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Genome-wide Analysis of Human Constitutive Androstane Receptor (CAR) Transcriptome in Wild-type and CAR-knockout HepaRG cells

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Abstract

The constitutive androstane receptor (CAR) modulates the transcription of numerous genes involving drug metabolism, energy homeostasis, and cell proliferation. Most functions of CAR however were defined from animal studies. Given the known species difference of CAR and the significant cross-talk between CAR and the pregnane X receptor (PXR), it is extremely difficult to decipher the exact role of human CAR (hCAR) in gene regulation, relying predominantly on pharmacological manipulations. Here, utilizing a newly generated hCAR-knockout (KO) HepaRG cell line, we carried out RNA-seq analysis of the global transcriptomes in wild-type (WT) and hCAR-KO HepaRG cells treated with CITCO, a selective hCAR agonist, phenobarbital (PB), a dual activator of hCAR and hPXR, or vehicle control. Real-time PCR assays in separate experiments were used to validate RNA-seq findings. Our results indicate that genes encoding drug-metabolizing enzymes are among the main clusters altered by both CITCO and PB. Specifically, CITCO significantly changed the expression of 135 genes in an hCAR-dependent manner, while PB altered the expression of 227 genes in WT cells of which 94 were simultaneously modulated in both cell lines reflecting dual effects of PB on hCAR/PXR. Notably, we found that many genes promoting cell proliferation and tumorigenesis were up-regulated in hCAR-KO cells, suggesting that hCAR may play an important role in cell growth that differs from mouse CAR. Together, our results reveal both novel and known targets of hCAR and support the role of hCAR in maintaining the homeostasis of metabolism and cell proliferation in the liver.

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Keywords

HepaRG; CAR; gene knockout; RNA-seq; phenobarbital; CITCO

1. Introduction

Mounting evidence has established the constitutive androstane receptor (CAR, NR1I3) as a hepatic sensor that regulates the expression of a large array of genes associated with drug metabolism/transport, energy homeostasis, and cell proliferation [1–4]. Activation of CAR not only affects the biotransformation and excretion of drugs and endobiotic molecules such as the chemotherapeutic cyclophosphamide, the anti-HIV efavirenz, and the heme catabolism breakdown bilirubin, it also influences the homeostasis of glucose, lipids, and fatty acids, holding the potential to function as a drug target for metabolic disorders [5–8]. Notably, many of these actions of CAR are shared with its closely related nuclear receptor, the pregnane X receptor (PXR, NR1I2) [9, 10]. Cross-talk between CAR and PXR through recognition of each other's response elements triggers reciprocal activation of a cluster of overlapping target genes that coordinate comprehensive hepatic defensive responses [11, 12]. For instance, CYP2B6, a prototypical target of human CAR (hCAR), can be efficiently induced by rifampicin (RIF), a selective agonist of human PXR (hPXR) [13]. On the other hand, phenobarbital (PB), a well-known inducer of CYP2B6, activates both CAR and PXR in humans [14]. Although cross-talk between CAR and PXR formulates a defensive network against xenobiotic exposure, it generates challenges toward the identification of hCAR specific function and target genes. Moreover, the current paradigm of cross-talk between CAR and PXR relies majorly on animal studies. Significant species differences exist between hCAR and its rodent counterparts [15, 16].

The specific role of mouse CAR (mCAR) in gene regulation was conclusively defined by using CAR-null mice. Induction of *cyp2b10* gene, the corresponding isozyme of human CYP2B6, by PB and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP, a selective agonist of mCAR) was completely abolished in CAR-null mice [17]. At the whole genome level, numerous genes associated with mCAR-dependent induction and repression were also identified in comparison of microarray data between wild-type (WT) and CAR-null mice [18]. Nevertheless, documented species differences of CAR prevent direct extrapolation of such data from mouse to humans. Notably, TCPOBOP activates mouse but not human CAR; on the other hand, the known hCAR agonist 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime (CITCO) selectively binds and activates hCAR only; while the antiemetic meclizine functions as an agonist of mCAR but inverse agonist of hCAR [16, 19, 20].

Unlike prototypical nuclear receptors, CAR can be activated through either direct ligand binding or ligand-independent mechanisms, involving protein kinase-mediated signaling pathways [21, 22]. Consistent with its initial designation, CAR is constitutively activated in immortalized cell lines and spontaneously accumulated in the nucleus without xenobiotic stimulation [23, 24]. In contrast, CAR is sequestered in the cytoplasm of the more physiologically relevant primary hepatocytes before activation [25]. In primary hepatocytes, CAR forms a cytoplasmic multi-protein complex with a number of chaperone proteins and

only translocates to the nucleus upon chemical-stimulated activation [26]. Thus, human primary hepatocytes (HPH) represent a valuable model for understanding the biological function of hCAR. Indeed, many drugs and environmental chemicals have been identified and validated as hCAR activators using HPH [27, 28]. However, both hCAR and hPXR are enriched and functionally intact in HPH, making delineation of the precise role of hCAR difficult. To this end, investigation of the function of hCAR relies predominantly on pharmacological manipulation of this receptor. The majority of the known hCAR activators also influence the activity of the promiscuous hPXR. Thus, the need for a human liver cell system with genetically modified hCAR is evident.

The HepaRG cell line, exhibiting hepatocyte-like morphology and functions, and expressing hepatic specific genes, has been recognized as a promising alternative to HPH [29, 30]. Most recently, we have shown that differentiated HepaRG cells retain hCAR in the cytoplasm, which can be translocated to the nucleus in response to PB treatment (manuscript under review), suggesting that functional hCAR signaling is preserved in these cells. To obtain hCAR-mediated global gene expression profiles, we carried out RNA sequencing (RNA-seq) and RT-PCR assays on WT and CAR knockout (KO) HepaRG cells treated with PB or CITCO. PB and CITCO induced a spectrum of overlapping and distinct genes. Both novel and known genes associated with hCAR activation have been recognized. Importantly, knockout of hCAR alone altered the expression of numerous genes, in particular, those related to cell proliferation and tumor development.

2. Material and methods

2.1. Reagents

PB was purchased from Sigma-Aldrich (St. Louis, MO). CITCO was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA). Human CAR antibody was acquired from Perseus Proteomics (Tokyo, Japan). HepaRG WT cells, HepaRG hCAR-KO cells, and related culture medium used were from Sigma-Aldrich.

2.2. HepaRG cell differentiation and RNA sequencing

HepaRG cell differentiation and induction were carried out following instructions from Sigma-Aldrich. HepaRG cells were seeded in 24-well plates in Maintenance Medium at the density of 0.4×10^6 cells/well on Day 0. Media was changed every 3 days until Day 14. Maintenance Medium was then replaced by Pre-induction Medium and incubated for another 3 days. On day 17, Pre-induction Medium was removed and Serum-Free Induction medium containing PB (1 mM), CITCO (1 μ M) or DMSO (0.1% v/v) was added to cell cultures for another 24 h. RNA samples from treated WT and hCAR-KO cells were isolated using the miRNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instruction. RNA samples from different treatments were analyzed at Beckman Coulter Genomics (Danvers, USA). In brief, Illumina TruSeq RNA Library Preparation Kit v2 was used in combination with Beckman Coulter Biomek liquid handling automation to perform cDNA synthesis and adaptor ligation. Sequencing was performed on the Illumina HiSeq2500 instrument at 2×100 base pair read length according to the manufacturer's

instruction. Default quality trimming was used for the respective instruments with removal of reads that fall below CASAVA's passing filter.

2.3. Real-Time RT-PCR

Total RNA was isolated from HepaRG cells using TRIzol reagent (Qiagen), and reverse transcription was performed by using the High Capacity cDNA Archive kit (Applied Biosystems, Foster, CA) according to the manufacturers' instructions. Quantitative real-time PCR assay was performed by using SYBR Green ROX™ qPCR Mastermix (Qiagen) on an ABI StepOnePlus™ System (Life Technologies, Grand Island, NY). Expression values were analyzed by the equation: $\text{Fold} = 2^{-\Delta\Delta C_t}$, where ΔC_t represents the differences in cycle threshold numbers between each of the target gene and GAPDH, and $\Delta\Delta C_t$ represents the relative change between compared groups. All primer sequences are listed in Table 1.

2.4. Western blot analysis

Whole cell lysate (40 µg total protein) was loaded and electrophoretically separated on SDS-PAGE gels. The proteins were transferred onto polyvinylidene fluoride membranes at 500 mA for 1h. Subsequently, membranes were blocked and incubated with antibody of hCAR (Perseus Proteomics) or β-actin (Santa Cruz Biotechnology) at 4 °C overnight. Membranes were developed with West Pico/Femto chemiluminescent substrate (Thermo-Scientific, Rockford, IL) after incubation with secondary antibody.

2.5. RNA-seq data analysis

Detailed sequencing data have been uploaded in the Gene Expression Omnibus (GEO) database that can be viewed with the accession number of GSE71446. Sequence mapping was performed using Tophat version 2.0.10 and Bowtie version 1.0.0 on the Ensembl annotation of human genome reference sequence GRCh38. For expression analysis, Cufflinks version 2.1.1 was used to detect genes and transcripts that are expressed by comparing the Tophat2 read mapping with the Ensembl gene models for the grch37 release of the human genome. The easyRNASeq R Bioconductor package was used to establish raw read counts from the Tophat2 generated BAM alignment files before using the edgeR R Bioconductor 2.12 package. A generalized linear model was used instead of a pairwise comparison approach to account for multiple experimental factors according to the edgeR Users Guide to determine differentially expressed genes. We used a 1.5-fold expression change cut-off combined with exclusion of those with *p*-value greater than 0.05 and logCPM less than -2 in comparison to define the differentially expressed genes. HemI (Heatmap Illustrator, version 1.0) was used to generate the heatmaps of differentially expressed genes. Database for Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics Resources 6.7 was used for the gene function categories and pathway analysis (<http://david.abcc.ncifcrf.gov/>) by selecting GOTERM_BP_FAT and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, respectively.

2.6. General data analysis

Results from real-time PCR are expressed as mean ± standard deviation of triplicate determinations. Statistical comparisons were made between vehicle control and drug

treatment or WT vs. hCAR-KO group using one-way analysis of variance followed by post hoc Dunnett's test and Students' *t* test where appropriate. The criterion of significance was set at $p < 0.05$ or $p < 0.01$.

3. Results

3.1. Validation of the hCAR-KO HepaRG cell line

The hCAR-KO HepaRG cell line obtained from Sigma-Aldrich is a newly generated cell line that yet requires full validation. Utilizing the Zinc Finger Nuclease technology, the start sequence of hCAR was targeted. Sequencing of the related DNA region revealed the lack of TGGCCAGTAGG in the knockout cells, which can be genotyped by PCR (Fig. 1A). This editing resulted in differential expression of hCAR protein between HepaRG-WT and hCAR-KO cells, where hCAR was undetectable in KO cells (Fig. 1B). To further validate the function of hCAR-KO cell, inductive expression of CYP2B6, the prototypical hCAR target gene, was measured at the mRNA and protein levels in WT and CAR-KO cells treated with PB and CITCO. As shown in Fig. 1C and 1D, PB, a dual activator of hCAR and hPXR, markedly induced the expression of CYP2B6 in both WT and hCAR-KO cells, while CITCO, a selective activator of hCAR, enhanced the expression of CYP2B6 in the WT cells only. These results clearly establish the hCAR-KO HepaRG cells as a novel research tool in elucidating specific functions associated with hCAR.

3.2. CITCO-mediated gene expression in WT and hCAR-KO HepaRG cells

CITCO represents one of the most selective agonists of hCAR identified thus far. Differentially expressed gene profiles were obtained by comparing RNA-seq data generated from CITCO and vehicle control treated WT and hCAR-KO HepaRG cells (Fig. 2, Supplementary Table S2 deposited in GEO dataset GSE71446). The numbers of genes induced by CITCO in WT and hCAR-KO cells are 77 and 41, respectively (Fig. 2A). There are also 13 genes induced by CITCO in both cell lines independent of hCAR. Functional categorization revealed that among the 77 genes induced in HepaRG WT cells, many are associated with DNA damage repair, including genes coding for bloom syndrome protein (BLM), histone H2A (H2AFX), and DNA (cytosine-5-)-methyltransferase 3 beta (DNMT3B) (Table 2). In addition, a group of genes related to the nuclear factor-like 2 (Nrf2) signaling pathway such as glutathione peroxidase 2 (GPX2) and Kruppel-like factor 2 (KLF2) were also enriched in this category. On the other hand, many of the 41 genes induced by CITCO in the absence of hCAR are functionally related to ion homeostasis and transmission of nerve impulse (Fig. 2B, Table 2). Among the 13 genes overexpressed in both cell lines, the majority of them encode drug-metabolizing enzymes such as CYP2B6, CYP3A4, and CYP1A1. It is noteworthy that although CYP2B6 was classified as a gene induced by CITCO in both lines in our RNA-seq analysis, CYP2B6 was induced 12-fold in WT cells while only increased around 1.8-fold in hCAR-KO cells. This phenomenon is consistent with previous reports and supports the predominant selectivity of CITCO in hCAR activation and CYP2B6 induction.

In addition to induction, CITCO treatment also resulted in the repression of 58 genes in WT and 33 genes in hCAR-KO cells (Fig. 2C). Many genes down-regulated in a hCAR-

dependent manner are related to carbohydrate metabolism and ion transport, including glucokinase (GCK), prostaglandin-endoperoxide synthase 2 (PTGS2), and receptor-type tyrosine-protein phosphatase-like N (PTPRN). The 33 genes only repressed in hCAR-KO cells appear to be associated with cell-cell signaling, cell proliferation, and ossification (Table 2). Notably, there were only 3 genes repressed in both WT and hCAR-KO cells. Together, these results identify both known and novel hCAR target genes. CITCO treatment led to a limited overlap of differentially expressed genes between WT and hCAR-KO cells, consistent with its central action on hCAR.

3.3. PB-mediated gene expression in WT and hCAR-KO HepaRG cells

PB is a potent activator of both hCAR and hPXR and induces a large number of genes associated with drug metabolism and clearance. In this report, we observed a total of 190 genes being induced by PB in WT and/or hCAR-KO HepaRG cells (Fig. 3A), of which 49 genes were induced in a hCAR-dependent manner, 69 genes were induced only in hCAR-KO cells, while 72 genes were overexpressed in both cell lines. As shown in Table 3, genes typically induced by PB via the activation of hCAR and hPXR such as CYP2B6 and CYP3A4 are among the 72 genes. Furthermore, other cytochrome P450 enzymes including CYP3A7, CYP3A43, CYP2C8, CYP2A13, CYP2A6, CYP2C18, CYP1A1, CYP3A5, and CYP4F8 were also induced in both cell lines (Fig. 3B). In addition to P450 enzymes, a group of efflux and uptake transporter proteins such as ATP-binding cassette, sub-family B member 1 (ABCB1), ABCC2, solute carrier family 5 member 12 (SLC5A12), and SLC51B were identified as hCAR and hPXR shared targets. The function of most genes in this group are related to cellular biosynthetic and metabolic processes including the metabolism of drugs, steroids, fatty acids, and hormones, as well as the biosynthesis of carboxylic acids, organic acids, and nitrogen compounds (Table 3). Of the 49 genes induced only in WT cells by PB, a large number of which are associated with hormone metabolism including dehydrogenase/reductase SDR family member 9 (DHRS9), 3-oxo-5- α -steroid 4-dehydrogenase 2 (SRD5A2), UDP-glucuronosyltransferase (UGT2B28), and CYP19A1, suggesting that hCAR may play an important role in modulating the hormone balance in humans. Functional categorization of the 69 genes specifically induced in hCAR-KO cells revealed that most genes are involved in regulation of gene transcription and RNA metabolism (Table 3).

In contrast to induction, 106 genes were repressed by PB in WT cells, while 84 of them were down-regulated in hCAR-dependent fashion (Fig. 3C). Functional classification revealed that a number of genes down-regulated by PB in an hCAR-dependent manner involving cell adhesion and cell-cell interactions, such as the G protein-coupled receptor 56 (GPR56), apolipoprotein A4 (APOA4), and angiopoietin-1 receptor (TEK) (Fig. 3B, Table 4). APOA4 and TEK were also reported to have relationship with liver regeneration and cell growth [31]. Genes repressed in the absence of hCAR upon PB treatment appear to be associated with the oxidation and reduction process, embryonic organ morphogenesis, as well as the Wnt signaling pathway (Table 3). We have also observed 22 genes that are repressed by PB in both WT and hCAR-KO cell lines, which modulate skeletal system development and cell growth. Collectively, these results support a role of PB in human liver cell growth by inducing or repressing genes downstream of hCAR.

3.4. Comparison of gene expression between PB and CITCO treatment

We next analyzed the transcription profiles between PB and CITCO treated WT and hCAR-KO HepaRG cells. As shown in Figure 4, the number of differentially expressed genes upon PB treatment was markedly greater than that of affected by CITCO exposure. Among the 180 genes up-regulated by PB and/or CITCO in HepaRG-WT cells, 31 genes were found to be induced by both drugs and nearly half of them are involved in the metabolism of xenobiotics, hormones, or vitamins (Fig. 4A, Table 4). Many drug-metabolizing enzymes induced by PB and CITCO, such as CYP3A4, CYP3A7, and CYP2B6 are known targets shared by hCAR and hPXR (Fig. 4B). PB and CITCO treatment also led to the down-regulation of 106 and 60 genes in WT cells, respectively. Of the down-regulated genes, 15 were suppressed by both drugs, including genes associated with the promotion of cell proliferation such as TEK, PTGS2, and the regulator of cell cycle (RGCC). Combined, these results are consistent with the promiscuous role of PB in gene regulation that involves both hCAR and hPXR.

3.5. Differential gene expression in WT and hCAR-KO HepaRG cells

Besides responding to xenobiotic stimulation, CAR may hold inherent endobiotic functions that cannot be appropriately elucidated through pharmacological manipulations. The pair of WT and hCAR-KO HepaRG cells provides a unique model to investigate the importance of endogenous hCAR expression on gene expression. We compared the differential transcriptomes between WT and hCAR-KO cells and identified a great number of genes shown significant differences at the transcriptional level, suggesting endogenously expressed “quiescent” hCAR is of particular physiological importance via gene regulation. A total of 2057 genes were up-regulated in WT HepaRG cells (Fig. 5A, Supplementary Table S5 deposited in GEO dataset). Subsequent pathway analysis revealed that a large number of these genes belong to the families of cytochrome P450, UGT, glutathione S-transferases, ABC transporters, and alcohol dehydrogenases, which are involved in the metabolism of xenobiotics, carbohydrates, lipids, and amino acids. Therefore, hCAR itself is important in the maintenance and modulation of the basal expression of metabolizing enzymes in human liver cells.

Additionally, up to 2009 genes were repressed in WT cells in comparison to hCAR-KO cells (Fig. 5B). It is worth noting that genes associated with cancer biology, cell cycle, and cardiovascular diseases are among the most enriched pathways down-regulated. Among these genes, 51 are known targets in cancer-related signaling pathways including BCL2-like 1 (BCL2L1), insulin-like growth factor 1 (IGF1), jun proto-oncogene (JUN), and epidermal growth factor receptor (EGFR) and, 19 play important check-point roles at different phases of cell cycle such as cyclin-dependent kinase CDK1, CDK2, cell division cycle 20 (CDC20), cyclin B1 (CCNB1), and cyclin E2 (CCNE2), suggesting that hCAR may inhibit cell cycle progression at its native status. Pathway analysis also revealed that several critical cancerous pathways including the TGF- β , p53, and Jak-STAT signaling were repressed in the presence of hCAR (Fig. 5B). Together, these results indicate that even without the presence of xenobiotic agonists or antagonists, hCAR functions as an important modulator in maintaining the homeostasis of metabolism, cell cycle and proliferation in the liver cells.

3.6. Validation of RNA-seq results in HepaRG cells

To validate the RNA-seq findings, we performed real-time PCR to examine a select set of genes differentially expressed in HepaRG WT and hCAR-KO cells in the presence or absence of chemical stimulation. CYP3A4, the most abundant human hepatic CYP, is a representative target of hPXR with hCAR playing a moderate role in its inductive expression. In agreement with the RNA-seq data, our PCR results indicated that expression of CYP3A4 was markedly induced by PB, the dual activator hPXR/CAR, in both cell lines, while only affected by CITCO to a minimal extent (Fig. 6A). Intriguingly, PB-mediated induction of CYP3A4 in hCAR-KO cells was markedly higher than that in WT cells, which was also observed with a number of other hPXR activators (data not shown), suggesting the role of hPXR might be enhanced in the absence of hCAR competition. GPX2 is a downstream target of the Nrf2 signaling pathway, which can be activated by oxidative stress [32]. We identify GPX2 as a novel hCAR target gene that was induced by CITCO in WT but not hCAR-KO cells (Fig. 6B). CYP1A1 is a prototypical target of the aryl hydrocarbon receptor (AhR) and catalyzes the metabolism of many drugs and environmental chemicals [33]. Both our RNA-seq and PCR data demonstrated that expression of CYP1A1 was significantly increased by CITCO regardless of the presence or absence of hCAR (Fig. 6C). Other than induction, a number of genes selectively repressed by PB and/or CITCO were also examined using RT-PCR. Consistent with RNA-seq findings, GCK, which is critical in glucose metabolism, was repressed by CITCO in WT only (Fig. 6D). GPR56, a member of G-protein coupled receptor family that is functional associated with tumor cell growth suppression and apoptosis induction, was selectively repressed in WT HepaRG cells upon PB treatment [34, 35] (Fig. 6E); whereas GSTA5, a glutathione S-transferase which can catalyze the conjugation of carcinogens, was induced by PB only in hCAR-KO cells (Fig. 5F). Furthermore, a batch of genes differentially expressed in WT and hCAR-KO cells without chemical stimulation were subjected to PCR validation. As shown in Figure 7, knockout of hCAR increased the expression of genes known to be involved in cell cycle and tumorigenesis, including CCNB1, CDK1, DMBT1, EPO, IGF1, IGFBP1, MAFB, MAGEA9, NLGN4X, NXF3 and TRIML2. On the other hand, genes associated with metabolism such as ALB, APOA1, and GSTA5 were confirmed to be down-regulated. Collectively, these results reconfirmed that hCAR exhibits extensive regulatory roles ranging from xenobiotic/endobiotic metabolism to energy homeostasis, cell proliferation and cancer development.

4. Discussion

Despite major progress in deciphering the role of rodent CAR by using genetically modified animal models, the function of hCAR has been investigated predominantly via the use of chemical modulators which often lack specificity [36, 37]. In this report, we have examined the hCAR-associated transcriptome employing a newly generated hCAR-KO HepaRG cell line in comparison to its WT parent cells. RNA-seq was used to obtain global expression profiles between WT and hCAR-KO HepaRG cells challenged with PB, CITCO, or vehicle control. Our data revealed numerous new and previously reported genes that are associated with the presence or activation of hCAR in human liver cells. Consistent with previous reports, the main function of hCAR in the liver involves up-regulation of drug metabolism

upon PB and CITCO stimulation. Activation of hCAR is also involved in the regulation of important physiological and/or pathophysiological functions including cell proliferation, hormone homeostasis, energy metabolism, and oxidative stress. Notably, we found that endogenous expression of hCAR alone was sufficient to alter the expression of many genes known to be involved in critical biological functions.

HepaRG cells, a human liver cell line, originally derived from human hepatocellular carcinoma [38], exhibit prototypical human primary hepatocyte-like features such as the inductive expression of major P450s, the efflux function of transporters, and the physiologically relevant expression of a number of liver enriched transcription factors. Notably, these hepatic characteristics are lost in widely used human hepatoma cell lines including HepG2 cells, making HepaRG cells an attractive surrogate for HPH [39, 40]. CITCO, a compound that activates hCAR with relatively high specificity, was chosen as the model agonist for the investigation of hCAR and its prototypical target gene, CYP2B6. Our data demonstrated that the expression of CYP2B6 was robustly induced in HepaRG cells by CITCO to an extent which challenges that of HPH. Whereas such induction was disrupted at both mRNA and protein levels when hCAR was knocked out in HepaRG cells, establishing this line an excellent model for deciphering hCAR functions in a human hepatic cellular environment.

We first determined the global transcriptomes between WT and hCAR-KO HepaRG cells in the presence or absence of CITCO stimulus. Clearly, a significantly higher number of genes were up- or down-regulated by CITCO in WT over hCAR-KO cells, reflecting the overall high selectivity of CITCO for hCAR. Interestingly, other than the known drug metabolism-coding genes, a group of genes associated with oxidative stress response were induced by CITCO treatment in a hCAR-dependent manner. Among others, expression of GPX2, an antioxidant and anti-inflammatory enzyme, was enhanced by CITCO in WT cells only. It has been known that GPX2 is transcriptionally regulated by Nrf2 and Wnt/ β -catenin pathways. GPX2 promoter harboring multiple binding sites for Nrf2 and β -catenin/TCF was induced by prototypical Nrf2 activators such as sulforaphane and t-butyl hydroquinone [41, 42]. Notably, the Nrf2 activator Oltipraz was reported to activate CAR and induce cyp2b10 expression in mice [43], while Nrf2 knockout mice exhibited decreased expression of CAR target genes [44]. Whether CITCO-induced expression of GPX2 relies on the interplay between CAR and Nrf2 is an intriguing question that needs be address next. In addition to hCAR-dependent gene regulation, CITCO also altered the expression of a number of genes independent of hCAR. For instance, CYP1A1, a prototypical target gene of AhR, was potently induced by CITCO in both WT and hCAR-KO HepaRG cells (Fig. 6C). Likewise, mCAR-independent induction of CYP1A1 and/or CYP1A2 by pyrene and TCPOBOP was observed in both WT and CAR-KO mice [45, 46]. Whereas cell-based CYP1A1 luciferase reporter assays revealed that CITCO does not active AhR (Data not shown). Thus, CITCO may induce CYP1A1 in a yet unknown mechanism(s) that requires future investigation.

Although PB is a selective CAR activator in animal models, it induces the expression of CYP2B6 and CYP3A4 through activation of both hCAR and hPXR [47]. Consistent with the known cross-talk between hCAR and hPXR and dual effects of PB on these receptors, we have observed a set of largely overlapping genes induced by PB in both WT and hCAR-

KO cells. PB also influences the expression of a significantly greater number of genes than CITCO, which is more selective towards hCAR. In line with the reported central roles of these two compounds, genes up-regulated by both PB and CITCO in WT HepaRG cells are enriched majorly in drug and steroid metabolism, including CYP3A4, CYP2B6, CYP2A6, AKR1B10, and SLC5A12. On the other hand, differentially expressed genes related to glucose and lipid metabolism were limited after PB or CITCO treatment in our RNA-seq analysis, which differs significantly from studies in mice. Previous microarray studies revealed that PB represses multiple genes associated with fatty acid oxidation and glucose synthesis in a CAR-dependent manner [18]. The beneficial effects of CAR activation on obesity and diabetes were functionally confirmed in WT and CAR-KO mice using another mCAR activator, TCPOBOP, in which activation of mCAR down-regulates the process of gluconeogenesis, lipogenesis, and fatty acid synthesis [48, 49]. Studies using HPH however indicated that although activation of hCAR remarkably repressed the expression of genes corresponding to gluconeogenesis, its role on lipogenesis and fatty acid synthesis in human liver is contentious [50]. Thus, it appears that modulation of CAR may exhibit greater species selectivity in energy homeostasis than in drug metabolism.

Emerging evidence reveals that CAR has evolved into a dual xenobiotic/endobiotic sensor, dictating both prototypical agonists/antagonists and endogenous signaling molecules [17, 51]. Although the biological function of CAR relies predominantly on chemical modulation, expression of CAR itself may affect the downstream regulation of its target genes. Positive correlation between the expression of hCAR and CYP2B6 was observed in human liver samples [52]. In animal studies, fasting and caloric restriction increased the expression and activity of mCAR leading to diminished energy expenditure, while such compensatory adjustment was lost in CAR-KO animals [53]. By comparing the transcriptomes of HepaRG WT and hCAR-KO cells, we found that absence of hCAR suppressed many drug-metabolizing enzymes, while unexpectedly promoted cell proliferation. Among others, the basal level of CYP2E1 was drastically repressed, while expression of CYP3A4 and CYP2B6 was largely unchanged in hCAR-KO cells, suggesting maintenance of CYP2E1 expression requires the presence of hCAR. Interestingly, many genes encoding energy metabolizing enzymes were also down-regulated in the absence of hCAR, indicating the importance of hCAR in energy metabolism and homeostasis. A total of 2009 genes were up-regulated in hCAR-KO cells. To our surprise many genes associated with cell proliferation and tumorigenesis were enriched in this category. Considering that activation of hCAR by both CITCO and PB also repressed the expression of cell growth promoting genes such as TEK and RGCC, hCAR could have a role in suppressing cell proliferation and potential tumor development in human liver. Indeed, activation of hCAR by CITCO was reported to cause cell cycle arrest and enhanced apoptosis in human brain tumor stem cells [54], whereas overexpression of hCAR in human embryonic stem cells increased albumin secretion and promoted the differentiation and maturation of hepatic-like cells [55]. Nonetheless, these findings in human are in contrast to what has been reported previously in animal models, in which the tumor promotion effects of TCPOBOP and PB rely predominantly on a functional mCAR [17, 56]. Collectively, these studies support a presumptive role of hCAR in cell proliferation that is contrary to that of mCAR, further emphasizing the significance of species variations between hCAR and its rodent counterparts.

In summary, our results demonstrate that selective modulation of hCAR both pharmacologically and genetically lead to altered expression of numerous genes involving drug metabolism and transportation, oxidative stress, and energy balance, as well as cell proliferation and differentiation. Overlapping, distinct, and even opposite functions were shown between human and mouse CAR, with the differences lying mainly in the areas of energy metabolism and cell proliferation. These findings warrant more detailed investigation to eventually define the biological functions of human CAR and explore its possibilities as a therapeutic drug target.

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Abbreviations

CAR	constitutive androstane receptor
CITCO	6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime
CYP	cytochrome P450
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPR56	G protein-coupled receptor 56
GPX2	glutathione peroxidase 2
HPH	human primary hepatocytes
Nrf2	nuclear factor (erythroid-derived 2)-like 2
PB	phenobarbital
PXR	pregnane X receptor
TCPOBOP	1,4-bis[2-(3,5-dichloropyridyloxy)]benzene
TEK	angiopoietin-1 receptor

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Figure 1.

Validation of HepaRG hCAR-KO cells. (A) DNA sequencing revealed the deletion of TGGCCAGTAGG from exon 2 of hCAR in the knockout line. The hCAR-KO cells can be genotyped by PCR using primers shown in Table 1. (B) Expression of hCAR protein was analyzed using Western blotting in HepaRG WT and hCAR-KO cells. Induction of CYP2B6 was examined by Real-time PCR (C) and Western blotting (D) in HepaRG WT and hCAR-KO cells treated with PB (1 mM), CITCO (1 μ M) or DMSO (0.1% v/v) for 24 h as detailed in Materials and Methods. Each bar represents the mean $\pm$ SD (n = 3). **, $p < 0.01$.

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Figure 2. CITCO-mediated gene expression in WT and hCAR-KO HepaRG cells. (A) Venn diagram of up-regulated genes by CITCO in WT and hCAR-KO cells. (B) Heat map illustrating differentially expressed genes in WT and hCAR-KO cells treated with CITCO or vehicle control. (C) The number of genes down-regulated by CITCO in WT and hCAR-KO cells.

Figure 3. PB-mediated gene expression in WT and hCAR-KO HepaRG cells. (A) Venn diagram of up-regulated genes by PB in WT and hCAR-KO cells. (B) Heat map illustrating differentially expressed genes in WT and hCAR-KO cells treated with PB or vehicle control. (C) The number of genes down-regulated by PB in WT and hCAR-KO cells.

Figure 4.

Comparison of gene expression between PB and CITCO treatment. (A) Up- and down-regulated genes in HepaRG WT cells exposure to PB or CITCO. (B) Heat maps illustrating genes co-regulated by both PB and CITCO in WT and hCAR-KO cells. (C) Up- and down-regulated genes in hCAR-KO cells exposure to PB or CITCO.

Figure 5.

Differential gene expression in WT and hCAR-KO HepaRG cells. The gene expression profiles between WT and hCAR-KO cells were compared without activator stimulation. (A) The number of genes up-regulated and enriched pathways in WT HepaRG cells. (B) The number of genes up-regulated and enriched pathways in hCAR-KO cells.

Figure 6.

Real-time PCR validation of genes altered in RNA-seq analysis. WT and hCAR-KO HepaRG cells were cultured and treated with vehicle control (0.1% DMSO), CITCO (1 μ M) or PB (1 mM) for 24 h. Real-time PCR was used to analyze expression of CYP3A4 (A), GPX2 (B), CYP1A1 (C), GSK (D), GPR56 (E), and GSTA5 (F) as detailed in Materials and Methods. Data are represented as mean $\pm$ SD (n = 3). *, $p < 0.05$, **, $p < 0.01$.

Figure 7. Real-time PCR validation of differentially expressed genes in WT and hCAR-KO cells. Total RNA was harvested in WT and hCAR-KO cells in the absence of chemical treatment. A panel of selected genes exhibited altered expression in RNA-seq analysis was subjected to real-time PCR validation, which include (A) up-regulated genes and (B) down-regulated genes as indicated in the figures. Data are represented as mean $\pm$ SD ($n = 3$). *, $p < 0.05$, **, $p < 0.01$.

Table 1

Primer sequences for genomic DNA PCR and Real-time PCR

	Forward primer sequence 5'-3'	Reverse primer sequence 5'-3'
Primers for genomic DNA		
CAR sequencing	AGGCTGAAGTGGAGGATTGC	CCTCTGTTATGCCACCAGTT
hCAR exon1	AAGCAGCAGCTTCCAATGAG	ACTCCTGGGCTCAAGCGATC
hCAR exon2	AACACGTGACGTCATGGCCAG	CCTCTGTTATGCCACCAGTT
Primers for Real-time PCR		
ALB	GAGACCAGAGGTTGATGTGATG	AGTTCCGGGGCATAAAAGTAAG
APOA1	CCCTGGGATCGAGTGAAGGA	CTGGGACACATAGTCTCTGCC
CCNB1	AATAAGGCGAAGATCAACATGGC	TTTGTTACCAATGTCCCAAGAG
CDK1	GGATGTGCTTATGCAGGATTCC	CATGTACTGACCAGGAGGGATAG
DMBT1	CAAGGACTACAGACTACGCTTCA	TCCGAGGGAAATGGAGAACCT
EPO	AGGCCCTGTGGTCAACTCT	GCAGTGATTGTTCCGAGTGGA
GCK	CCTGGGTGGCACTAACTTCAG	TAGTCGAAGAGCATCTCAGCA
GSTA5	CATGAAGGAGAGAGCCCTGA	TGGCATCTCTTCTCTGCT
IGF1	GCTCTTCAGTTCGTGTGTGGA	GCCTCCTTAGATCACAGCTCC
IGFBP1	TTGGGACGCCATCAGTACCTA	TTGGCTAAACTCTCTACGACTCT
MAFB	TCAAGTTCGACGTGAAGAAGG	GTTCATCTGCTGGTAGTTGCT
MAGEA9	TTGGCCTCTCGTGCGATAG	AACGCTTCCCAGATAACCTCT
NLGN4X	GGTTTACCGCCAATTGGATACT	CCGTGGGCACGTAGATGTT
NXF3	AGCAGTAGGTCTGAACCTGTC	CTGCCTTCCGATTATAGGGTG
SERPINB2	CAGCACCGAAGACCAGATGG	CCTGCAAAATCGCATCAGGATAA
TRIML2	GCCACCGAGCTAGAGGAGAT	CTTGAGCAATGCCAAGGTGC
GPR56	TAGTCCCAGGTTTCTCTCT	CTCTCCTACGTGGGCTGTGT
CYP2B6	AGACGCCTTCAATCCTGACC	CCTTCACCAAGACAAATCCGC
CYP3A4	GTGGGGCTTTTATGATGGTCA	GCCTCAGATTCTCACCAACACA
hGAPDH	CCCATCACCATCTTCCAGGAG	GTGTGCATGGATGACCTTGGC
GPX2	GCCTCCTTAAAGTTGCCATA	GCCCAGAGCTTACCCA
CYP1A1	AGGCTGAGGTCTGTGATAAGCA	GCTAAGCAGTTCTTGGTGGTTG

Table 2

Functional categorization of CITCO-mediated gene expression

	Term	Count	%	PValue	Genes
Induced in WT cells only	GO:0010212~response to ionizing radiation	3	4.69	0.02	BLM, H2AFX, DNMT3B
	GO:0009314~response to radiation	4	6.25	0.03	SLC1A2, BLM, H2AFX, DNMT3B
	GO:0006310~DNA recombination	3	4.69	0.04	BLM, MND1, H2AFX
	GO:0051052~regulation of DNA metabolic process	3	4.69	0.05	BLM, PIF1, H2AFX
	GO:0000724~double-strand break repair via homologous recombination	2	3.13	0.06	BLM, H2AFX
	GO:0045934~negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	5	7.81	0.08	BLM, CCDC85B, TBX20, ST18, DNMT3B
Induced in KO cells only	GO:0019226~transmission of nerve impulse	4	12.12	0.02	SCN1A, PDE7B, TAC1, STX1B
	GO:0050801~ion homeostasis	4	12.12	0.03	SCN1A, GIPR, SLC7A8, TAC1
	GO:0042592~homeostatic process	5	15.15	0.04	SCN1A, GIPR, SLC7A8, TAC1, MB
	GO:0055065~metal ion homeostasis	3	9.09	0.05	GIPR, SLC7A8, TAC1
	GO:0007268~synaptic transmission	3	9.09	0.1	PDE7B, TAC1, STX1B
Both induced in WT and KO cells	GO:0055114~oxidation reduction	7	63.64	0	CYP3A43, CYP3A4, CYP3A7, CYP1A1, CYP2B6, CYP1A2, ALDH3A1
	GO:0017144~drug metabolic process	3	27.27	0	CYP3A4, CYP1A1, CYP1A2
	GO:0006805~xenobiotic metabolic process	3	27.27	0	CYP3A4, CYP1A1, CYP1A2
	GO:0009410~response to xenobiotic stimulus	3	27.27	0	CYP3A4, CYP1A1, CYP1A2
	GO:0018894~dibenzo-p-dioxin metabolic process	2	18.18	0	CYP1A1, CYP1A2
Repressed in WT cells only	GO:0009746~response to hexose stimulus	3	6.38	0	PTGS2, GCK, PTPRN
	GO:0034284~response to monosaccharide stimulus	3	6.38	0	PTGS2, GCK, PTPRN
	GO:0009743~response to carbohydrate stimulus	3	6.38	0.01	PTGS2, GCK, PTPRN
	GO:0010959~regulation of metal ion transport	3	6.38	0.01	ATP2B2, PTGS2, GCK
	GO:0043269~regulation of ion transport	3	6.38	0.01	ATP2B2, PTGS2, GCK
Rpressed in KO cells only	GO:0007267~cell-cell signaling	5	17.24	0.01	PGR, GRIK4, GRIK5, STC1, IHH
	GO:0001503~ossification	3	10.34	0.01	STC1, IHH, IGFBP5
	GO:0042127~regulation of cell proliferation	4	13.79	0.09	PGR, SFTPD, IHH, IGFBP5

Table 3

Function categorization of PB-mediated gene expression

Term	Count	%	PValue	Genes
Induced in WT cells only	6	13.04	0	ISYNA1, DHRS9, SRD5A2, PCYT1B, LPCAT2, CYP19A1
	3	6.52	0.03	ISYNA1, PCYT1B, LPCAT2
	3	6.52	0.03	DHRS9, RELN, SRD5A2
Induced in KO cells only	4	6.9	0.01	ADRB1, KCNJ8, NPR2, EPO
	10	17.24	0.04	MSX1, NR6A1, TGIF2LX, SSX6, ZNF597, CENPK, HDX, ZNF724P, EPO, FOXN4
	6	10.34	0.05	ADRB1, TRPM8, NPC1L1, GAL3ST1, MB, EPO
	20	31.25	0	CYP3A4, CYP3A5, CYP3A7, CYP1A1, CYP2C18, CYP2C9, CYP2B6, CYP2C8, etc
Both induced in WT and KO cells	8	12.5	0	CYP3A4, AKR1C2, CYP3A5, AKR1B15, CYP1A1, SULT2A1, AKR1B10, UGT2B4, AKR1D1
	5	7.81	0	AKR1C2, AKR1B15, CHIA, SULT2A1, AKR1B10, AKR1D1
	4	6.25	0	CYP3A4, CYP1A1, CYP2C9, NECAB2
	4	6.25	0	GLYAT, CYP1A1, EPHX1, SLC7A11
	4	6.25	0.01	SULT2A1, PRODH2, PAH, AKR1D1
	4	6.25	0.02	PRODH2, PAH, ASNS, AKR1D1
	10	13.51	0	APOA4, CLCA2, CLDN19, ITGAX, PDPN, TEK, GPR56, VCAN, POSTN, AMICA1
Repressed in WT cells only	8	10.81	0.01	APOA4, CTSK, PMCH, GIPR, SERPINE1, SFTPD, LGI4, EPO
	7	9.46	0.02	APOA4, PDPN, SFTPD, DCDC2, AFAP1L2, MST1R, NLRP1
	7	9.46	0.05	RARG, RARRES1, TEK, SERPINE1, SFTPD, MST1R, EPO
	6	13.04	0.03	NOX4, FMO5, HSD3B1, ALOXE3, FMO2, LOXL1
Rpressed in KO cells only	3	6.52	0.05	MAFB, CLIC5, GLI1
	3	6.52	0.05	NKD2, RSP03, RSP02
	3	6.52	0.08	MAFB, CLIC5, GLI1
	3	13.64	0.03	MATN3, CTGF, IGFBP3
Both repressed in WT and KO cells	2	9.09	0.09	CTGF, IGFBP3

Table 4

Classification of genes differentially expressed in PB- and CITCO-treated HepaRG WT cells

	Term	Count	%	PValue	Genes
PB induced only	GO:0055114~oxidation reduction	16	19.5	1.03E-07	NOX4, CYP3A5, CYP2C18, CYP2C9, CYP2C8, DHRS9, PAH, CYB5A, POR, GPX2, etc
	GO:0008202~steroid metabolic process	8	9.8	3.29E-05	AKR1C2, CYP3A5, SULT2A1, DHRS9, UGT2B4, SRD5A2, AKR1D1, CYP19A1
	GO:0006706~steroid catabolic process	4	4.9	0.00011	SULT2A1, UGT2B4, SRD5A2, AKR1D1
	GO:0008209~androgen metabolic process	3	3.7	0.001072	DHRS9, SRD5A2, AKR1D1
CITCO induced only	GO:0010212~response to ionizing radiation	3	6.4	0.008809	BLM, H2AFX, DNM3B
	GO:0009314~response to radiation	4	8.5	0.01152	SLC1A2, BLM, H2AFX, DNM3B
	GO:0006310~DNA recombination	3	6.4	0.025441	BLM, MND1, H2AFX
Both induced by PB and CITCO	GO:0055114~oxidation reduction	8	28.6	1.55E-05	CYP3A43, CYP3A4, CYP2A13, AKR1B15, CYP3A7, CYP1A1, CYP2B6, AKR1B10, CYP2A6
	GO:0017144~drug metabolic process	3	10.7	2.22E-04	CYP3A4, CYP1A1, NECAB2
	GO:0042359~vitamin D metabolic process	2	7.1	1.12E-02	CYP3A4, CYP1A1
	GO:0008202~steroid metabolic process	3	10.7	3.21E-02	CYP3A4, AKR1B15, CYP1A1, AKR1B10
	GO:0006775~fat-soluble vitamin metabolic process	2	7.1	4.54E-02	CYP3A4, CYP1A1
	GO:0007155~cell adhesion	9	10.7	0.006228	APOA4, CLCA2, PDPN, CTGF, TEK, GPR56, VCAN, POSTN, etc
PB repressed only	GO:0009611~response to wounding	7	8.3	0.018475	PDPN, CTGF, SERPINE1, VCAN, AFAP1L2, IGFBP1, EPO
	GO:0031099~regeneration	3	3.6	0.030943	SERPINE1, VCAN, IGFBP1
	GO:0042127~regulation of cell proliferation	8	9.5	0.035969	RARG, RARRES1, TEK, SERPINE1, SFTPD, MST1R, IGFBP3, EPO
CITCO repressed only	GO:0009743~response to carbohydrate stimulus	3	1.1	0.003848	PTGS2, GCK, PTPRN
Both repressed by PB and CITCO	GO:0019226~transmission of nerve impulse	2	16.7	9.96E-02	CLDN19, LGI4

Table 5
Classification of genes differentially expressed in PB- and CITCO-treated hCAR-KO cells

Term	Count	%	PValue	Genes
PB induced only	16	15.4	8.59E-07	CYP3A5, MDH1B, CYP2C18, CYP2C9, CYP2C8, PAH, CYP5A, POR, GPX2, CYP2A13, CYP4F8, AKR1C2, etc
	8	7.7	8.84E-05	AKR1C2, CYP3A5, AKR1B15, SULT2A1, AKR1B10, APOF, NPC1L1, UGT2B4, AKR1D1
	6	5.8	0.000113	AKR1C2, AKR1B15, PNLIIPRP2, SULT2A1, AKR1B10, NPC1L1, AKR1D1
	3	2.9	0.005347	SULT2A1, UGT2B4, AKR1D1
	5	4.8	0.012916	AZGP1P1, PNLIIPRP2, SULT2A1, UGT2B4, AKR1D1
	2	1.9	0.015664	SULT2A1, AKR1D1
CITCO induced only	5	27.8	0.001247	CYP3A43, CYP3A4, CYP3A7, CYP1A1, CYP2B6
	2	11.1	0.006488	CYP3A4, CYP1A1
Both induced by PB and CITCO	4	15.4	0.016162	SCN1A, PDE7B, TAC1, STX1B
	4	15.4	0.024352	SCN1A, GIPR, SLC7A8, TAC1
	3	11.5	0.039729	GIPR, SLC7A8, TAC1
	7	11.5	0.014917	NOX4, FMO5, HSD3B1, ALOXE3, FMO2, CYP4Z1, LOXL1
PB repressed only	5	8.2	0.018185	MATN3, CTGF, ACAN, IGFBP3, GLI1
	3	4.9	0.051608	CTGF, IGFBP3, GLI1
CITCO repressed only	4	16	0.026514	PGR, GRIK4, STC1, IHH
	2	8	0.075936	PGR, IHH