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Epidermal Growth Factor represses Constitutive Androstane Receptor expression in primary human hepatocytes and favors regulation by Pregnane X Receptor

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Abbreviations:

CAR, Constitutive Adrostone Receptor; CITCO, 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl) oxime ; CYP, Cytochrome P450; EGF, Epidermal Growth Factor; PHH, Primary Human Hepatocytes; PXR, Pregnane X Receptor; TCBOBOP, 1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene, 3,3',5,5'-Tetrachloro-1,4-bis(pyridyloxy) benzene; RXR, Retinoic X Receptor.

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Abstract

Growth factors have key roles in liver physiology and pathology, particularly by promoting cell proliferation and growth. Recently, it has been shown that in mouse hepatocytes, Epidermal Growth Factor Receptor (EGFR) plays a crucial role in the activation of the xenosensor Constitutive Androstane Receptor (CAR) by the antiepileptic drug phenobarbital. Due to the species-selectivity of CAR signaling, here, we investigated Epidermal Growth Factor (EGF) role in CAR signaling in primary human hepatocytes. Primary human hepatocytes were incubated with CITCO, a human CAR agonist, or with phenobarbital, an indirect CAR activator, in the presence or absence of EGF. CAR-dependent gene expression modulation and PXR involvement in these responses were assessed upon siRNA-based silencing of the genes that encode CAR and PXR. EGF significantly reduced CAR expression and prevented gene induction by CITCO and, to a lower extent, by phenobarbital. In the absence of EGF, phenobarbital and CITCO modulated the expression of 144 and 111 genes, respectively, in primary human hepatocytes. Among these genes, only 15 were regulated by CITCO and one by phenobarbital in a CAR-dependent manner. Conversely, in the presence of EGF, CITCO and phenobarbital modulated gene expression only in a CAR-independent and PXR-dependent manner. Overall, our findings suggest that in primary human hepatocytes, EGF suppresses specifically CAR signaling mainly through transcriptional regulation, and drives the xenobiotic response towards a Pregnane X Receptor (PXR)-mediated mechanism.

Introduction

Liver is a metabolically active organ that biotransforms a myriad of exogenous and endogenous compounds essentially through the metabolic functions of hepatocytes. These activities are finely regulated by a coordinated network of transcription factors. Among them, two nuclear receptors are known to control the expression of key detoxication enzymes: pregnane X receptor (PXR, NR1I2) and constitutive androstane receptor (CAR, NR1I3) (Wang et al., 2012). They may also participate in cancer development and drug resistance (De Mattia et al., 2016). Moreover, increasing evidences indicate that they modulate numerous endogenous signals, and cross-talk with signaling pathways involved in the regulation of the homeostasis of bile acids, lipids, hormones, glucose, inflammation, vitamins and other endobiotics (Pascussi et al., 2008; Wada et al., 2009; Wagner et al., 2005). PXR activation may worsen steatosis, obesity and insulin resistance (Li et al., 2012; Zhou et al., 2008; Zhou et al., 2006). Conversely, CAR activation is often described as beneficial, because it allows maintaining the homeostasis of cholesterol and bile acids and reducing blood glucose and steatosis. However, CAR effect on lipid metabolism was not reproduced in human primary hepatocytes (Lynch et al., 2014).

Considering the central role of CAR and PXR in transforming xenobiotics and regulating cell metabolism, much research has focused on the identification and development of molecules to modulate their activities. However, the study of PXR and CAR function is hindered by several factors. Indeed, the cross-talk between CAR and PXR through recognition of common responsive elements triggers the reciprocal activation of overlapping target genes that coordinate the hepatic response (Xie et al., 2000). For instance, *CYP2B6* is a target of human CAR, but can be also efficiently induced by rifampicin (RIF), a specific PXR activator (Goodwin et al., 2001). Similarly

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CAR can regulate *CYP3A4*, the prototypical PXR target gene (Maglich et al., 2002). Moreover, CAR and PXR operate as heterodimers with retinoic X receptor (RXR, NR2B1), and share co-activators, such as SRC-1 and PGC-1 α , and co-repressors, such as SMRT and NCoR (Oladimeji et al., 2016). It is therefore challenging to identify human CAR specific functions and target genes.

Species-specific direct CAR agonists have been found, such as 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl) oxime (CITCO) (Maglich et al., 2003) for human CAR and 1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene, 3,3',5,5'-Tetrachloro-1,4-bis(pyridyloxy) benzene (TCBOBOP) (Tzamelis et al., 2000) for mouse CAR. Conversely, phenobarbital (PB) is an indirect CAR activator that does not directly interact with the CAR ligand binding domain and thus does not show species specificity. Pregnenolone 16 α -carbonitrile specifically activates mouse PXR, while the antibiotic RIF activates human, but not mouse PXR. Human PXR can also be activated by PB via a receptor binding mechanism, in contrast to the receptor binding-independent mechanism involved in PB-mediated CAR activation (Moore et al., 2000). CAR activation by PB is the key molecular initiating event and promotes hepatocarcinogenesis in rodents. No carcinogenic effect of PB has been reported in humans (Elcombe et al., 2014).

Accumulating evidences indicate that xenobiotic metabolism is repressed during hepatic regeneration and cancer. This stimulated investigations on the involvement of growth factors and cytokines in the downregulation of CYP enzymes, due to their important role in liver regeneration and diseases (Berasain et al., 2014). For instance, EGF represses *CYP2B* gene induction by PB in mouse and rat hepatocytes (Bachleda et al., 2009; Kawamura et al., 1999; Koike et al., 2007). Moreover, EGF-mediated *CYP2B6* inhibition in human hepatocytes is accompanied by a strong decrease of CAR

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mRNA expression (Bachleda et al., 2009). The recent finding of a cross-talk between CAR and EGFR (Mutoh et al., 2013) further highlights the connection between cell proliferation/survival and liver detoxification/metabolism. Early and recent studies demonstrated that PB activates CAR transcriptional activity by preventing EGFR phosphorylation (Mutoh et al., 2009; Negishi et al., 2017). Very recently, it was found that EGF stimulates CAR homodimerization, thus forcing CAR in its inactive form (Shizu et al., 2017). Indeed, CAR activation by CAR ligands (CITCO) and indirect activators (PB) is regulated through homodimer-monomer conversion (Shizu et al., 2017). Moreover, PB competes with EGF for binding to EGFR in human cell lines and mouse hepatocytes (Mutoh et al., 2013).

To date EGF impact on CAR signaling has not been studied in primary human hepatocytes (PHHs). This model presents advantages over human liver cell lines, such as the physiological expression of transcription factors and coregulatory molecules and human CAR localization in the cytoplasm. We investigated EGF effect on CAR transcriptional activity in PHHs, using the human CAR agonist CITCO and the indirect human CAR activator PB. We also downregulated CAR with specific siRNAs to identify specific targets/responses. We found that EGF reduced transcription of CAR target genes, as expected, and also decreased CAR, but not PXR expression. EGF greatly influenced PHH response to direct (CITCO) or indirect (PB) activators and prevented CAR-dependent gene expression. Finally, we demonstrated that in the presence of EGF, CAR target genes are not regulated by PB via CAR, but via PXR.

Materials and Methods

Primary human hepatocyte isolation and culture. PHHs were provided by Biopredic International (Rennes, France), or isolated, as described previously (Pichard et al., 2006), from donor organs unsuitable for transplantation or from liver resections performed in adult patients for medical reasons unrelated to our research program. Liver samples were obtained from the Biological Resource Center of Montpellier University Hospital (CRB-CHUM; <http://www.chu-montpellier.fr>; Biobank ID: BB-0033-00031) and this study benefitted from the expertise of Dr Jeanne Ramos (hepatogastroenterology sample collection) and Prof Sylvain Lehmann (CRB-CHUM manager). The patients' clinical characteristics are presented in **Table 1**. The procedure was approved by the French Ethics Committee and written or oral consent was obtained from the patients or their families.

PHHs were seeded at confluency ($4 \cdot 10^5$ cells/well) in collagen-coated 24-well dishes and cultured in 5% CO₂ humidified atmosphere at 37°C in hepatocyte growth medium (HGM: WME medium supplemented with 5 µg/ml insulin, 0.1 µM hydrocortisone, 10 µg/ml transferrin, 250 µg/ml ascorbic acid, 3.75 mg/ml fatty acid-free bovine serum albumin, 2 mM glutamine, penicillin and streptomycin). When indicated, HGM was supplemented with 10 ng/ml EGF (Peprotech, France) (Ismail et al., 1991; Katsura et al., 2002).

siRNA transfection. Adherent PHHs were transfected with 10 nM scrambled siRNA (siCT) or ON-TARGET plus SMARTpool siRNAs (pool of four siRNAs) specific for human *NR1I3* (the gene encoding CAR) or human *NR1I2* (the gene encoding PXR) (Dharmacon, Lafayette, CO) using Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA) at day 2 and 5 after seeding. At day 5 post-seeding, PHHs were

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incubated with 0.5 mM PB, 1 μ M CITCO, 10 μ M RIF (Sigma) or vehicle (DMSO) for 24h.

RNA isolation and quantitative PCR. RNA was extracted using the TRIzol reagent (Invitrogen/Life Technologies, Carlsbad, CA), and then 300 ng of total RNA was reverse transcribed (RT) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR (qPCR) was performed using the Roche SYBER Green reagent and a LightCycler 480 apparatus (Roche Diagnostic, Meylan, France) with the following program: one step at 95°C for 10 minutes, and then 50 cycles of denaturation at 95°C for 10 seconds, annealing at 65°C for 15 seconds, and elongation at 72°C for 15 seconds. The primer sequences are listed in **Table 2**.

Microarray data acquisition. Total RNA (500 ng) was amplified and labeled for hybridization on GeneChip HT HG-U133+ PM Array plates. Data were acquired following the manufacturer's instructions (Affymetrix, UK). For each condition, five biological replicates were used. To obtain the intensity value signal, scanned plates were processed using the Affymetrix GCOS 1.4 software, and the Affymetrix CEL files were normalized using the Robust Multichip Average (RMA) implementation of the algorithm. The raw microarray data in .CEL files are available in the Gene Expression Omnibus with accession number GSE68493 at <http://www.ncbi.nlm.nih.gov/geo/>.

Microarray data analysis. Microarray data were analyzed using the web tool Genomicscape (genomicscape.com) (Kassambara et al., 2015). Briefly, paired significance analysis of microarrays (SAM) was performed using the 15,000 probe sets

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with the highest standard deviation. Significance was calculated using the Wilcoxon test and the list of significantly deregulated genes (fold change, FC, >1.3; false discovery rate, FDR, <5%) was extracted. Venn diagrams were generated using the Venny tool (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>). Heat maps of the identified genes of interest were produced using Cluster 3.0 and Treeview. Finally, pathway enrichment was assessed using KEGG (Kyoto Encyclopedia for Genes and Genomes (KEGG) and the STRING web tool (<http://string-db.org>).

Protein analysis. Total proteins were extracted from PHH cultures using RIPA lysis buffer supplemented with a protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA). Protein concentration was determined using the bicinchoninic acid method, according to the manufacturer's instructions (Pierce Chemical, Rockford, IL). Proteins (20 µg/sample) were resolved on 10% Precast SDS-polyacrylamide gels (Bio-Rad Laboratories, Marnes la Coquette, France) and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories). Membranes were incubated with antibodies against CYP2B6 and GAPDH (sc-67224 and sc-32233, Santa Cruz Biotechnology). Immunocomplexes were detected with a horseradish peroxidase-conjugated mouse secondary antibody (Sigma) followed by enhanced chemiluminescence (Millipore, Molsheim, France). Signals were acquired using a ChemiDoc-XRS+ apparatus (Bio-Rad Laboratories) and quantified with the Image Lab software (version 4.1)

DigiWest. The NuPAGE SDS-PAGE gel system (Life Technologies™) was used for protein separation and blotting. Proteins (18 µg per sample) were separated using 4-12% Bis-Tris gels according to the manufacturer's instructions. Blotting onto PVDF

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membranes (Millipore) was performed in standard conditions. For high content western blot analysis, the DigiWest procedure was performed as described (Treindl et al., 2016).

Statistical analysis. Quantitative PCR results were expressed as the mean \pm standard error of the mean (SEM). Results were compared using the paired t-test. Differences were considered statistically significant when $P < 0.05$.

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Results

EGF strongly affects gene expression in PHHs

First, the EGFR pathway activity in PHHs was monitored after long-term incubation (from D0 to D5) with 10 ng/ml EGF. Phosphorylation of ERK1/2 (at T202 and Y204), of MEK1/2 (at S217 and S221) and of the immediate downstream kinase RSK1 (p90RSK T573) demonstrated the sustained activation of the EGFR pathway upon incubation with the ligand (**Fig. 1A**).

Then, a global transcriptome analysis was performed in PHHs incubated or not with EGF for the same period (D0 to D5). Compared with control PHHs (without EGF), 328 genes (187 upregulated and 141 downregulated) were deregulated in EGF-treated cultures. KEGG pathway enrichment analysis revealed the contribution of the differentially expressed genes to specific biological processes. Genes upregulated following EGF incubation were mostly classified in 14 KEGG pathways mainly associated with cell proliferation and cancer (Komposch et al., 2015) (**Fig. 1B**). Conversely, genes downregulated upon incubation with EGF belonged mostly to KEGG pathways related to metabolism. “Drug metabolism cytochrome P450” and “Metabolism of xenobiotics by cytochrome P450” were among the five most deregulated signaling cascades (**Fig. 1C**). Unsupervised hierarchical clustering of the most significantly deregulated genes (35 up- and 35 downregulated genes) clearly showed that despite the inter-individual variability, PHH samples segregated according to whether they had been incubated or not with EGF (**Fig. 1D**). *NR1I3* (asterisk in Fig. 1D) that encodes CAR, the master regulator of *CYP2B6* gene induction, was among the genes downregulated upon incubation with EGF (FC= 0.31, q=0.009).

EGF reduces the expression of CAR and CAR-target genes

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To determine the role of EGF in CAR expression and signaling in PHHs, cells were incubated with CITCO, a CAR agonist, or with PB, an indirect CAR activator, for 24h (from D2 to D3 post-seeding), in the presence or absence of 10 ng/ml EGF. CITCO was used at a low concentration that does not activate PXR (Maglich et al., 2003). To rule out an indirect effect due to long-term treatment, EGF was added before (D0) or concomitantly (D2) with the activators. RT-qPCR analysis confirmed that, compared with untreated cells (UT), EGF specifically and reproducibly decreased CAR mRNA level (**Fig. 2A**), in the presence or not of CITCO and PB, but had a minor effect on PXR mRNA expression (not shown). Next, as PB can activate both CAR and PXR (Lehmann et al., 1998; Moore et al., 2000), the expression of their prototypical target genes *CYP2B6* (Sueyoshi et al., 1999) and *CYP3A4* was monitored. In the absence of EGF, PB and CITCO incubation increased *CYP2B6* and *CYP3A4* mRNA level compared with UT cells. Conversely, incubation with EGF before or concomitantly with the CAR activators strongly and significantly reduced *CYP2B6* upregulation by CITCO and PB (**Fig. 2B**) and of *CYP3A4* by CITCO, and to a lesser extent by PB (**Fig. 2C**). Of note, *CYP2B6* basal level (UT samples) was also significantly affected by the presence of EGF in the medium (**Fig. 2B**).

These findings suggest that EGF targets rapidly and more specifically CAR signaling in PHHs by modulating its expression and possibly its activity.

EGF affects CYP2B6 induction via CAR

To better understand EGF effect on CAR signaling, CAR expression was downregulated using specific small interfering RNAs (siCAR) and a non-targeting siRNA as a negative control (siCT). After siRNA transfection at D2 and D5, CAR mRNA expression was reduced by about 50% independently of incubation with EGF (from D1

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to D5) and CITCO or PB addition at D5 for 24h (**Fig. 3A**). In parallel, CAR inhibition reduced *CYP2B6* basal expression by two-fold (**Fig. 3B**), as observed in the presence of EGF (**Fig. 2B**). In the absence of EGF, *CYP2B6* mRNA was induced ~15- and ~17-fold following CITCO and PB incubation, respectively, in siCT cells. This induction was reduced by more than 50% in CAR-depleted PHHs (**Fig. 3B, left part**). In the presence of EGF, CAR downregulation abolished *CYP2B6* mRNA induction mediated by CITCO, whereas it did not significantly affect induction by PB (**Fig. 3B, right part**), although EGF presence greatly affected *CYP2B6* mRNA level. Western blot analysis of *CYP2B6* expression confirmed these results (**Fig. 3C**). The different effects observed following incubation of PHHs with CITCO and PB and the impact of CAR downregulation suggest that EGF affects *CYP2B6* induction via CAR.

EGF differentially affects the response to CITCO and PB

Our previous observation was limited to *CYP2B6*, the prototypical CAR target gene. Therefore, EGF effect on CAR transcriptional activity in PHHs was assessed by transcriptomic analysis. First, to identify genes affected by EGF, the transcriptomic profiles of siCT-transfected PHHs incubated with CITCO or PB in the presence or absence of EGF were analyzed (**Fig. 4A-B**). Independently of EGF presence/absence, more genes were deregulated following incubation with PB than with CITCO (no EGF: 144 versus 111; with EGF: 109 versus 58, respectively), in agreement with PB pleiotropic effects (Handschin et al., 2003). Moreover, genes were mostly upregulated (red) in response to PB or CITCO in the presence of EGF, whereas the effect was more heterogeneous (up- and down-regulation) in the absence of EGF. Further analysis revealed that only *CYP2B6* and *CYP2A6* (1.2% of all deregulated genes after CITCO incubation) were significantly deregulated by CITCO both in the presence and

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absence of EGF (**Fig. 4A**). However, the FC values were different in the presence and absence of EGF (FC=2.1 vs 10.5 for *CYP2B6*, and FC=1.4 vs 14.8 for *CYP2A6*). On the other hand, upon PB incubation, 45 genes (21.6% of all deregulated genes, including *CYP2A6* and *CYP2B6*) were affected both in the absence and presence of EGF (**Fig. 4B**), with a weak effect of EGF on their FC (data not shown). Functional characterization demonstrated that most of the 109 genes deregulated by CITCO only in the absence of EGF encoded drug-metabolizing enzymes and were associated with xenobiotic metabolic processes (**Table 3**). A similar pattern was observed for the 45 PB-deregulated genes that do not depend on EGF (**Table 4**). Surprisingly, among the genes deregulated by PB (64 genes) or CITCO (56 genes) only in the presence of EGF, many were associated with mRNA processing. Moreover, in the absence of EGF, most of the deregulated genes in response to PB were associated with lipid and steroid metabolism.

Finally, comparison of the KEGG pathway enrichment for the genes deregulated in siCT-transfected PHHs incubated with PB (**Fig. 5A**) or CITCO (**Fig. 5B**) for 24h with or without EGF (relative to UT cells) showed that in the absence of EGF, most of the enriched pathways belonged to metabolism, whatever the treatment. This profile was weakly affected by EGF in cultures incubated with PB. In contrast, in the presence of EGF, CITCO-deregulated genes were no more associated with any enriched KEGG pathway.

Identification of CAR target genes

The role of CAR in gene induction following incubation with CITCO or PB (with or without EGF) was then assessed in PHHs in which CAR was downregulated by siCAR transfection. On the basis of their unresponsiveness to CITCO or PB in siCAR cells

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compared with siCT cells, a list of CAR target genes was identified (**Table 5**). Among the 111 genes deregulated by CITCO in the absence of EGF in siCT cells (**Fig. 4A**), only 15 genes were identified as CAR-target genes. They included previously described CAR targets (*CYP2B6*, *-2A6/7*, *-2C8/9*, *3A4/7/43*, *EPHX1*, *ALAS1*, and *POR*) and four new potential up- (*RDH16*, *TSKU*, *CPEB3*) and downregulated (*TAGLN*) genes (**Table 5**). Among the 144 genes induced by PB in the absence of EGF in siCT cells (**Fig. 4B**), most (n=140) were regulated in a CAR-independent manner. Among the few CAR targets in PB-treated PHHs, *CYP2B6* and *CYP2A6/7* were also induced by CITCO, whereas *STEAP2* was specifically downregulated by PB. In the presence of EGF, among the 58 genes deregulated following CITCO treatment in siCT cells, only *CYP2B6* and *CYP2A6* were regulated by CITCO in a CAR-dependent manner. Conversely, upon incubation with PB in the presence of EGF, no gene was regulated in a CAR-dependent manner (**Table 5**).

These results were validated by qPCR analysis. In siCT cells, *CYP2A6* and *CYP2A7* (**Fig. 6A-B**) were similarly induced by PB and CITCO in the absence of EGF. This induction was strongly reduced following CAR downregulation (siCAR). In the presence of EGF, induction of *CYP2A6* and *CYP2A7* by CITCO and PB was strongly reduced in siCT cells. CITCO-mediated induction was abolished following CAR downregulation, while PB-mediated induction was less affected, as observed for *CYP2B6* (**Fig. 3B**). In the absence of EGF, *CYP3A4*, *CYP3A43*, *CYP3A7*, *CYP2C8*, *CYP2C9*, *TSKU*, *EPHX1* and *POR* (**Fig. 6C-J, Table 5**) upregulation by CITCO depended on CAR expression, whereas most of the PB-induced genes were CAR-independent, but for *CYP2C8* and *TSKU*. CAR contribution, monitored by the degree of siCAR-mediated inhibition of CITCO-induced response, varied among genes and was positively correlated with EGF inhibitory effect on gene expression. Addition of

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EGF did not change the FC induction of these genes by PB in controls (siCT) and CAR-depleted cells. Conversely, EGF abolished completely CITCO effect on the induction of these genes (**Fig. 6C-L**), as shown for *CYP2B6* (**Fig. 3B**). This pattern was not observed for *CPEB3* and *STEAP2* (**Fig. 6M-N**). These results suggest that EGF deeply affects the drug metabolism response in PHHs.

Analysis of EGF effect on PHH transcriptome profile highlighted important differences compared with EGF-free cultures. As CAR expression is inhibited by EGF, no effect of CITCO was expected in the presence of EGF. Surprisingly, 58 genes were still upregulated upon incubation of PHHs with CITCO in the presence of EGF (**Fig. 4A**), and 55 of these genes were deregulated also by PB (**Fig. 4B**).

Altogether PHH transcriptomic analysis—showed that: 1) EGF strongly affects the transcriptional activity of the xenosensor CAR; 2) PHH response to PB is much less sensitive to EGF than that to CITCO, suggesting that CAR-independent mechanisms are involved.

In the presence of EGF, PXR regulates CAR target genes

It is well known that PXR shares ligand and target genes with CAR in humans (Moore et al., 2000). Recently, Kandel et al. (Kandel et al., 2016) demonstrated, using human hepatocytes, that CAR and PXR activation by CITCO and RIF, respectively, deregulate the same pathways (including the drug metabolism pathway). Our results showed that EGF negatively affects CAR expression and that a set of genes can still be induced by PB in the presence of EGF in siCAR cells. Therefore, we hypothesized that in the presence of EGF, PXR can take the place of CAR. To test this hypothesis, the effect of CITCO and PB, in the presence or absence of EGF, was assessed in PHHs after siRNA-mediated downregulation of PXR (siPXR). In PHHs transfected with siPXR, a

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50% reduction of *PXR* mRNA was observed, independently of the presence of PB, CITCO or EGF (**Fig. 7A**). *CAR* mRNA level was not affected (**Fig. 7B**). Differently from what observed in si*CAR* cells, in the absence of EGF, PB- and CITCO-mediated *CYP2B6* induction was not inhibited upon *PXR* downregulation (**Fig. 7C**). Conversely, in the presence of EGF, PB-mediated *CYP2B6* induction was significantly reduced by *PXR* downregulation, while CITCO-mediated induction was not affected. *CYP3A4* mRNA was upregulated by CITCO only in the absence of EGF (**Fig. 7D** and **Fig. 2B**, **Table 5**), and its induction by PB depended on *PXR*, both in the presence or absence of EGF. These data suggest that in the presence of EGF, PB effect on *CYP2B6* and *CYP3A4* expression could be mediated via *PXR* rather than *CAR*. This was confirmed by siRNA-mediated downregulation of both *CAR* and *PXR* (**Fig. 7E**). Indeed, in the presence of EGF, induction of *CYP2B6*, *-3A4*, *-2C8*, *-2A6*, *-2A7*, *-3A43* and *TSKU* by PB (**Fig. 7F**) was strongly reduced when *PXR* (but not *CAR*) expression was downregulated. Moreover, this effect was increased in cells in which both *CAR* and *PXR* were silenced, particularly for *CYP2A6* and *CYP2A7*.

Finally, individual analysis of the effect of CITCO or PB (in the absence of EGF) in the different PHH cultures used for gene expression analysis (n=8) indicated that CITCO-mediated induction of *CYP2B6* mRNA expression was variable among samples (**Fig. 8A**). Moreover, it was significantly lower than the effect mediated by PB in Liv13 and Liv15, and almost no induction could be observed in Liv14 relative to UT cultures. Similarly, the relative *CAR* mRNA level in the different cultures was more heterogeneous than that of *PXR* (from 1 to 100 versus 1 to 10 respectively, not shown). Comparison of CITCO effect and of the *PXR*/*CAR* mRNA expression ratio in the eight samples showed that in the absence of EGF, when the *PXR*/*CAR* mRNA ratio was low (**Fig. 8B**) (i.e., high *CAR* mRNA expression), CITCO-mediated *CYP2B6* induction was

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high (**Fig. 8A**). Conversely, when the PXR/CAR mRNA ratio was high (i.e., low CAR mRNA expression), CITCO-mediated *CYP2B6* induction was low. In the presence of EGF that downregulates CAR expression, *CYP2B6* induction was lower after incubation with CITCO than with PB (**Fig. 8C**), and the PXR/CAR mRNA ratio values were higher (low CAR expression) (**Fig. 8D**). Moreover, siRNA-mediated CAR or PXR downregulation in two samples that poorly responded to CITCO (Liv13 and Liv15) showed that *CYP2B6* upregulation by PB depended on PXR activity (**Fig. 8E** and **Fig. 8F**), both in the presence and absence of EGF.

In conclusion, a switch from CAR to PXR dependency in PB-target gene induction is observed when CAR expression is low and in the presence of EGF. This observation could be critical for the development of target therapies.

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Discussion

Growth factors play crucial roles in liver physiology, and the involvement of some of them in CAR activity and expression has been widely studied. However, few data are available on EGF role in PHHs. Here, by using a direct and an indirect CAR activator, we show that EGF influences the expression of *CAR* and *CYP2B6*, the prototypical CAR target gene, in PHHs. We then found that few genes are strictly CAR-dependent in PHHs. Moreover, in this cell model, the indirect CAR activator PB modulates gene expression differentially in the absence or presence of EGF, but mostly in a CAR-independent and PXR-dependent manner. This may influence the design and interpretation of drug investigation studies.

Very few studies have analyzed PHH transcriptome profile following activation of human CAR in vitro. Kandel et al. performed a genome-wide comparison of the inducible transcriptomes of the nuclear receptors CAR, PXR and PPAR α in PHHs (Kandel et al., 2016). Li et al. studied genome-wide the transcriptome profiles of parental and HepaRG cells where *NR1I3* was knocked down (CAR-KO) (Li et al., 2015). Surprisingly, we identified only 15 CAR target genes among the 111 genes differentially regulated by CITCO and only four among the 144 genes affected by PB. Conversely, Li et al. found that in HepaRG cells, the expression of 135 genes and of more than 120 genes was affected by CITCO and by PB, respectively, in a CAR-dependent manner (Li et al., 2015). The difference in the number of deregulated genes could be linked to the cell type (HepaRG cells vs PHHs). Moreover, CAR silencing by siRNA led to a decrease of only approximately 50% of *CAR* mRNA expression in PHHs. Therefore, we cannot rule out that the remaining CAR protein is enough to regulate gene expression and that we only identified the major CAR targets. However,

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the observation that *CYP2B6* expression was significantly downregulated in CAR-depleted hepatocytes after treatment with CITCO suggests that the silencing strategy was effective. In addition, PB and CITCO off-target effects have been already reported (Li et al., 2015; Ueda et al., 2002). Therefore, our results are in agreement with PB non-specific and pleiotropic effects and its possible cross-activation of PXR, and also highlight the poorly described CAR-independent CITCO activity.

Most of the identified CAR target genes have been previously described (*ALAS1*, *POR*, *CYP2A6* -*2A7* -*2B6* -*2C8* -*2C9* -*3A4* -*3A7*, *EPHX1*) (Kandel et al., 2016) and we found only four new genes, of which three were validated by PCR (*TSKU*, *TAGLN*, *RDH16*). *CYP2B6*, *CYP2A6* and *CYP2A7* were the only genes modulated in a CAR-dependent manner by both PB and CITCO. *CYP2A6* and *CYP2A7* are located in close proximity of *CYP2B6* on chromosome 19q13.2, and common modulation mechanisms could be involved. Accordingly, several studies found that *CYP2A6* (Itoh et al., 2006; Maglich et al., 2003) and *CYP2A7* (Kandel et al., 2016) are induced by CITCO in PHHs. *TSKU* is induced by PB in HepaRG cells and PHHs (Lambert et al., 2009) and is regulated by PXR in osteoblasts (Ichikawa et al., 2006). *TAGLN*, one of the new genes identified as downregulated by CAR in the present study, encodes transgelin that is mainly expressed in stellate cells and fibroblasts (Petkov et al., 2004). We cannot rule out that PHHs may be contaminated by non-parenchymal cells. Retinol Dehydrogenase 16 (*RDH16*) is involved in retinol synthesis. Its expression increases following PPAR α activation in PHHs (Kandel et al., 2016). In silico analysis revealed the presence of putative DR3 and DR4 motifs on its regulatory region, suggesting a possible regulation by CAR (Ebert et al., 2016). Our results demonstrate that in PHHs, only very few genes are strictly CAR-dependent, suggesting that complementary mechanisms can promote

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the expression of xenobiotic-related genes. This could explain in part the difference between humans and mice. Studying CAR transcriptional regulation in rodents, using in vivo and in vitro approaches, could help understanding whether our findings are specific to PHHs, or biased by the 2D culture model. Indeed, gene expression (Lauschke et al., 2016b) and energy metabolism (Fu et al., 2013) patterns rapidly change when human/rodent hepatocytes are seeded in culture. Therefore, although PHHs are the most relevant cell model for human studies, data obtained in vitro using this system should be interpreted with caution. During the last decade, cell culture strategies to mimic in vivo situation have been developed. Specifically hepatocytes have been cultured in 3D spheroids (Bachmann et al., 2015; Bell et al., 2016; Vorrink et al., 2017) in the presence or not of stromal cells (Khetani et al., 2008), and in association or not with medium flux (Lauschke et al., 2016a; Lin et al., 2015; Vinci et al., 2011). These conditions favor the long-term maintenance of cell viability and an in vivo-like hepatic phenotype, as indicated by transcriptomic, proteomic and metabolomic analyses. This is accompanied by stable expression of phase I and phase II enzymes, phase III transporters, xenoreceptors (CAR and PXR) and transcription factors (HNF4), as well as improved cell to cell contacts and bile canalicular structure and activity compared with 2D culture conditions. Therefore, moving from monolayer to 3D cell culture systems can greatly improve nuclear receptor expression and gene-mediated regulation (Vorrink et al., 2017) as well as the in vitro assessment of drug metabolism and toxicity (Lin et al., 2016).

It has been shown that in the liver of EGF2B transgenic mice, the expression of genes predisposing to malignancy is increased, while that of enzymes associated with retinoic acid and xenobiotic metabolism is downregulated (Borlak et al., 2005). Interestingly,

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we obtained similar results in PHHs. KEGG pathway analysis showed that in the presence of EGF, upregulated genes mostly belonged to the cell cycle and carcinogenesis pathways (Komposch et al., 2015). In addition, in the presence of EGF, pathways associated with drug metabolism–cytochrome P450 were downregulated (**Fig. 1C**). These pathways are downregulated also in CAR-KO HepaRG cells [37], suggesting that the EGF-mediated phenotype could be explained, at least in part, by CAR signaling deregulation (Li et al., 2015). This hypothesis is reinforced by the finding that the same KEGG pathways are upregulated in PHHs incubated with CITCO (this study and Kandel et al. (Kandel et al., 2016)). This CITCO-mediated effect was almost completely abrogated by the presence of EGF (**Fig. 5B**).

In our model, we observed that EGF affects similarly *CYP2B6* induction by CITCO and PB in short-term culture (Day 2) (**Fig. 2**). In addition, after 6 days of culture in the presence of EGF, CITCO-mediated *CYP2B6* induction was lost, in contrast to the induction mediated by PB (**Fig. 3**). Our transcriptome analysis clearly revealed the strong EGF effect on CAR signaling when using CITCO as activator. In contrast, PB response was almost not affected by the presence of EGF. This is in agreement with PB non-specific action, but also suggests a secondary compensatory mechanism.

CAR activity regulation involves multiple mechanisms, including the AMPK, insulin, EGFR and MEK-ERK signaling pathways (Yang et al., 2014; Yasujima et al., 2016). Conversely, CAR transcriptional regulation has been poorly studied. The hepatotrophic factor augments of liver regeneration (ALR) reduces P450 enzyme activities in human hepatocytes partially through NF- κ B activation and CAR downregulation (Dayoub et al., 2006). In a mouse model in which mouse HGF is overexpressed, CAR but not PXR mRNA expression is reduced (Kakizaki et al., 2007). It would be important to assess whether other growth factors and/or serum have a similar inhibitory effect in PHHs and

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in HepaRG cells (Aninat et al., 2006). In addition, EGF and serum downregulate CAR, but not PXR and RXR expression in HepG2 cells (Osabe et al., 2009) via the SAPK signaling pathway. This may also occur in PHHs.

HNF-4 α , PGC-1 α and glucocorticoid receptor are considered to be the main regulators of CAR promoter activity (Ding et al., 2006a; Pascussi et al., 2003). Insulin-like growth factor-1 receptor (IGF-1R) inhibitor (Li et al., 2012) and thyroid hormone (Ooe et al., 2009) also modulate CAR expression. In addition, post-transcriptional regulation by miR-34-a (Lamba et al., 2014) and miR-137 (Chen et al., 2014) leads to *NR1I3* downregulation. HNF4 α regulates both CAR transcriptional activity (Gonzalez et al., 2008) and expression (Ding et al., 2006b; Kamiyama et al., 2007). However, HNF4 α gene expression was not affected by EGF in our model. Direct HNF4 α phosphorylation is another possible mechanism that could contribute to EGF effect in hepatocytes (De Boussac et al., 2010; Vetó et al., 2017).

Importantly, we found that in the presence of EGF, CAR does not modulate anymore the expression of its target genes (but for *CYP2B6* and *CYP2A6*, to a lower extent). Using a gene silencing strategy, we could show that PB preferentially induces *CYP2B6* by selective CAR activation, but can switch to PXR activation when CAR is present in limited amount (in the presence of EGF, and when CAR is artificially downregulated). The finding that many genes modulated by PB independently of CAR in the presence of EGF are known PXR target genes (e.g., *CYP2B6*, *CYP3A7*, *CYP2C8*, *CYP3A4*, *EPHX1*, *CYP3A43*, *CYP2C9*, *THRSP*, *ALAS1*, *ABCC2*, *POR*, *CYP3A5* and *ABCB1*) further supports the existence of a PXR-mediated compensatory mechanism that might allow overcoming the lack of response to potential threats. This conclusion is consistent with the enhanced role of human PXR in the absence of CAR competition observed in CAR-KO HepaRG cells (Li et al., 2015), and-also with the asymmetrical

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regulation of *CYP3A4* and *CYP2B6* by CAR or PXR previously reported by Faucette et al. (Faucette et al., 2006). We also observed that induction of drug metabolism enzymes depends on the PXR/CAR ratio. Similarly, a previous report using reporter genes in the mouse, showed a crucial role of the PXR/CAR ratio in *Cyp2b10* modulation (Ding et al., 2005). Considering the inter-individual variability of CAR and PXR expression, this observation could have a critical impact in drug development.

Altogether, our findings bring new insights into EGF effect on CAR activity in PHHs that might be physiologically relevant and highlight the importance of transcriptional regulation in CAR biology. CAR transcriptional regulation may play an underestimated role in drug metabolism and transport, energy homeostasis and cell proliferation. Furthermore, unlike the results described in rodents, our work shows that the indirect CAR activator PB modulates gene expression predominantly in a CAR-independent manner, although EGF significantly affects PHH response to this drug. It would be interesting to evaluate this effect using the most recent microfluidic and 3D spheroid culture systems in which nuclear receptor expression is maintained (Lauschke et al., 2016a; Vorrink et al., 2017). Finally, the switch between CAR and PXR for gene modulation in CAR-depleted cells highlights the cross-talk between these xenosensors in the presence of EGF. This cross-talk provides a mechanism for amplifying the body detoxification response to a broad range of chemicals.

Besides their primary role in drug metabolism, CAR and PXR are also potential pharmacological targets for the treatment of cancers (De Mattia et al., 2016) and metabolic diseases, such as cholestasis, non-alcoholic fatty liver disease and diabetes (Gao et al., 2012; Kakizaki et al., 2007). As PXR and CAR may differentially affect metabolic pathways, it is important to selectively target these receptors. In this context,

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controlling the microenvironment and culture conditions of the hepatic cells used for investigating CAR activity is crucial.

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Authorship Contribution:

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Contributed new reagent and analytic tools: Fabre, Herrero, Ramos, Maurel

Performed data analysis: de Boussac, Gerbal-Chaloin, Daujat-Chavanieu

Wrote or contributed to the writing of the manuscript: de Boussac, Gerbal-Chaloin, Daujat-Chavanieu

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References

Aninat, C., Piton, A., Glaise, D., Le Charpentier, T., Langouet, S., Morel, F., Guguen-Guillouzo, C. and Guillouzo, A. (2006). Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. *Drug Metab Dispos* **34**(1): 75-83.

Bachleda, P., Vrzal, R. and Dvorak, Z. (2009). Activation of MAPKs influences the expression of drug-metabolizing enzymes in primary human hepatocytes. *Gen Physiol Biophys* **28**(3): 316-320.

Bachmann, A., Moll, M., Gottwald, E., Nies, C., Zantl, R., Wagner, H., Burkhardt, B., Sanchez, J. J., Ladurner, R., Thasler, W., Damm, G. and Nussler, A. K. (2015). 3D Cultivation Techniques for Primary Human Hepatocytes. *Microarrays (Basel)* **4**(1): 64-83.

Bell, C. C., Hendriks, D. F., Moro, S. M., Ellis, E., Walsh, J., Renblom, A., Fredriksson Puigvert, L., Dankers, A. C., Jacobs, F., Snoeys, J., Sison-Young, R. L., Jenkins, R. E., Nordling, A., Mkrtchian, S., Park, B. K., Kitteringham, N. R., Goldring, C. E., Lauschke, V. M. and Ingelman-Sundberg, M. (2016). Characterization of primary human hepatocyte spheroids as a model system for drug-induced liver injury, liver function and disease. *Sci Rep* **6**: 25187.

DMD # 78683

Berasain, C. and Avila, M. A. (2014). The EGFR signalling system in the liver: from hepatoprotection to hepatocarcinogenesis. *J Gastroenterol* **49**(1): 9-23.

Borlak, J., Meier, T., Halter, R., Spanel, R. and Spanel-Borowski, K. (2005). Epidermal growth factor-induced hepatocellular carcinoma: gene expression profiles in precursor lesions, early stage and solitary tumours. *Oncogene* **24**(11): 1809-1819.

Chen, S., He, N., Yu, J., Li, L., Hu, Y., Deng, R., Zhong, S. and Shen, L. (2014). Post-transcriptional regulation by miR-137 underlies the low abundance of CAR and low rate of bilirubin clearance in neonatal mice. *Life Sci* **107**(1-2): 8-13.

Dayoub, R., Thasler, W. E., Bosserhoff, A. K., Singer, T., Jauch, K. W., Schlitt, H. J. and Weiss, T. S. (2006). Regulation of polyamine synthesis in human hepatocytes by hepatotrophic factor augments liver regeneration. *Biochem Biophys Res Commun* **345**(1): 181-187.

De Boussac, H., Ratajewski, M., Sachrajda, I., Köblös, G., Tordai, A., Pulaski, L., Buday, L., Váradi, A. and Arányi, T. (2010). The ERK1/2-hepatocyte nuclear factor 4 α axis regulates human ABCC6 gene expression in hepatocytes. *Journal of Biological Chemistry* **285**(30): 22800-22808.

De Mattia, E., Cecchin, E., Roncato, R. and Toffoli, G. (2016). Pregnane X receptor, constitutive androstane receptor and hepatocyte nuclear factors as emerging players in cancer precision medicine. *Pharmacogenomics* **17**(14): 1547-1571.

DMD # 78683

Ding, X., Lichti, K., Kim, I., Gonzalez, F. J. and Staudinger, J. L. (2006a). Regulation of constitutive androstane receptor and its target genes by fasting, cAMP, hepatocyte nuclear factor alpha, and the coactivator peroxisome proliferator-activated receptor gamma coactivator-1alpha. *J Biol Chem* **281**(36): 26540-26551.

Ding, X., Lichti, K., Kim, I., Gonzalez, F. J. and Staudinger, J. L. (2006b). Regulation of constitutive androstane receptor and its target genes by fasting, cAMP, hepatocyte nuclear factor α , and the coactivator peroxisome proliferator-activated receptor γ coactivator-1 α . *Journal of Biological Chemistry* **281**(36): 26540-26551.

Ding, X. and Staudinger, J. L. (2005). The ratio of constitutive androstane receptor to pregnane X receptor determines the activity of guggulsterone against the Cyp2b10 promoter. *Journal of Pharmacology and Experimental Therapeutics* **314**(1): 120-127.

Ebert, B., Kisiela, M. and Maser, E. (2016). Transcriptional regulation of human and murine short-chain dehydrogenase/reductases (SDRs) - an in silico approach. *Drug Metab Rev* **48**(2): 183-217.

Elcombe, C. R., Pepper, R. C., Wolf, D. C., Bailey, J., Bars, R., Bell, D., Cattley, R. C., Ferguson, S. S., Geter, D., Goetz, A., Goodman, J. I., Hester, S., Jacobs, A., Omiecinski, C. J., Schoeny, R., Xie, W. and Lake, B. G. (2014). Mode of action and human relevance analysis for nuclear receptor-mediated liver toxicity: A case study

DMD # 78683

with phenobarbital as a model constitutive androstane receptor (CAR) activator. *Crit Rev Toxicol* **44**(1): 64-82.

Faucette, S. R., Sueyoshi, T., Smith, C. M., Negishi, M., LeCluyse, E. L. and Wang, H. (2006). Differential regulation of hepatic CYP2B6 and CYP3A4 genes by constitutive androstane receptor but not pregnane X receptor. *Journal of Pharmacology and Experimental Therapeutics* **317**(3): 1200-1209.

Fu, D., Mitra, K., Sengupta, P., Jarnik, M., Lippincott-Schwartz, J. and Arias, I. M. (2013). Coordinated elevation of mitochondrial oxidative phosphorylation and autophagy help drive hepatocyte polarization. *Proc Natl Acad Sci U S A* **110**(18): 7288-7293.

Gao, J. and Xie, W. (2012). Targeting xenobiotic receptors PXR and CAR for metabolic diseases. *Trends Pharmacol Sci* **33**(10): 552-558.

Gonzalez, F. J. (2008). Regulation of hepatocyte nuclear factor 4 α -mediated transcription. *Drug metabolism and pharmacokinetics* **23**(1): 2-7.

Goodwin, B., Moore, L. B., Stoltz, C. M., McKee, D. D. and Kliewer, S. A. (2001). Regulation of the human CYP2B6 gene by the nuclear pregnane X receptor. *Mol Pharmacol* **60**(3): 427-431.

DMD # 78683

Handschin, C. and Meyer, U. A. (2003). Induction of drug metabolism: the role of nuclear receptors. *Pharmacol Rev* **55**(4): 649-673.

Ichikawa, T., Horie-Inoue, K., Ikeda, K., Blumberg, B. and Inoue, S. (2006). Steroid and xenobiotic receptor SXR mediates vitamin K2-activated transcription of extracellular matrix-related genes and collagen accumulation in osteoblastic cells. *J Biol Chem* **281**(25): 16927-16934.

Ismail, T., Howl, J., Wheatley, M., McMaster, P., Neuberger, J. M. and Strain, A. J. (1991). Growth of normal human hepatocytes in primary culture: effect of hormones and growth factors on DNA synthesis. *Hepatology* **14**(6): 1076-1082.

Itoh, M., Nakajima, M., Higashi, E., Yoshida, R., Nagata, K., Yamazoe, Y. and Yokoi, T. (2006). Induction of human CYP2A6 is mediated by the pregnane X receptor with peroxisome proliferator-activated receptor-gamma coactivator 1alpha. *J Pharmacol Exp Ther* **319**(2): 693-702.

Kakizaki, S., Yamazaki, Y., Kosone, T., Horiguchi, N., Sohara, N., Sato, K., Takagi, H., Yoshinari, K. and Mori, M. (2007). Gene expression profiles of drug-metabolizing enzymes and transporters with an overexpression of hepatocyte growth factor. *Liver Int* **27**(1): 109-119.

Kamiyama, Y., Matsubara, T., Yoshinari, K., Nagata, K., Kamimura, H. and Yamazoe, Y. (2007). Role of human hepatocyte nuclear factor 4 α in the expression of drug-

DMD # 78683

metabolizing enzymes and transporters in human hepatocytes assessed by use of small interfering RNA. *Drug metabolism and pharmacokinetics* **22**(4): 287-298.

Kandel, B. A., Thomas, M., Winter, S., Damm, G., Seehofer, D., Burk, O., Schwab, M. and Zanger, U. M. (2016). Genomewide comparison of the inducible transcriptomes of nuclear receptors CAR, PXR and PPARalpha in primary human hepatocytes. *Biochim Biophys Acta* **1859**(9): 1218-1227.

Kassambara, A., Reme, T., Jourdan, M., Fest, T., Hose, D., Tarte, K. and Klein, B. (2015). GenomicScape: an easy-to-use web tool for gene expression data analysis. Application to investigate the molecular events in the differentiation of B cells into plasma cells. *PLoS Comput Biol* **11**(1): e1004077.

Katsura, N., Ikai, I., Mitaka, T., Shiotani, T., Yamanokuchi, S., Sugimoto, S., Kanazawa, A., Terajima, H., Mochizuki, Y. and Yamaoka, Y. (2002). Long-term culture of primary human hepatocytes with preservation of proliferative capacity and differentiated functions. *J Surg Res* **106**(1): 115-123.

Kawamura, A., Yoshida, Y., Kimura, N., Oda, H. and Kakinuma, A. (1999). Phosphorylation/dephosphorylation steps are crucial for the induction of CYP2B1 and CYP2B2 gene expression by phenobarbital. *Biochemical and biophysical research communications* **264**(2): 530-536.

DMD # 78683

Khetani, S. R. and Bhatia, S. N. (2008). Microscale culture of human liver cells for drug development. *Nat Biotechnol* **26**(1): 120-126.

Koike, C., Moore, R. and Negishi, M. (2007). Extracellular signal-regulated kinase is an endogenous signal retaining the nuclear constitutive active/androstane receptor (CAR) in the cytoplasm of mouse primary hepatocytes. *Mol Pharmacol* **71**(5): 1217-1221.

Komposch, K. and Sibilio, M. (2015). EGFR Signaling in Liver Diseases. *Int J Mol Sci* **17**(1).

Lamba, V., Ghodke, Y., Guan, W. and Tracy, T. S. (2014). microRNA-34a is associated with expression of key hepatic transcription factors and cytochromes P450. *Biochem Biophys Res Commun* **445**(2): 404-411.

Lambert, C. B., Spire, C., Claude, N. and Guillouzo, A. (2009). Dose- and time-dependent effects of phenobarbital on gene expression profiling in human hepatoma HepaRG cells. *Toxicol Appl Pharmacol* **234**(3): 345-360.

Lauschke, V. M., Hendriks, D. F., Bell, C. C., Andersson, T. B. and Ingelman-Sundberg, M. (2016a). Novel 3D Culture Systems for Studies of Human Liver Function and Assessments of the Hepatotoxicity of Drugs and Drug Candidates. *Chem Res Toxicol* **29**(12): 1936-1955.

DMD # 78683

Lauschke, V. M., Vorrink, S. U., Moro, S. M., Reyazee, F., Nordling, A., Hendriks, D. F., Bell, C. C., Sison-Young, R. L., Park, B. K., Goldring, C. E., Ellis, E., Johansson, I., Mkrtchian, S., Andersson, T. B. and Ingelman-Sundberg, M. (2016b). Massive rearrangements of cellular miRNA signatures are key drivers of hepatocytes dedifferentiation. *Hepatology*.

Lehmann, J. M., McKee, D. D., Watson, M. A., Willson, T. M., Moore, J. T. and Kliewer, S. A. (1998). The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest* **102**(5): 1016-1023.

Li, D., Mackowiak, B., Brayman, T. G., Mitchell, M., Zhang, L., Huang, S. M. and Wang, H. (2015). Genome-wide analysis of human constitutive androstane receptor (CAR) transcriptome in wild-type and CAR-knockout HepaRG cells. *Biochem Pharmacol* **98**(1): 190-202.

Li, L., Sinz, M. W., Zimmermann, K. and Wang, H. (2012). An insulin-like growth factor 1 receptor inhibitor induces CYP3A4 expression through a pregnane X receptor-independent, noncanonical constitutive androstane receptor-related mechanism. *Journal of Pharmacology and Experimental Therapeutics* **340**(3): 688-697.

Lin, C., Ballinger, K. R. and Khetani, S. R. (2015). The application of engineered liver tissues for novel drug discovery. *Expert Opin Drug Discov* **10**(5): 519-540.

DMD # 78683

Lin, C. and Khetani, S. R. (2016). Advances in Engineered Liver Models for Investigating Drug-Induced Liver Injury. *Biomed Res Int* **2016**: 1829148.

Lynch, C., Pan, Y., Li, L., Heyward, S., Moeller, T., Swaan, P. W. and Wang, H. (2014). Activation of the constitutive androstane receptor inhibits gluconeogenesis without affecting lipogenesis or fatty acid synthesis in human hepatocytes. *Toxicology and applied pharmacology* **279**(1): 33-42.

Maglich, J. M., Parks, D. J., Moore, L. B., Collins, J. L., Goodwin, B., Billin, A. N., Stoltz, C. A., Kliewer, S. A., Lambert, M. H., Willson, T. M. and Moore, J. T. (2003). Identification of a novel human constitutive androstane receptor (CAR) agonist and its use in the identification of CAR target genes. *J Biol Chem* **278**(19): 17277-17283.

Maglich, J. M., Stoltz, C. M., Goodwin, B., Hawkins-Brown, D., Moore, J. T. and Kliewer, S. A. (2002). Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. *Molecular pharmacology* **62**(3): 638-646.

Moore, L. B., Parks, D. J., Jones, S. A., Bledsoe, R. K., Consler, T. G., Stimmel, J. B., Goodwin, B., Liddle, C., Blanchard, S. G., Willson, T. M., Collins, J. L. and Kliewer, S. A. (2000). Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J Biol Chem* **275**(20): 15122-15127.

DMD # 78683

Mutoh, S., Osabe, M., Inoue, K., Moore, R., Pedersen, L., Perera, L., Rebollosa, Y., Sueyoshi, T. and Negishi, M. (2009). Dephosphorylation of threonine 38 is required for nuclear translocation and activation of human xenobiotic receptor CAR (NR1H3). *J Biol Chem* **284**(50): 34785-34792.

Mutoh, S., Sobhany, M., Moore, R., Perera, L., Pedersen, L., Sueyoshi, T. and Negishi, M. (2013). Phenobarbital indirectly activates the constitutive active androstane receptor (CAR) by inhibition of epidermal growth factor receptor signaling. *Sci Signal* **6**(274): ra31.

Negishi, M. (2017). Phenobarbital Meets Phosphorylation of Nuclear Receptors. *Drug Metab Dispos* **45**(5): 532-539.

Oladimeji, P., Cui, H., Zhang, C. and Chen, T. (2016). Regulation of PXR and CAR by protein-protein interaction and signaling crosstalk. *Expert Opin Drug Metab Toxicol* **12**(9): 997-1010.

Ooe, H., Kon, J., Oshima, H. and Mitaka, T. (2009). Thyroid hormone is necessary for expression of constitutive androstane receptor in rat hepatocytes. *Drug Metab Dispos* **37**(9): 1963-1969.

Osabe, M., Sugatani, J., Takemura, A., Kurosawa, M., Yamazaki, Y., Ikari, A. and Miwa, M. (2009). Up-regulation of CAR expression through Elk-1 in HepG2 and SW480 cells by serum starvation stress. *FEBS Lett* **583**(5): 885-889.

DMD # 78683

Pascussi, J. M., Busson-Le Coniat, M., Maurel, P. and Vilarem, M. J. (2003). Transcriptional analysis of the orphan nuclear receptor constitutive androstane receptor (NR1I3) gene promoter: identification of a distal glucocorticoid response element. *Mol Endocrinol* **17**(1): 42-55.

Pascussi, J. M., Gerbal-Chaloin, S., Duret, C., Daujat-Chavanieu, M., Vilarem, M. J. and Maurel, P. (2008). The tangle of nuclear receptors that controls xenobiotic metabolism and transport: crosstalk and consequences. *Annu Rev Pharmacol Toxicol* **48**: 1-32.

Petkov, P. M., Zavadil, J., Goetz, D., Chu, T., Carver, R., Rogler, C. E., Bottinger, E. P., Shafritz, D. A. and Dabeva, M. D. (2004). Gene expression pattern in hepatic stem/progenitor cells during rat fetal development using complementary DNA microarrays. *Hepatology* **39**(3): 617-627.

Pichard, L., Raulet, E., Fabre, G., Ferrini, J. B., Ourlin, J. C. and Maurel, P. (2006). Human hepatocyte culture. *Methods Mol Biol* **320**: 283-293.

Shizu, R., Osabe, M., Perera, L., Moore, R., Sueyoshi, T. and Negishi, M. (2017). Phosphorylated Nuclear Receptor CAR Forms a Homodimer To Repress Its Constitutive Activity for Ligand Activation. *Mol Cell Biol* **37**(10).

DMD # 78683

Sueyoshi, T., Kawamoto, T., Zelko, I., Honkakoski, P. and Negishi, M. (1999). The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. *J Biol Chem* **274**(10): 6043-6046.

Treindl, F., Ruprecht, B., Beiter, Y., Schultz, S., Dottinger, A., Staebler, A., Joos, T. O., Kling, S., Poetz, O., Fehm, T., Neubauer, H., Kuster, B. and Templin, M. F. (2016). A bead-based western for high-throughput cellular signal transduction analyses. *Nat Commun* **7**: 12852.

Tzamelis, I., Pissios, P., Schuetz, E. G. and Moore, D. D. (2000). The xenobiotic compound 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene is an agonist ligand for the nuclear receptor CAR. *Mol Cell Biol* **20**(9): 2951-2958.

Ueda, A., Hamadeh, H. K., Webb, H. K., Yamamoto, Y., Sueyoshi, T., Afshari, C. A., Lehmann, J. M. and Negishi, M. (2002). Diverse roles of the nuclear orphan receptor CAR in regulating hepatic genes in response to phenobarbital. *Mol Pharmacol* **61**(1): 1-6.

Vető, B., Bojcsuk, D., Bacquet, C., Kiss, J., Sipeki, S., Martin, L., Buday, L., Bálint, B. L. and Arányi, T. (2017). The transcriptional activity of hepatocyte nuclear factor 4 alpha is inhibited via phosphorylation by ERK1/2. *PloS one* **12**(2): e0172020.

Vinci, B., Duret, C., Klieber, S., Gerbal-Chaloin, S., Sa-Cunha, A., Laporte, S., Suc, B., Maurel, P., Ahluwalia, A. and Daujat-Chavanieu, M. (2011). Modular bioreactor for

DMD # 78683

primary human hepatocyte culture: medium flow stimulates expression and activity of detoxification genes. *Biotechnol J* **6**(5): 554-564.

Vorrink, S. U., Ullah, S., Schmidt, S., Nandania, J., Velagapudi, V., Beck, O., Ingelman-Sundberg, M. and Lauschke, V. M. (2017). Endogenous and xenobiotic metabolic stability of primary human hepatocytes in long-term 3D spheroid cultures revealed by a combination of targeted and untargeted metabolomics. *FASEB J* **31**(6): 2696-2708.

Wada, T., Gao, J. and Xie, W. (2009). PXR and CAR in energy metabolism. *Trends Endocrinol Metab* **20**(6): 273-279.

Wagner, M., Halilbasic, E., Marschall, H. U., Zollner, G., Fickert, P., Langner, C., Zatloukal, K., Denk, H. and Trauner, M. (2005). CAR and PXR agonists stimulate hepatic bile acid and bilirubin detoxification and elimination pathways in mice. *Hepatology* **42**(2): 420-430.

Wang, Y. M., Ong, S. S., Chai, S. C. and Chen, T. (2012). Role of CAR and PXR in xenobiotic sensing and metabolism. *Expert Opin Drug Metab Toxicol* **8**(7): 803-817.

Xie, W., Barwick, J. L., Simon, C. M., Pierce, A. M., Safe, S., Blumberg, B., Guzelian, P. S. and Evans, R. M. (2000). Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR. *Genes Dev* **14**(23): 3014-3023.

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Yang, H. and Wang, H. (2014). Signaling control of the constitutive androstane receptor (CAR). *Protein Cell* **5**(2): 113-123.

Yasujima, T., Saito, K., Moore, R. and Negishi, M. (2016). Phenobarbital and Insulin Reciprocate Activation of the Nuclear Receptor Constitutive Androstane Receptor through the Insulin Receptor. *J Pharmacol Exp Ther* **357**(2): 367-374.

Zhou, J., Febbraio, M., Wada, T., Zhai, Y., Kuruba, R., He, J., Lee, J. H., Khadem, S., Ren, S., Li, S., Silverstein, R. L. and Xie, W. (2008). Hepatic fatty acid transporter Cd36 is a common target of LXR, PXR, and PPARgamma in promoting steatosis. *Gastroenterology* **134**(2): 556-567.

Zhou, J., Zhai, Y., Mu, Y., Gong, H., Uppal, H., Toma, D., Ren, S., Evans, R. M. and Xie, W. (2006). A novel pregnane X receptor-mediated and sterol regulatory element-binding protein-independent lipogenic pathway. *J Biol Chem* **281**(21): 15013-15020.

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Footnotes

SGC and MDC are co-authors.

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Legends:

Fig. 1. Differential gene expression in PHHs following incubation with EGF. (A) DIGIWest analysis of protein expression in PHHs (Liv10, Table 1) following incubation or not with 10ng/ml EGF for 5 days. (B) Number of upregulated genes and enriched pathways in EGF-treated PHHs ($FDR=4.7 \times 10^{-18}$ to 1.9×10^{-5}). (C) Number of downregulated genes and enriched pathways in EGF-treated PHHs ($FDR=8.8 \times 10^{-10}$ to 0.008). (D) Heat map showing the genes differentially expressed in PHHs (n=5) following incubation with EGF.

Fig. 2. EGF influences specifically the expression of CAR and its target genes in PHHs. PHHs from two donors (Liv1 and Liv2, Table 1) were incubated with 1 μ M CITCO, 0.5mM PB or DMSO (UT) for 24h in the absence or presence of 10ng/ml EGF. EGF was added before (D0) or together (D2) with the inducers. The relative expression of *CAR* (A), *CYP2B6* (B) and *CYP3A4* (C) mRNA was measured by qPCR and normalized to *RPLP0*. Results are expressed relative to control (UT) in medium without EGF; * $p < 0.05$ and ** $p < 0.01$, relative to control (no EGF) in the same treatment group; # $p < 0.05$ and ## $p < 0.01$, relative to control (UT).

Fig. 3. CAR depletion inhibits *CYP2B6* induction by PB and CITCO only in the absence of EGF. PHHs from five donors (Liv3-7) were transfected with control (siCT) or CAR-targeting siRNAs (siCAR). Then, they were incubated with 0.5mM PB, 1 μ M CITCO or DMSO (UT) for 24h, with or without 10ng/ml EGF (from D0). The relative expression of *CAR* (A) and *CYP2B6* (B) mRNA was measured by qPCR and normalized to *RPLP0*. Results are expressed relative to UT in siCT-transfected cells in the absence

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of EGF. (C) CYP2B6 protein level was assessed by western blotting (representative experiment using PHHs from Liv12). GAPDH protein expression was used as loading control. Results are expressed relative to control (UT) in medium without EGF; *** $p < 0.005$, relative to control (siCT) in the same treatment group; ### $p < 0.005$, relative to control (UT).

Fig. 4. EGF differentially affects the response to CITCO and PB. Venn diagrams showing the overlap of genes that are differentially expressed in PHHs from five donors (Liv3-7) upon incubation with 1 μ M CITCO (A), or 0.5mM PB (B) in the absence or presence of 10 ng/ml EGF (from D0). The number of up- and down-regulated genes in each experimental condition is indicated in red and green, respectively.

Fig. 5. KEGG pathway enrichment, relative to untreated cells, of siCT-transfected PHHs from five donors (Liv3-7) incubated with 0.5mM PB (A) or 1 μ M CITCO (B) for 24h in the presence or absence of 10 ng/ml EGF (from D0).

Fig. 6. PHHs from two donors (Liv6 and Liv7, Table 1) were transfected with control (siCT) or CAR-targeting siRNAs (siCAR). They were then incubated with 0.5mM PB, 1 μ M CITCO, or DMSO (UT) for 24h, in the presence or not of 10 ng/ml EGF (from D0). The relative expression of *CYP2A6* (A), *CYP2A7* (B), *CYP3A4* (C), *CYP3A43* (D), *CYP3A7* (E), *CYP2C8* (F), *CYP2C9* (G), *TSKU* (H), *EPHX1* (I), *POR* (J), *ALAS1* (K), *TAGLN* (L), *STEAP2* (M) and *CEBP3* (N) mRNA was measured by qPCR and normalized to *RPLP0*. Results are expressed relative to not treated siCT-transfected PHHs (UT) in medium without EGF. * $p < 0.05$ and ** $p < 0.01$, relative to control in the same treatment group; ## $p < 0.01$ and ### $p < 0.005$, relative to control (UT).

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Fig. 7. PHHs from five donors (Liv3-7, Table 1) were transfected with control (siCT), CAR-targeting (siCAR) or PXR-targeting (siPXR) siRNAs. Then, they were incubated with 0.5mM PB, 1 μ M CITCO or DMSO (UT) for 24h, in the presence or not of 10 ng/ml EGF (from D0). The relative expression of *PXR* (A), *CAR* (B), *CYP2B6* (C) or *CYP3A4* (D) mRNA was measured by qPCR and normalized to *RPLP0*. Results are expressed relative to not treated siCT-transfected PHHs (UT) in medium without EGF.

PHHs from Liv16 were transfected with siCT, siCAR and/or siPXR siRNAs. Then, they were incubated with 0.5mM PB or DMSO for 24h, in the presence of 10 ng/ml EGF (from D0). The relative expression of *CAR and PXR* (E), *CYP2B6*, *CYP3A4*, *TSKU*, *CYP2C8*, *CYP2A6*, *CYP2A7*, *CYP3A43* (F) mRNA was measured by qPCR and normalized to *RPLP0* expression level. Results are expressed relative to not treated siCT-transfected PHHs in medium with EGF (white bars). $p^* < 0.05$; $p^{**} < 0.01$; $p^{***} < 0.001$ relative to control in the same treatment group.

Fig. 8. PHHs from eight donors (Liv3-7 and 13-15, Table 1) were incubated with 0.5mM PB, 1 μ M CITCO or DMSO (UT) for 24h, in the presence or not of 10 ng/ml EGF (from D0). The relative expression of *CYP2B6* was measured by qPCR and normalized to *RPLP0* in the absence (A), or presence (C) of EGF. *PXR/CAR* mRNA expression ratio in the absence (B), or presence (D) of EGF (expressed as LOG10). PHHs from two donors (Liv13 and Liv15, Table 1) (E and F) were transfected with control (siCT), CAR-targeting (siCAR) or PXR-targeting (siPXR) siRNAs. They were then incubated with 0.5mM PB, 1 μ M CITCO or DMSO (UT) for 24h, in the presence or not of 10 ng/ml EGF (from D0). The relative expression of *CYP2B6* was measured by qPCR and normalized to *RPLP0* in the absence or presence of EGF. Results are expressed relative to not

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treated siCT-transfected PHHs (UT) in medium without EGF. $p^* < 0.05$; $p^{**} < 0.01$;
 $p^{***} < 0.001$.

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Table 1: Liver donors' data

Liver	Origin	Age (years)	Sex	Pathology
Liv1	Donor	41	M	Anoxia
Liv2	Biopredic Intl	78	F	Hepatocellular carcinoma
Liv3	Resection	66	F	Metastasis from colon cancer
Liv4	Donor	69	F	Renal cyst
Liv5	Donor	53	M	Stroke
Liv6	Resection	76	M	Metastasis from colon cancer
Liv7	Resection	45	F	Polycystic liver disease
Liv10	Biopredic Intl	72	M	Hepatocellular carcinoma
Liv12	Resection	78	F	Cholangiocarcinoma
Liv13	Donor	59	M	Meningeal hemorrhage
Liv14	Donor	60	M	Stroke
Liv15	Donor	55	M	Stroke
Liv16	Resection	67	F	Metastasis from colon cancer

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Table 2: Primer sequences.

Gene	Forward Primer	Reverse Primer
ALAS1	GATGTCAGCCACCTCAGAGAAC	CATCCACGAAGGTGATTGCTCC
CAR	TGCTGCCTCTGGTCACACAC	CCCGCAGAGGAAGTTTTGTG
CPEB3	GGTGTGTTGTTGGAGGACTTCCTC	CCTCTTGGAACAGCAGAAAGGC
CYP2A6	GTCAATCTCCTCATGGACCTTGG	CCTGGTGATGACCACGTTGAAC
CYP2A7	CGCTATGGCTTCTTGCTGCTCA	CTCCATGTAGGGCATCTTGCTC
CYP2B6	ATGGGGCACTGAAAAAGACTGA	AGAGGCGGGGACACTGAATGAC
CYP2C8	GAGACAACAAGCACCACTCTGAG	CAGTGTAAGGCATGTGGCTCCT
CYP2C9	TCCTATCATTGATTACTTCCCG	AACTGCAGTGTTTTCCAAGC
CYP3A4	GCCTGGTGCTCCTCTATCTA	GGTGTTGACCATCATAAAG
CYP3A43	CTGCCTATGACACAACCTAGCACC	TACCAGGGCATCGTAGGTGACA
CYP3A7	AAGTCTGGGGTATTTATGACT	CGCTGGTGAATGTTGGAGAC
EPHX1	GTTTTCCACCTGGACCAATACGG	TGGTGCCTGTTGTCCAGTAGAG
POR	ACTCTGCTCTCGTCAACCAGCT	TGGGTGCTTCTTGTTGGACTCC
PXR	GGACCAGCTGCAGGAGCAAT	CATGAGGGGCGTAGCAAAGG
RDH16	TATGGCGTGGAAGCCTTCTCTG	GGTCCCAAATCTCCAGGAAGCT
RPLP0	TCGACAATGGCAGCATCTAC	GCCTTGACCTTTTCAGCAAG
STEAP2	CCTCTGCTTACCGATGAGAAGG	CAGGAGGGAAAGTAAGCCAAGG
TAGLN	TCCAGGTCTGGCTGAAGAATGG	CTGCTCCATCTGCTTGAAGACC
TSKU	AGTCGCTTGACCTCAGCCACAA	TCGTGAAGGCAGACACTGAGAC

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Table 3: Enriched biological processes after incubation of PHHs with CITCO

GO number	Biological Process	Count	p value	Genes
Deregulated by CITCO in the absence of EGF only				
GO.0042738	Exogenous drug catabolic process	6	1.09e-05	CYP1A2, CYP2A7, CYP2C19, CYP2C8, CYP2C9, CYP3A4
GO.0016098	Monoterpenoid metabolic process	4	4.66e-05	CYP1A2, CYP2C19, CYP2C9, CYP3A4
GO.0019373	Epoxygenase P450 pathway	5	0.000103	CYP1A2, CYP2A7, CYP2C19, CYP2C8, CYP2C9
GO.0042221	Response to chemical	41	0.000103	ADCY2, AKR1B1, ANXA3, CA3, CPEB3, CREB3L3, CTGF, CXCL5, CYP1A2, CYP2A7, CYP2C19, CYP2C8, CYP2C9, CYP3A4, CYP3A43, CYP3A7, EPHX1, ETV1, F2R, GSTA1, IL18, ITGA2, KCNA5, KRT19, MATN2, MICALL1, MRC1, MYL9, OR1C1, OR51B4, PANX1, PF4V1, POR, SERPINE1, SLC23A1, SOCS2, SOX2, TGFB2, TMEM67, TNFSF11, WNT6
GO.0050896	Response to stimulus	58	0.000166	ADCY2, AK7, AMOTL1, ANO1, BOC, CA3, CPE, CPEB3, CRIM1, CXCL5, CYP1A2, CYP2A7, CYP2C19, CYP2C8, CYP2C9, CYP3A4, CYP3A43, CYP3A7, DKK3, DST, EPHX1, ETV1, F2R, GSTA1, HRH4, IL18, ITGA2, KCNA5, KRT19, LRRN4, MAP4K5, MATN2, MCTP2, MICALL1, MRC1, MYL9, NABP1, OR1C1, OR51B4, PF4V1, PLK2, PNMA1, POR, RASEF, RRAD, RRM1, SERPINE1, SLC23A1, SOCS2, STK17A, SUS4, TACSTD2, TGFB2, TMEM67, TNC, TNFSF11, VSIG4, WNT6
GO.0097267	Omega hydroxylase P450 pathway	4	0.000166	CYP1A2, CYP2C19, CYP2C8, CYP2C9
GO.0006805	Xenobiotic metabolic process	9	0.000299	CYP1A2, CYP2A7, CYP2C19, CYP2C8, CYP2C9, CYP3A4, CYP3A43, CYP3A7, GSTA1
GO.0071466	Cellular response to xenobiotic stimulus	9	0.00033	CYP1A2, CYP2A7, CYP2C19, CYP2C8, CYP2C9, CYP3A4, CYP3A43, CYP3A7, GSTA1
GO.0070989	Oxidative demethylation	4	0.00102	CYP1A2, CYP2C8, CYP2C9, CYP3A4
GO.1901700	Response to oxygen-containing compound	20	0.00389	ADCY2, AKR1B1, CA3, CPEB3, CTGF, CXCL5, CYP1A2, F2R, IGFBP1, IL18, KCNA5, MRC1, PANX1, PAX2, PF4V1, POR, SERPINE1, SOCS2, TGFB2, WNT6
Deregulated by CITCO in the presence of EGF only				
GO.0006397	mRNA processing	9	0.00486	DHX9, HNRNPC, MBNL2, RBM22, RBM27, SON, SRRM2, SYNCRIP, THRAP3
GO.0048255	mRNA stabilization	4	0.00486	DHX9, HNRNPC, SYNCRIP, THRAP3
GO.0016071	mRNA metabolic process	10	0.00533	DHX9, EIF4G1, HNRNPC, MBNL2, RBM22, RBM27, SON, SRRM2, SYNCRIP, THRAP3
GO.0008380	RNA splicing	8	0.0061	DHX9, HNRNPC, MBNL2, RBM22, SON, SRRM2, SYNCRIP, THRAP3

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Table 4: Enriched biological processes following incubation of PHHs with PB

GO number	Biological Process	Count	p value	Genes
Deregulated by PB only in medium without EGF				
GO.0008202	Steroid metabolic process	10	0.00081	ABCG1, AKR1B1, AKR1B10, CYP1A2, DHCR24, HMGCS1, HSD17B2, NSDHL, SLC27A2, TM7SF2
GO.0033993	Response to lipid	17	0.00081	ABCG1, ANKRD1, ANXA3, BAX, CDO1, CTGF, CXCL5, CYP1A2, HMGCS1, HSD17B2, INHBA, KLF4, KRAS, KRT19, MRC1, PELI1, SOCS3
GO.0006694	Steroid biosynthetic process	7	0.00273	AKR1B1, DHCR24, HMGCS1, HSD17B2, NSDHL, SLC27A2, TM7SF2
GO.0006629	Lipid metabolic process	18	0.0028	ABCG1, AKR1B1, AKR1B10, ANKRD1, BAX, CAV1, CTGF, CYP1A2, CYP2A7, DHCR24, ELOVL6, FADS1, HSD17B2, ME1, NSDHL, PNPLA3, RDH16, TM7SF2
GO.0044281	Small molecule metabolic process	26	0.0028	ABCG1, AKR1B10, ANKRD1, CAV1, CTGF, CTPS1, CYP1A2, CYP2A7, DHCR24, ELOVL6, ENTPD5, FADS1, GCAT, GLYAT, GSTA1, GSTA3, HMGCS1, HOGA1, MAT1A, ME1, NSDHL, PNPLA3, SLC23A1, SLC25A15, TM7SF2, UGT1A6
GO.0009725	Response to hormone	16	0.00289	ABCG1, AKR1B1, ANXA3, BAX, CAV1, CDO1, CTGF, CYP1A2, DHCR24, HMGCS1, IGFBP1, INHBA, KRT19, ME1, STEAP2, UGT1A6
GO.0042221	Response to chemical	35	0.00529	ABCG1, AKR1B1, ANKRD1, ANXA3, CAV1, CCL16, CDO1, CPEB3, CTGF, CTPS1, CUX2, CXCL5, CYP1A2, CYP2A7, DHCR24, FSTL3, GLYAT, GSTA1, GSTA3, HMGCS1, HSD17B2, KLF4, KRT19, MAT1A, MATN2, ME1, MRC1, MT1M, MYL9, NTN4, PELI1, SLC23A1, SLC47A1, STEAP2, UGT1A6
Deregulated by PB only in medium with EGF				
GO.0048255	mRNA stabilization	4	0.00728	DHX9, HNRNPC, SYNCRIP, THRAP3
Deregulated by PB in the presence and absence of EGF				
GO.0006805	Xenobiotic metabolic process	13	1.5e-13	CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP3A4, CYP3A43, CYP3A5, CYP3A7, CYP4A11, SULT2A1, UGT1A1, UGT1A8
GO.0071466	Cellular response to xenobiotic stimulus	13	1.5e-13	CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP3A4, CYP3A43, CYP3A5, CYP3A7, CYP4A11, SULT2A1, UGT1A1, UGT1A8
GO.0017144	Drug metabolic process	9	9.51e-13	CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP3A4, CYP3A5, UGT1A1, UGT1A8
GO.0042737	Drug catabolic process	7	8.5e-11	CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP3A4, CYP3A5
GO.0019373	Epoxygenase P450 pathway	6	4.24e-09	CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP4A11
GO.0042738	Exogenous drug catabolic process	6	7.24e-09	CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP3A4
GO.0032787	Monocarboxylic acid metabolic process	13	9.67e-09	AKR1D1, CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP4A11, FASN, PDK4, POR, SULT2A1, UGT1A1, UGT1A8
GO.0044281	Small molecule metabolic process	22	1.24e-08	ABCB1, AKR1D1, ALAS1, CYB5A, CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP3A4, CYP3A43, CYP3A5, CYP3A7, CYP4A11, DIO1, FASN, PDK4, POR, PRODH2, SULT2A1, UGT1A1, UGT1A8
GO.0019752	Carboxylic acid metabolic process	15	2.28e-08	AKR1D1, CYB5A, CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP4A11, FASN, PDK4, POR, PRODH2, SULT2A1, UGT1A1, UGT1A8
GO.0008202	Steroid metabolic process	10	4.36e-08	AKR1D1, CYP2A6, CYP2B6, CYP2C19, CYP2C9, CYP3A4, CYP3A5, SULT2A1, UGT1A1, UGT1A8

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Table 5: List of genes differentially regulated by CITCO and PB in a CAR-dependent manner.

	siCT								siCAR							
	- EGF				+EGF				- EGF				+EGF			
	CITCO		PB		CITCO		PB		CITCO		PB		CITCO		PB	
	FC	q-value	FC	q-value	FC	q-value	FC	q-value	FC *	q-value	FC **	q-value	FC *	q-value	FC **	q-value
TAGLN	0.6	0.0	0.6	0.0	1.4	7.5	1.1	ns	1.4	0.0		ns		ns		ns
RDH16	1.3	0.0	1.4	0.0	0.9	ns	1.1	ns	0.7	0.0		ns		ns		ns
ALAS1	1.4	0.0	3.3	0.0	1.1	ns	2.3	0.0	0.7	0.0		ns		ns		ns
POR	1.5	0.0	2.6	0.0	1.1	ns	2.4	0.0	0.7	0.0		ns		ns		ns
CYP2C9	1.5	0.0	1.8	0.0	1.1	ns	1.8	0.0	0.7	0.0		ns	0.7	5.3		ns
CPEB3	1.6	0.0	2.7	0.0	1.0	ns	1.3	ns	0.7	0.0		ns		ns		ns
CYP2C8	1.7	0.0	2.3	0.0	1.1	ns	3.7	0.0	0.7	0.0		ns		ns		ns
CYP3A43	1.9	0.0	5.4	0.0	1.0	ns	2.3	0.7	0.6	0.0		ns		ns		ns
EPHX1	2.1	0.0	2.3	0.0	1.3	ns	1.7	0.4	0.6	0.0		ns		ns		ns
TSKU	2.4	0.0	3.8	0.0	1.2	ns	3.1	0.0	0.5	0.0		ns		ns		ns
CYP3A7	2.4	0.0	6.0	0.0	0.9	ns	11.4	1.0	0.5	0.0		ns		ns		ns
CYP2A7	3.5	0.0	3.6	0.0	1.1	ns	1.3	ns	0.5	0.0	0.6	0.0		ns		ns
CYP3A4	3.8	0.0	14.4	0.0	1.1	ns	23.8	0.0	0.4	0.0		ns		ns		ns
CYP2B6	10.5	0.0	10.7	0.0	2.1	1.3	5.6	0.0	0.3	0.0	0.5	0.0	0.7	5.3		ns
CYP2A6	14.8	0.0	15.4	0.0	1.4	0.7	1.9	0.0	0.2	0.0	0.3	0.0	0.7	5.3		ns
STEAP2	0.8	ns	0.6	0.0	0.9	ns	0.8	ns		ns	1.4	2.4		ns		ns

FC: Fold change (siCT-CITCO/siCT-DMSO or siCT-PB/siCT-DMSO; *siCAR-CITCO/siCT-CITCO; **siCAR-PB/siCT-PB); ns: not significant; siCT: control siRNA; siCAR, anti-CAR siRNA.

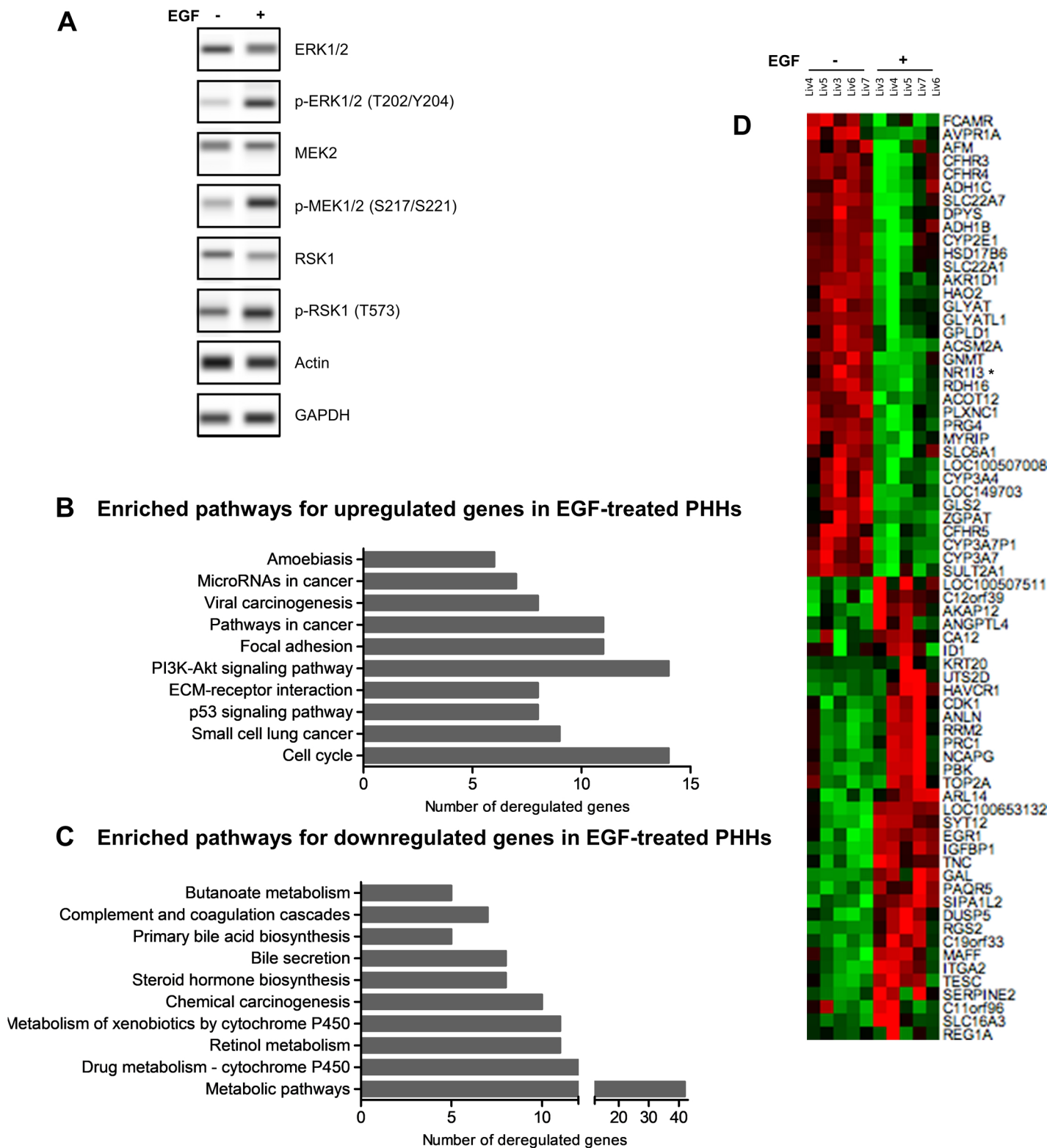


Figure 1

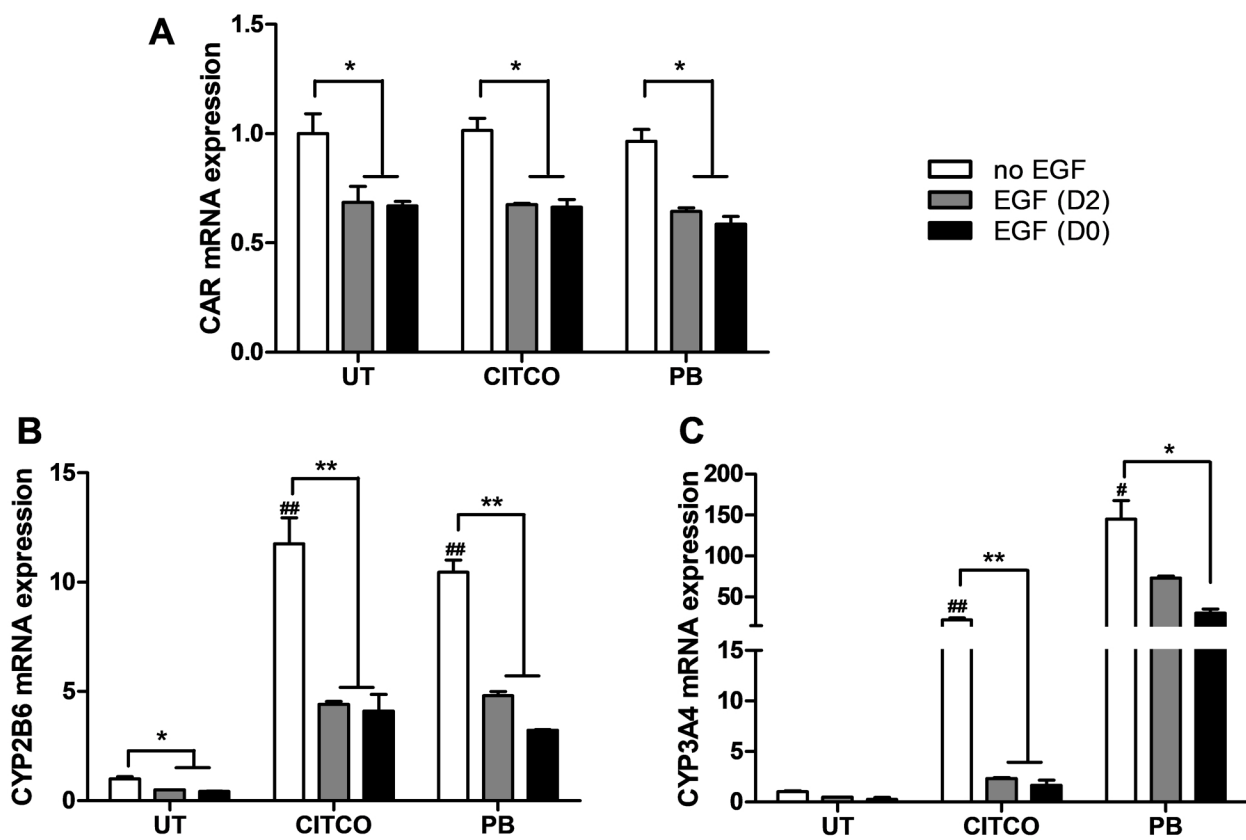


Figure 2

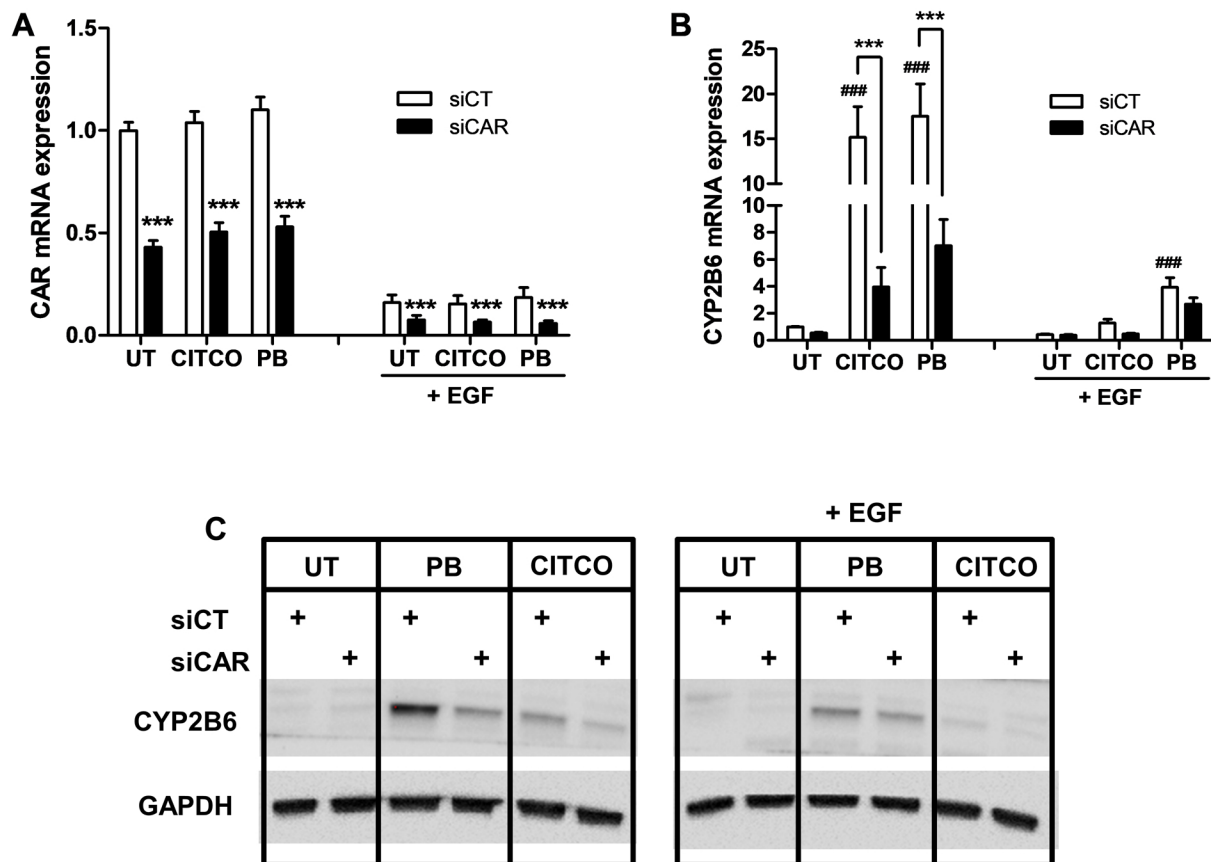


Figure 3

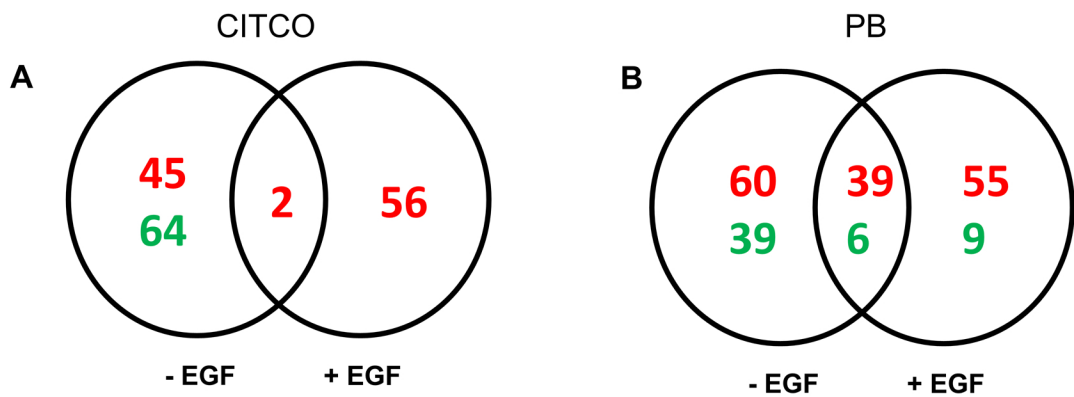


Figure 4

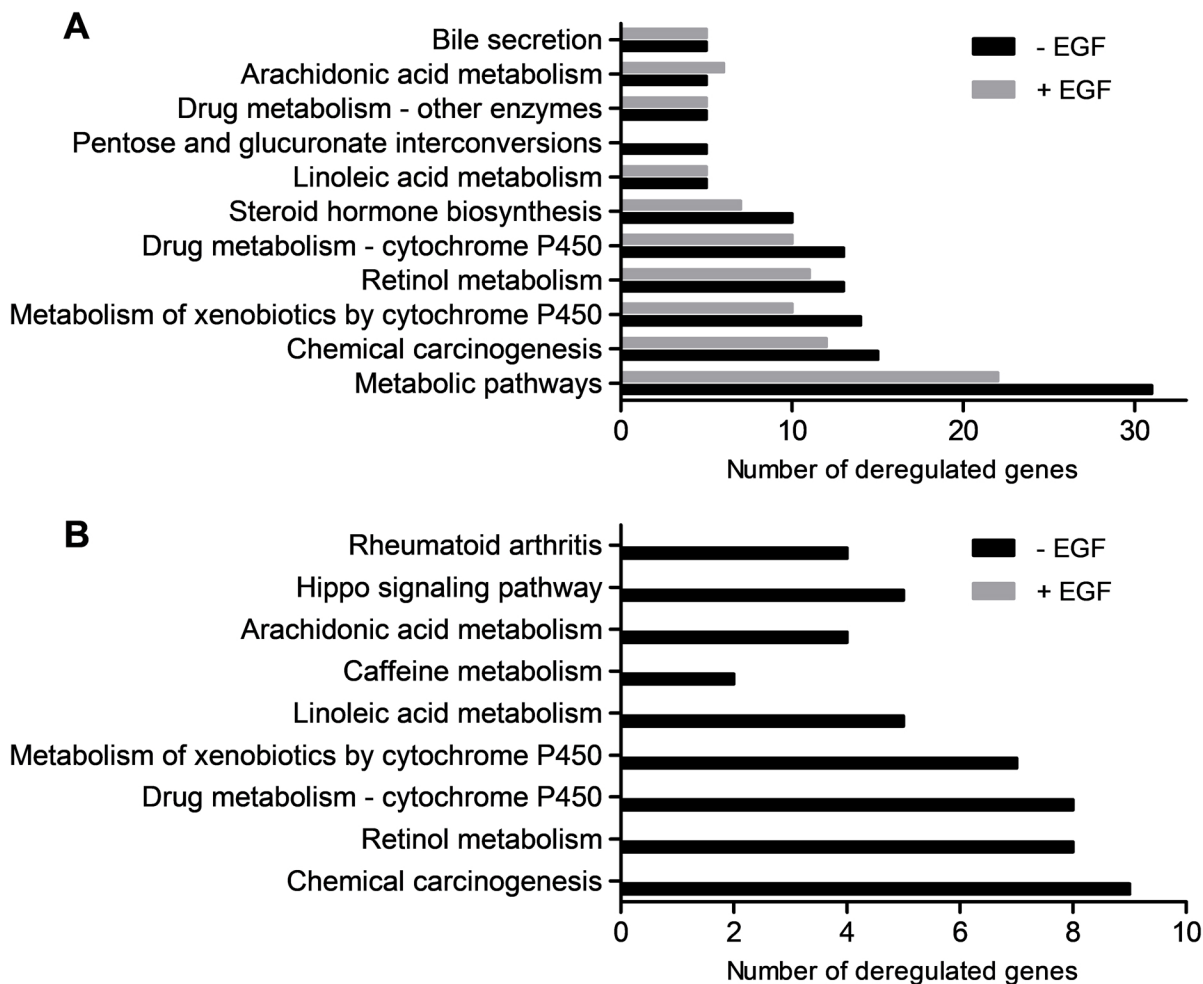


Figure 5

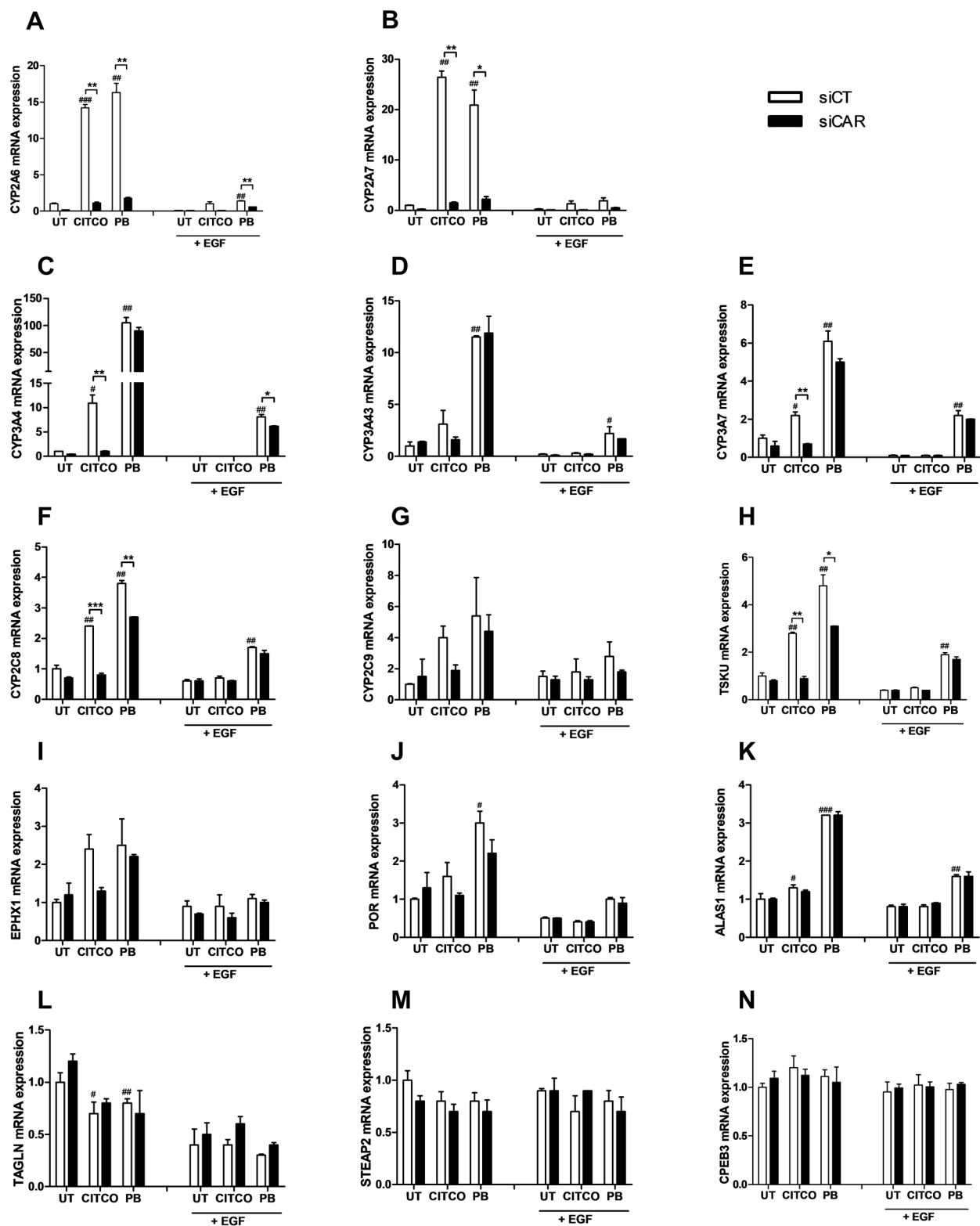


Figure 6

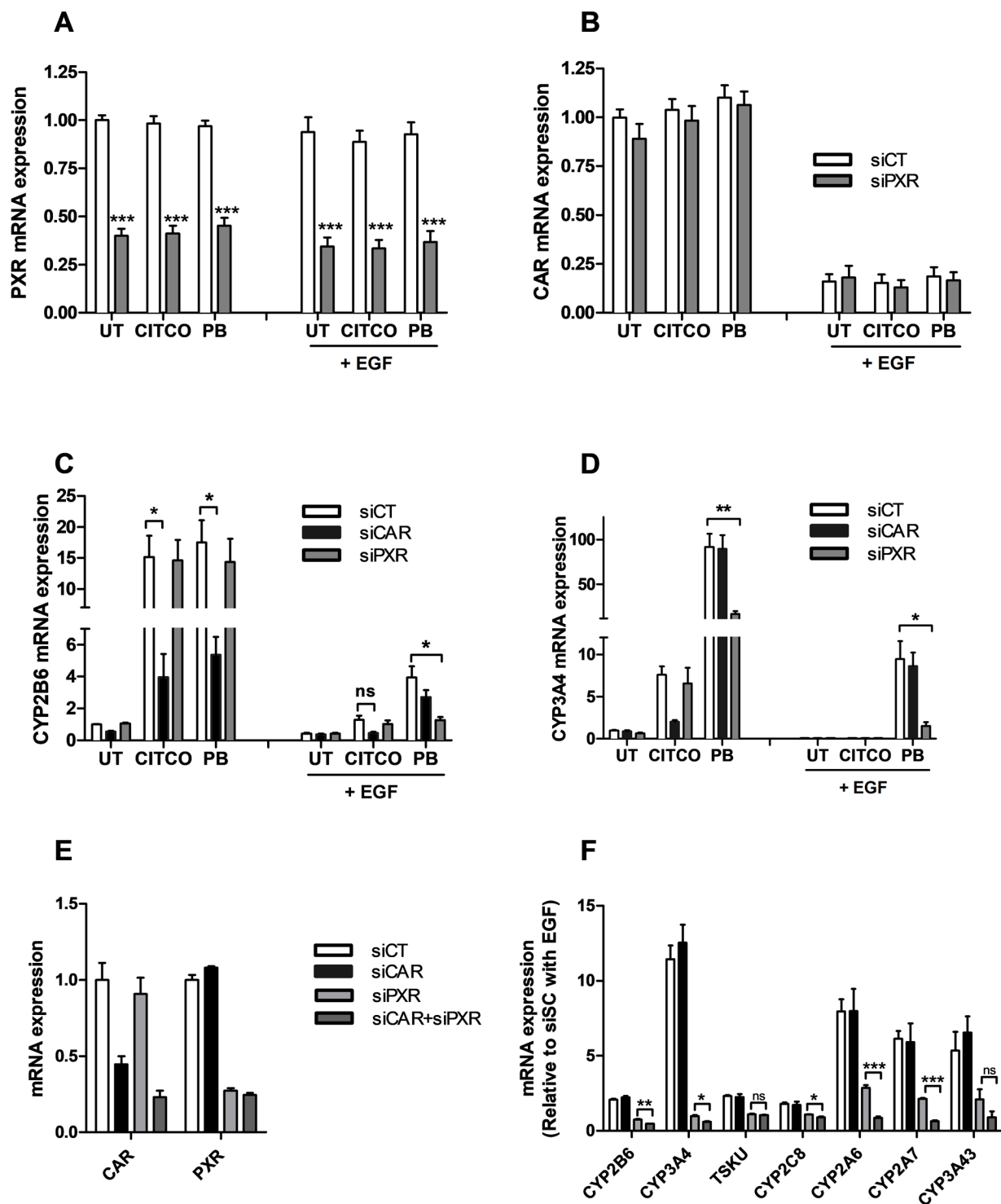


Figure 7

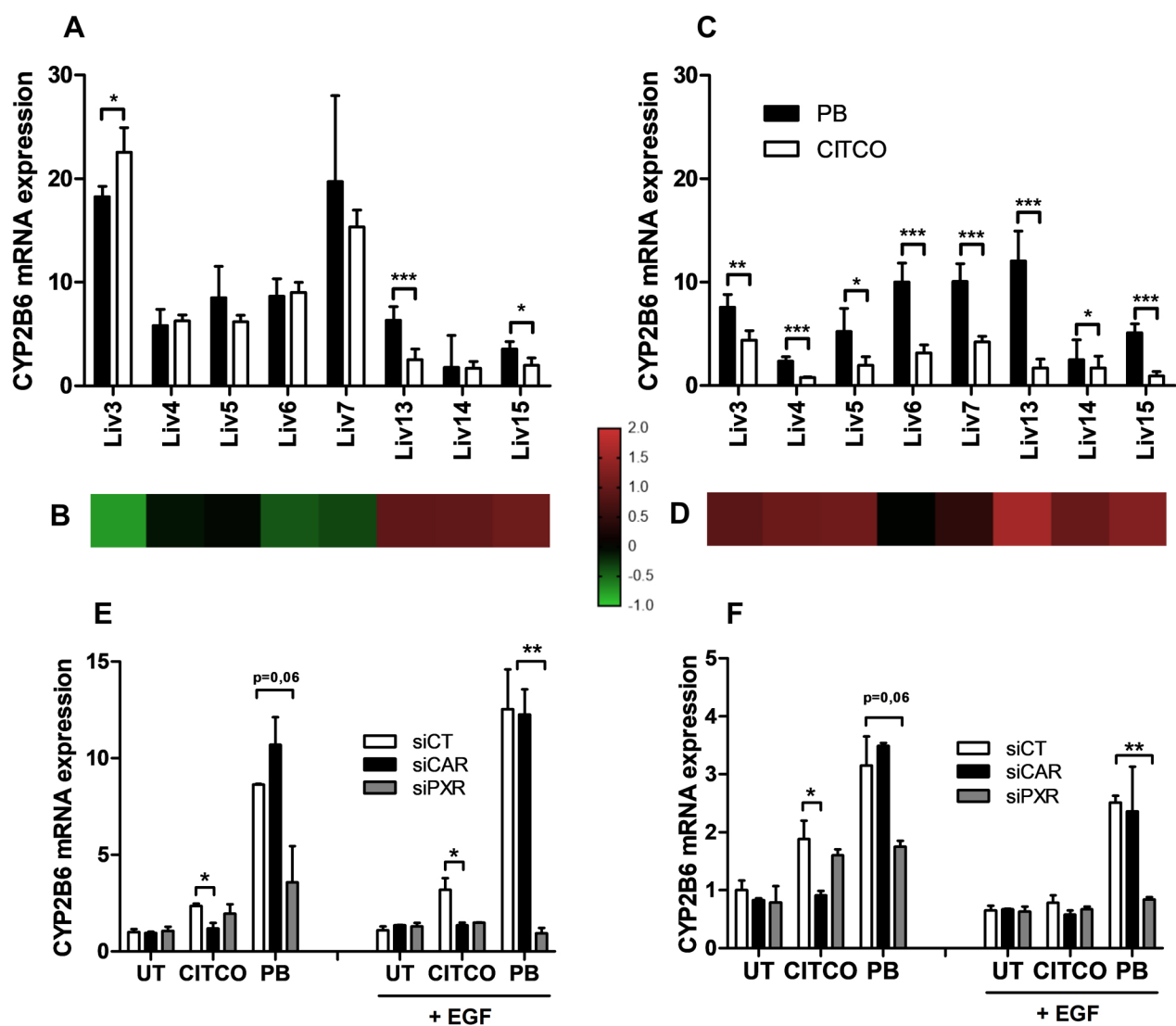


Figure 8