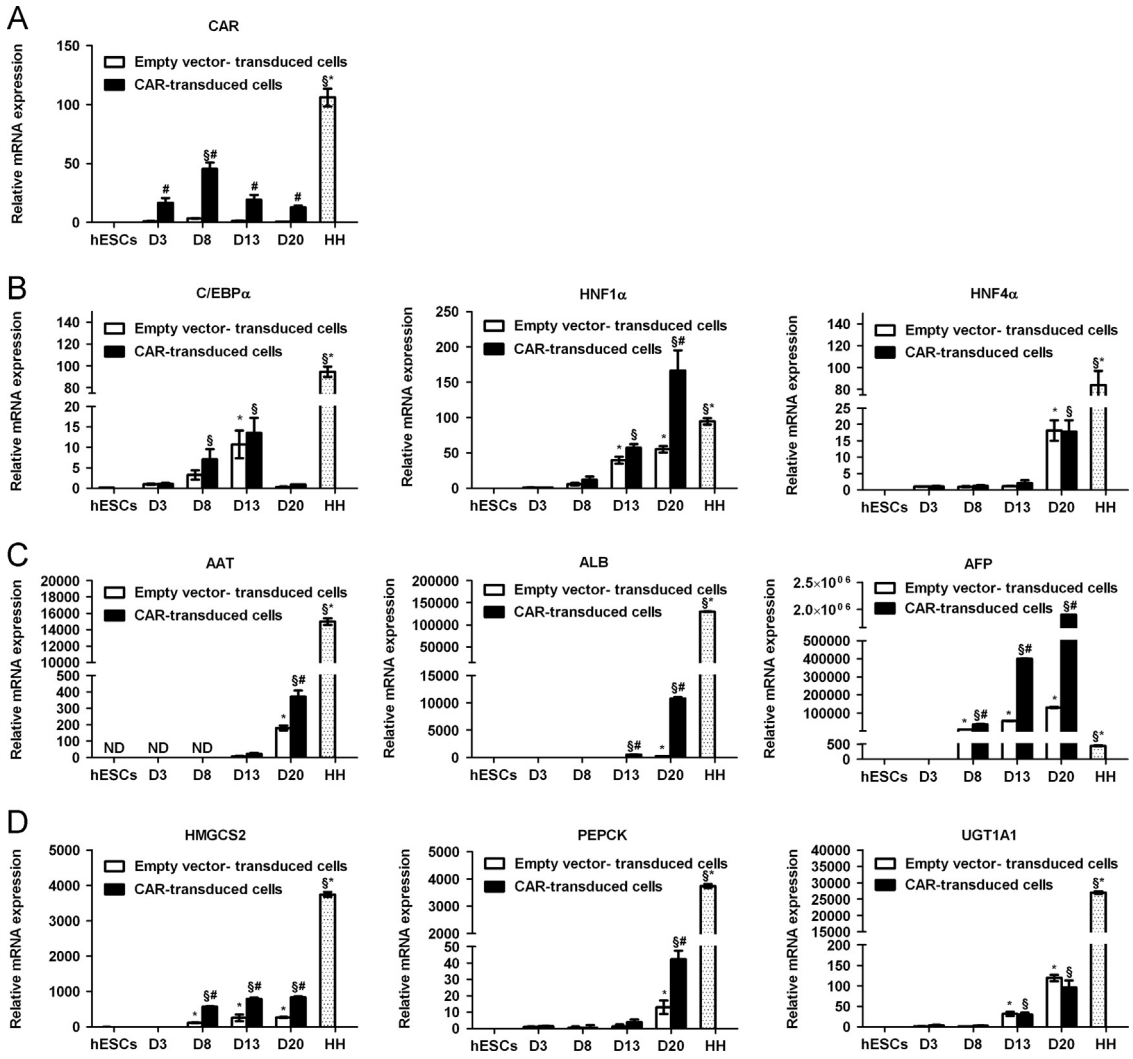

Fig. 2. CAR expression enhances hepatic differentiation of hESCs. hESCs were transduced with either empty lentiviral vectors or lentiviral vectors expressing CAR. Quantitative real-time PCR was performed to determine mRNA levels relative to empty vector-transduced cells at day 3 for the following target genes: (A) CAR, (B) the hepatic transcription factors, C/EPBα, HNF1α, and HNF4α and, (C) the plasma proteins, alpha-1 antitrypsin, albumin, and α-fetoprotein, and (D) the metabolic enzymes, HMGCS2, PEPCK, and UGT1A1. Data are from at least two independent trials using hESCs from different passages. * $p < 0.05$, compared with empty vector-transduced cells at day 3; $^{\$}p < 0.05$, compared with CAR-transduced cells at day 3; $^{\#}p < 0.05$, compared with empty vector-transduced cells at the same indicated day; ND, not detected.

day 20 of culture (Fig. 2C). As well, the liver-selective metabolic enzymes, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (HMGCS2) and phosphoenolpyruvate carboxykinase (PEPCK) were similarly enhanced by CAR expression, representing endpoints primarily expressed during hepatoblast expansion and hepatic maturation (Fig. 2D).

Effect of CAR expression on basal and inducible CYP450 mRNA expression

Cytochrome P450s (CYP, phase I monooxygenase enzymes), in particular CYP2B and CYP3A family members, are known as gene targets of CAR and PXR. Further, the extent of CYP expression and activity reflect in a functional respect the degree of liver maturation. In cultured hESCs induced to differentiate along a hepatic lineage, lentiviral transduction with exogenous CAR led to significantly increased basal expression of CYP450 mRNA expression, compared to empty vector transduced cells. On day 20 of the differentiation protocol, CYP2B6 mRNA levels was increased 15-fold in CAR transduced cells compared with approximately 6-fold for the empty vector cells (Fig. 3A). Further, after 20 days of differentiation CYP3A4 and CYP3A7 expression were 39- and 30-fold elevated, respectively, in the CAR transduced hepatic-like cells compared with non-transduced cells (Fig. 3B and C). To study the effect of NR activator treatment on CYP mRNA expression, differentiated hESCs with or without CAR transduction, were exposed at day 20 in culture to NR activators for 24 h. CYP2B6 levels were significantly increased following treatment with the human CAR activator CITCO; however, CYP2B6 inducibility was only modestly altered in CAR-transduced cells, relative to already enhanced levels detected in the empty vector transduced cells (Fig. 3D). Notably, CITCO significantly induced 3A4 expression in the CAR transduced cells. Relative to the treated empty vector-transduced cells, CITCO exposures increased CYP3A4 expression approximately 13.6-fold (Fig. 3E). Treatment with RIF, a human PXR activator, appeared to reverse the effect of CAR transduction on CYP3A4 expression. CYP2C9 expression was not affected by CITCO treatment, while RIF increased CYP2C9 expression approximately 4-fold, regardless of whether the cells were CAR-transduced or uninfected (Fig. 3F).

CAR expression enhances hepatic specific function of hepatic-like cells

To further characterize the impact of CAR expression on hepatic differentiation, hepatic-specific functional endpoints including

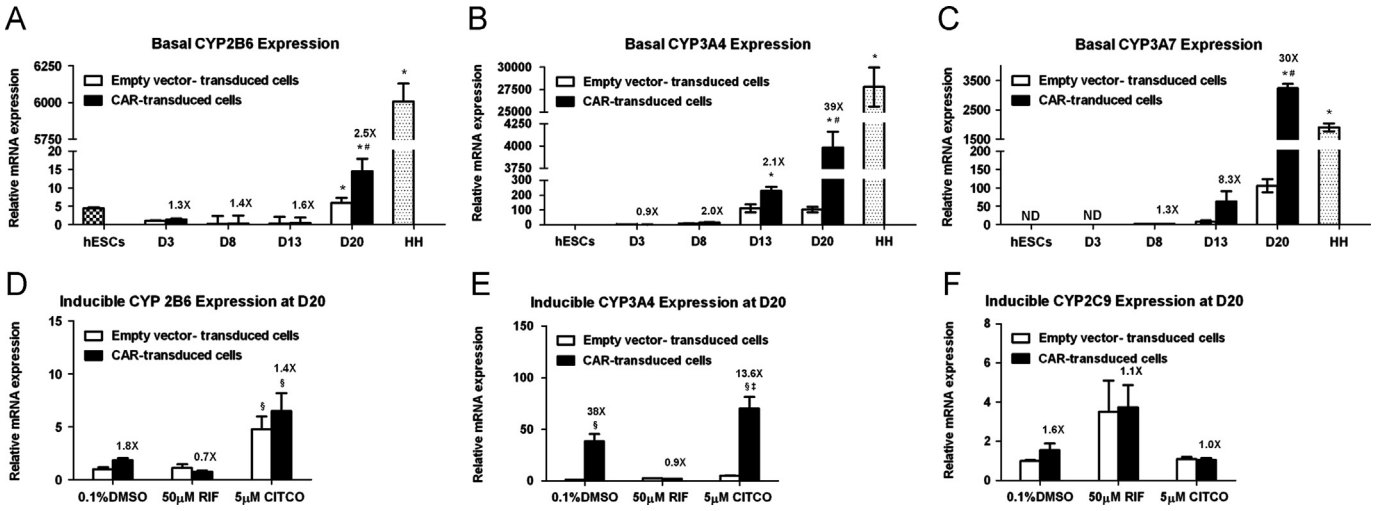

Fig. 3. Effect of CAR expression on basal and inducible CYP450 mRNA expression. (A–C) hESCs were transduced with either empty lentiviral vectors or CAR lentiviral vectors. Basal CYP450 mRNA expression at indicated days was detected using real-time PCR during hepatic differentiation. mRNA levels relative to empty lentiviral vector-transduced cells at day 3 was calculated for the following target genes: (A) CYP2B6, (B) CYP3A4 and, (C) CYP3A7. hESCs serve as a negative control and pooled samples from eight primary human hepatocyte donors (HH) serve as a positive control. (D–F) empty vector (Hepatic-like) or CAR-transduced cells (Hepatic-like+CAR) at day 20 were treated with 0.1% DMSO (solvent control), 50 μM rifampicin or 5 μM CITCO for 24 h. Real-time PCR was used to determine fold change levels relative to empty vector-transduced cells with DMSO treatment for the following target genes: (D) CYP2B6, (E) CYP3A4, and (F) CYP2C9, respectively. Numerical values denote target gene expression levels of CAR-transduced cells relative to empty vector-transduced cells. $^{*}p < 0.05$, compared with empty vector-transduced cells at day 3; $^{\#}p < 0.05$, compared with empty vector-transduced cells at the same indicated day.

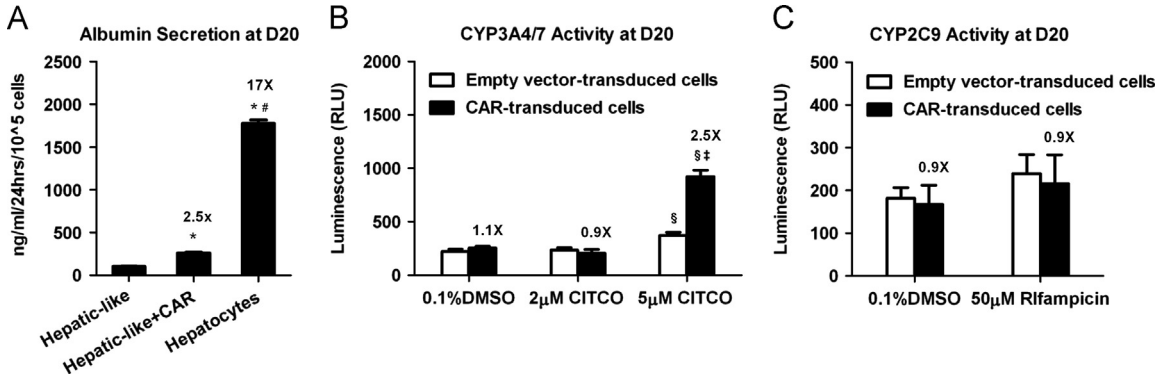

Fig. 4. CAR expression enhances albumin secretion and cytochrome P450 activity of differentiated hepatic-like cells. (A) The concentration of human albumin secreted at day 20 was assessed by ELISA. (B and C) Transduced cells at day 20 were treated with 0.1% DMSO (solvent control), the indicated concentrations of CITCO or 50 μM rifampicin for 24 h then CYP3A4/7 or CYP2C9 activity is measured and expressed as relative light units (RLU). Numerical values denote target gene activity of CAR-transduced cells relative to empty vector-transduced cells. * $p < 0.05$, compared with empty vector-transduced cells at day 3; $^{\#}p < 0.05$, compared with empty vector-transduced cells at the same indicated day.

albumin secretion and CYP activity assays were performed. CAR transduced cells exhibited a 2.5-fold increase in albumin secretion at day 20 when compared to uninfected cells (Fig. 4A). Inducible activity of CYP3A7 by 5 μM CITCO in CAR over-expressing cells was also significantly higher than that of non-transduced cells (Fig. 4B). Treatment with 50 μM rifampicin slightly increased the CYP2C9 inducible activity, while CAR overexpression did not further enhance CYP2C9 inducibility (Fig. 4C).

siRNA-mediated CAR reduction attenuates hepatic differentiation of hESCs

To further confirm the role of CAR in hepatic differentiation, we determined whether a reduction in CAR expression might attenuate the differentiation capacity of hESCs induced to differentiate along a hepatic lineage. Prior to hepatic induction using our hepatic differentiation protocol, hESCs were transduced with lentiviral particles expressing a siRNA targeted to CAR (Zamule et al., 2008). CAR mRNA was substantially reduced in hESCs infected with siRNA-expressing virus, compared to the cell populations infected with empty virus (Fig. 5A). The siRNA-mediated reduction of CAR resulted in corresponding reductions of the differentiation-induced enhancements in expression levels of the hepatic transcription factors, C/EBPα, HNF1α, HNF4α, and forkhead box A1 (FOXA1) (Fig. 5B), as well as for the plasma proteins, α-fetoprotein and transthyretin (Fig. 5C).

PXR expression does not enhance hepatic differentiation of hESCs

CAR and PXR share overlapping interactions with certain chemical activators as well as gene targets, and are related in primary sequence, sharing 40% sequence homology in the DBD and 45% in the LBD (Timsit and Negishi, 2007). Thus, we examined the ability of PXR to enhance the differentiation capacity of hESCs induced to differentiate along a hepatic lineage. In contrast to CAR (Fig. 1A), PXR mRNA expression was not increased in hESC-derived hepatic-like cells (Fig. 6A). Further, PXR mRNA expression in human fetal liver tissue samples was negligible (Fig. 6B), findings confirmed by microarray expression data from a panel of human tissue generated using U133A and GNF1H chips (publicly available

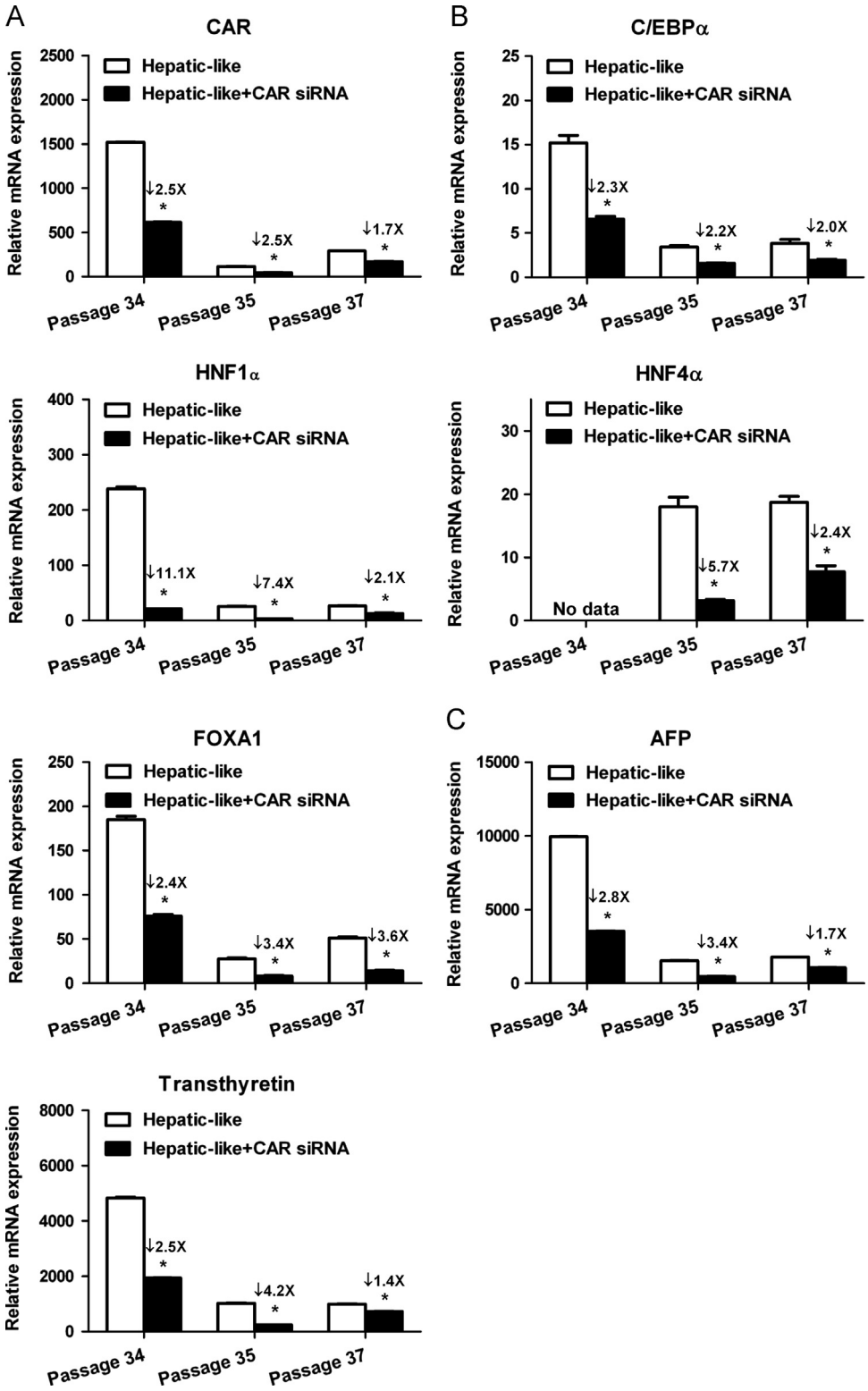

Fig. 5. siRNA-mediated reduction in CAR attenuates hepatic differentiation of hESCs. Prior to induction of hepatic differentiation by culturing for 10 days on collagen-coated plates, hESCs were transduced with either empty lentiviral vectors (Hepatic-like) or lentiviral vectors expressing a siRNA targeted to CAR (Hepatic-like + CAR siRNA). Real-time PCR was performed to determine expression levels relative to low passage number hESCs cultured on hFF feeder layers in hESC media of the following target genes: (A) CAR, (B) C/EBPα, HNF1α, HNF4α, and FOXA1 and (C) plasma proteins α-fetoprotein and transthyretin. Numerical values denote target gene expression levels of CAR siRNA cells relative to empty vector-transduced cells. * $p < 0.05$. Data are from at least two independent trials using hESCs from different passages.

through the UCSC Genome Browser website http://genome.ucsc.edu/ (Karolchik et al., 2008; Kent et al., 2002)).

More directly, we determined whether PXR, like CAR, can function to enhance hepatic differentiation capacity. To this end, hESCs were transduced with lentiviral vectors expressing PXR and subsequently stimulated to differentiate along a hepatic lineage. Indeed, PXR mRNA expression was substantively enhanced in hESCs infected with PXR-expressing virus, compared to cells

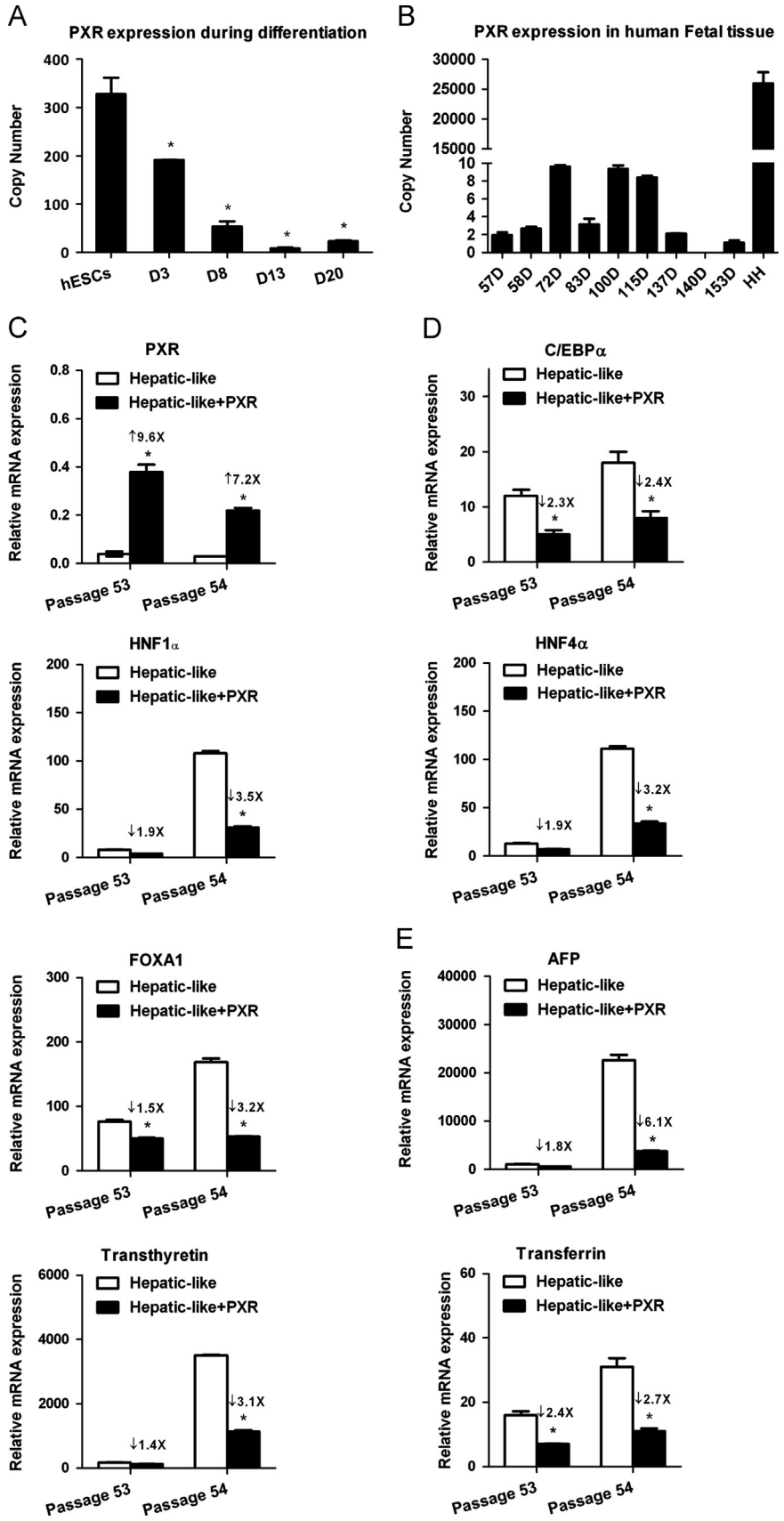

Fig. 6. PXR mRNA is expressed at very low levels during hepatic differentiation and PXR does not enhance hepatic differentiation of hESCs. Real-time PCR was performed using cDNA prepared from (A) HESCs or differentiated stem cells at indicated days, and (B) Human fetal liver tissue or pooled samples from eight primary human hepatocyte donors (HH). Copy number was calculated based on the standard curve generated by amplifying a dilution series of a standard plasmid DNA containing PXR. (C–E) Real-time PCR was carried out to determine expression levels relative to low passage number hESCs cultured on hFF feeder layers in hESC media of the following target genes: (C) PXR, (D) C/EBPα, HNF1α, HNF4α, FOXA1 and (E) α-fetoprotein, transthyretin, and transferrin. Numerical values denote target gene expression levels of PXR-transduced cells relative to empty vector-transduced cells. * $p < 0.05$. Data are from two independent trials using hESCs from different passages.

infected with empty virus (Fig. 6C). However, in contrast to CAR, hESCs infected with PXR-expressing virus and subject to hepatic differentiation actually exhibited decreased expression levels of the hepatic transcription factors C/EBPα, HNF1α, HNF4α, and FOXA1 (Fig. 6D) as well as decreased levels of the liver-generated plasma proteins α-fetoprotein, transthyretin, and transferrin (Fig. 6E). Therefore the differentiation endpoints impacted by CAR appeared highly specific to this receptor.

Discussion

These investigations establish an important functional role for CAR in human hepatic differentiation. In contrast to the relative lack of detection and functional role of the related nuclear receptor, PXR, CAR exhibited maximal expression during the hepatic specification and hepatoblast expansion phases of hESC differentiation and in fetal liver tissue achieved maximal levels during the first and second trimesters of gestation.

Previous reports have similarly suggested that CAR expression is increased in hepatic-like cells derived from hESCs (Ek et al., 2007; Zamule et al., 2011). In addition, CAR activation has been shown to participate in mammalian postnatal development. Transient activation of CAR by exposure to mouse CAR-specific agonist TCPOBOP on the third day after birth resulted in long-term epigenetic memory, permanently inducing mouse liver expression of the CAR target gene, CYP2b10, suggesting that CAR activation during development permanently alters the capacity for drug metabolism, and therefore may affect therapeutic responses (Chen et al., 2012). CAR activation is also necessary for proliferation and differentiation of postnatal hepatic progenitor cells in mice, contributing to liver regeneration and recovery after injury (Yamazaki et al., 2011). To further define the mechanistic and functional roles for CAR in the hepatic differentiation process, we used lentiviral transduction schemes to stably express CAR in hESCs for extended times in culture. We demonstrate for the first time that stable CAR expression during the early phases of the hepatic differentiation process activates a variety of hepatic markers, including the expression of HNF1α, HNF4α, albumin and CYP enzymes, and also enhances hepatic functional endpoints such as albumin secretion (Figs. 2 and 4). In these respects, after 24 h of stimulation with 5 μM CITCO, CAR over-expressing cells elicit a much stronger CYP3A4 induction response (both mRNA and activity) than empty vector- transduced controls (Figs. 3A and 4B). Conversely, CAR knockdown attenuates the differentiation-dependent increases in expression of a host of hepatic markers (Fig. 5).

CAR's specificity as an activator of the hepatogenesis process was substantiated by the demonstration that PXR is expressed at only very low levels during human hepatic differentiation or in human fetal liver (Fig. 6, A and B) and that neither CYP2C9 mRNA nor CYP2C9 activity were induced upon exposure to rifampicin, a prototypical human PXR ligand (Fig. 4C). Rather, PXR is highly expressed selectively in undifferentiated and early differentiated stem cells (Fig. 6A). It is interesting that in experiments where PXR was transduced into developing cells primed for the hepatic lineage, expression of hepatic differentiation markers was reverted (Fig. 6, C–E). In these respects, PXR appears to function in the maintenance of the immature phenotype, rather than a promoter of stem cell differentiation, as is the case for CAR. This finding appears consistent with several previous reports demonstrating that PXR suppresses the proliferation of cancer cells by up-regulating cyclin-dependent kinase (CDK) inhibitor p21, that ectopic PXR expression in neuroblastoma cells results in growth suppression (Misawa et al., 2005), and that PXR activation alone has no effect on cell proliferation in mouse liver (Shizu et al., 2013).

The data provided in the current report suggest that CAR is a newly identified factor important in promoting the differentiation and maturation of hepatic-like cells from human embryonic stem cells (hESCs), naturally pluripotent cells. In contrast, the Yamanaka factors, Oct3/4, Sox2, c-Myc, and Klf4, are typically transduced via viral vectors and then transiently expressed in terminally differentiated cells, often in fibroblasts, where they promote somatic cell reprogramming to induce pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). These iPSCs exhibit morphology and growth properties of embryonic stem cells and express embryonic cell marker genes (Takahashi and Yamanaka, 2006). In these respects, CAR appears to function as a facilitator of the differentiation of hESCs into hepatic-like cells, while Yamanaka factors are characterized for their ability to participate in generating iPSCs derived from terminally differentiated cells.

Although it is noted that CAR's effects as presented in the current study resulted from the over-expression of CAR and not its chemical activation, overall the data generated support the contention that in human development from stem cell precursors, the expression of CAR specifically functions to integrate and drive the differentiation and maturation of hepatic-like cells.

Interestingly, data published in abstract form derived from ChIP-seq analyses conducted in human HepG2 cells indicated that CAR interacts with several known transcription factor motifs including HNF4, CEBP-α, AP1 and FOXA (Xiao et al., 2012). Moreover, cistromes of CAR overlap with those of RXRα, HNF-4α, CEBP-β, and Jun-D in HepG2 cells, suggesting that CAR may collaborate with other transcription factors to promote gene-specific regulatory cascades. Further studies will be necessary to precisely delineate these interactions, in particular in the differentiation context where hepatic transcription factors including HNF1, HNF4 and FOXA have been established for their importance in contributing to hepatic differentiation and maturation.

Given the apparently important role of CAR as an integrator of human hepatocyte development, it is curious that CAR-knockout mice exhibit no overt morphological abnormalities of the liver (Wei et al., 2000). This observation suggests that functional redundancy exists within the complex scheme of hepatic differentiation, such that compensatory mechanisms are in place to substitute for CAR's deficiency in the null mice. Alternatively, CAR's role in hepatic differentiation may be species-specific. In these respects, the review by Jeong and Mangelsdorf (2009) summarizes advances in the study of the roles of NRs in 'stemness' and early cell lineage commitment in a variety of species. The discussion provides support for the latter hypothesis, noting significant interspecies differences in the expression profiles and roles of members of the NR superfamily in the maintenance of pluripotency and differentiation, concluding that NR functions in these roles is highly species-specific. The study of Xie et al. (2009) further supports this hypothesis, demonstrating that while CAR is increasingly expressed during embryoid body differentiation in both H1 and H9 human ESC lines, expression of CAR in mouse ESCs is not detectable at any developmental stage. Microarray expression data from panels of human and mouse tissue, available through the UCSC Genome Browser website (http://genome.ucsc.edu/) (Karolchik et al., 2008; Kent et al., 2002), also document robust levels of CAR expression in human fetal liver tissue, as corroborated herein, whereas CAR expression in the mouse embryo appears negligible. Together, these data offer strong support for a species-selective role of CAR's function as a regulator of human hepatocyte development from the stem cell lineage.

In summary, this study defines a novel role for CAR as a key regulator of human hepatic differentiation – a complex and multifaceted process which is yet to be fully elucidated.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.10.012.

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