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Human *CYP2C8* is a Target of Peroxisome Proliferator-activated Receptor α in Human Liver

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Running Title: CYP2C8 is a Novel Target of PPARα in Human Liver

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Abbreviations: CYP, cytochrome P450; PPAR, Peroxisome proliferator-activated receptor; PPRE, PPAR response element; BF, bezafibrate; CAR, constitutive androstane receptor; PXR, pregnane X receptor; ChIP, chromatin immunopreciptation assay; EMSA; electrophoretic mobility shift assay; ACOX, acyl-CoA oxidase 1; CPT1A,Carnithine Palmitoyl transferase 1A; PXR, pregnane X receptor; qPCR, Quantitative real time PCR; PANK1, pantothenate kinase 1; miRNA, microRNA; HMGCR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; HNF4α, hepatocyte nuclear factor 4α; RXR, retinoid X receptor; WY14,643, 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio acetic acid.

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ABSTRACT

Human CYP2C enzymes metabolize ~30% of clinically prescribed drugs and various environmental chemicals. CYP2C8, an important member of this subfamily, metabolizes the anticancer drug paclitaxel, certain antidiabetic drugs, and endogenous substrates including arachidonic acid to physiologically active epoxyeicosatrienoic acids (EETs). Previous studies from our laboratory showed that microRNA 107 (miR107) and microRNA 103 down-regulate CYP2C8 post-transcriptionally. miR107 is located in intron 5 of the pantothenate kinase 1 (PANK1) gene. p53 has been reported to coregulate the induction of PANK1 and miR107. Here, we examine the possible down regulation of CYP2C8 by drugs capable of inducing miR107. Hypolipidemic drugs such as bezafibrate, known activators of the peroxisome proliferator-activated receptor α (PPARα), induce both the *PANK1* gene and miR107 (~2.5 fold) in primary human hepatocytes. Surprisingly, CYP2C8 mRNA and protein levels were induced by bezafibrate. *CYP2C8* promoter activity was increased by ectopic expression of PPARα in HepG2 cells with a further increase after bezafibrate (~ 18-fold), WY14643 treatment (~ 10-fold), or the antidiabetic drug rosiglitazone, all known PPAR activators. Promoter sequence analyses, deletion studies, mutagenesis studies and electrophoretic mobility shift assays (EMSAs) identify a PPARα response element (PPRE) located at position -2109 bp relative to the translation start site of CYP2C8. ChIP analysis confirmed recruitment of PPARα to this PPRE after bezafibrate treatment of human hepatocytes. Thus, we show for the first time that *CYP2C8* is transcriptionally regulated by PPARα suggesting the potential for drug-drug interactions due to up-regulation of CYP2C8 by PPAR activators.

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Introduction

CYP2C8 is the second most abundant CYP2C enzyme in human liver after CYP2C9 (Lai et al., 2009). It metabolizes the antidiabetic drugs rosiglitazone and troglitazone, the anticancer drug paclitaxel, the cholesterol-lowering drug cerivastatin, the antiarrhythmic drug amiodarone, the calcium channel blocker verapamil and the antimalarials amodiaquine and chloroquine (Totah and Rettie, 2005). CYP2C8 also metabolizes the endogenous molecule arachidonic acid to 11,12- and 14,15-EETs (Fisslthaler et al., 1999). It is highly expressed in human liver but also expressed in extrahepatic tissues such as the kidney, lung, nasal mucosa, arteries, endothelial mucosa and heart (Delozier et al., 2007; Ding and Kaminsky, 2003; Fisslthaler et al., 1999; Klose et al., 1999). Because CYP2C8 is expressed in endothelial cells, arteries and heart, and metabolizes arachidonic acid to physiologically active EETs, CYP2C8 has been proposed as an endothelial-derived hyperpolarizing factor synthetase (Fisslthaler et al., 1999; Zeldin, 2001).

Human *CYP2C8* is the most inducible of the *CYP2C* genes in human hepatocytes in response to microsomal inducers such as rifampicin, phenobarbital and CITCO (Chen and Goldstein, 2009; Ferguson et al., 2005; Gerbal-Chaloin et al., 2001; Lai et al., 2009). CYP2C8 is also induced by phenytoin, hyperforin, paclitaxel (a CYP2C8 substrate) and the synthetic glucocorticoid dexamethasone (Garcia-Martin et al., 2006; Raucy et al., 2002; Synold et al., 2001). Induction of CYP2C8 by xenobiotics contributes to the inter-individual variability in drug metabolism in human populations which can lead to a change in the half-life of drugs and result in drug tolerance or therapeutic failure. The induction of the *CYP2C8* gene by drugs and xenobiotics is mediated by the constitutive androstane receptor (CAR), pregnane X receptor (PXR) and glucocorticoid receptor (GR), while HNF4α appears to play a role in basal expression (Ferguson et al., 2005).

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Recently, our laboratory demonstrated that CYP2C8 protein levels are down regulated post-transcriptionally by miR107 and miR103 in human liver (Zhang et al., 2012). miRNAs play important roles in the regulation of target genes by binding to the 3'-untranslated region and promote mRNA degradation or repress mRNA translation (Bartel, 2004). miR107 and miR103 (paralogs) are encoded within the introns of three pantothenate kinase (*PANK)* genes located on separate chromosomes (Wilfred et al., 2007). PANK genes catalyze the rate limiting step in coenzyme A biosynthesis and are involved in the regulation of acetyl-CoA levels and lipid metabolism (Trajkovski et al., 2011; Wilfred et al., 2007). Although miR103 is not completely coregulated with the corresponding PANK genes (Wilfred et al., 2007), previous studies have shown that p53 coregulates PANK1 and miR107 in different cellular systems (Bohlig et al., 2011; Yamakuchi et al., 2010). PANK1 expression is upregulated by the PPARα agonist, bezafibrate (BF) in HepG2 cells, resulting in elevated CoA levels (Ramaswamy et al., 2004).

Peroxisome proliferator-activated receptors (PPARs) act as lipid sensors to control the expression of gene networks involved in lipid homeostasis and inflammatory responses (Lalloyer and Staels, 2010). There are three functional PPARs: PPARα, PPARβ and PPARγ. PPARα is highly expressed in the liver and functions primarily to regulate the expression of genes involved in peroxisomal and mitochondrial β-oxidation, and microsomal ω-hydroxylation (Gulick et al., 1994; Schoonjans et al., 1996). The activated PPARα heterodimerizes with the retinoic acid X receptor (RXR), and this complex binds to specific DNA sequences called peroxisome proliferator response elements (PPREs) located in the promoter regions of target genes to up-regulate their expression (Kliewer et al., 1992; Wahli and Michalik, 2012).

Until recently, CYP4 family members, which function as microsomal fatty acid ω-hydroxylases, were the only CYPs reported to be directly regulated by PPARα (Hsu et al., 2007;

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Waxman, 1999). However, the drug-metabolizing CYPs CYP3A4, CYP2B6, CYP2C8, CYP1A1 and CYP1A2 were recently reported to be induced by fibrates (Thomas et al., 2013). Studies using primary human hepatocytes and a *CYP3A4/3A7*-humanized mouse model showed that *CYP3A4* is directly regulated by PPARα (Thomas et al., 2013). However, the mechanism of regulation of *CYP2C8* by PPARα has not been investigated: it is not known whether the regulation of *CYP2C8* by PPARα agonists/ligands is modulated by transcriptional activation by PPARα or CAR/PXR or indirectly by changes in miR107 expression.

The purpose of this study was to examine the regulation of CYP2C8 expression by xenobiotics capable of inducing PANK1/miR107 in cultured primary human hepatocytes and whether PPARα affects *CYP2C8* transcription directly or indirectly. Surprisingly, the hypolipidemic fibrate, BF induced both *PANK1* and miR107 expression in primary human hepatocytes and also induced CYP2C8 expression. Here, we provide evidence to support the hypothesis that the *CYP2C8* gene is directly upregulated by PPARα in human hepatocytes.

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Materials and Methods

Chemicals and Reagents. BF, WY14643 and rosiglitazone were purchased from Sigma-Aldrich Company, Inc. (St Louis, MO). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (clone 6C5, MAB374) was purchased from Millipore (Temecula, CA). Rabbit polyclonal antibodies against PPARα (sc-9000), RXRα (sc-553) and RNA Pol II (sc-899) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The expression plasmids for human PPARα (pcDNA3-hPPARα) and human RXRα (pGEM-3T-RXRα) were kindly provided by Masahiko Negishi (Laboratory of Reproductive and Developmental Toxicology, NIEHS, NIH). The expression plasmids pSG5-PPARα (ID 22751), and pcDNA-Flag-PPARγ (ID 8895) and the luciferase construct, PPRE-X3-TK-Luc, containing three copies of the PPRE from rat ACOX cloned upstream of the TK gene promoter (ID 1015) were obtained from Addgene. The luciferase reporter constructs, *2C8*-3k, *2C9*-3k, *2C19*-2.7k, *2C8*-3k/-8.9-8.5 (-8.9 to -8.5 kb region of *CYP2C8* promoter containing the CAR site cloned upstream of the *2C8*-3k), *2C8*-2.5k, *2C8*-2k, *2C8*-1.5k, *2C8*-500, *2C8*-300, *2C8*-3k/ΔBgl II and *2C8*-2.5k/ΔBgl II (generated by digestion and ligation of Bgl II restriction sites at positions -2342 and -698) were previously described (Chen et al., 2009; Ferguson et al., 2005).

RNA Isolation and Quantitative RT-PCR

Total RNA containing small RNA were isolated from human primary hepatocytes and HepG2 cells using miRNeasy mini kit (Qiagen, Valencia, CA) , according to the manufacturer's instructions and reverse transcribed to cDNA using the SuperScript III First Strand Synthesis system for RT-PCR kit (Invitrogen) with Oligo (dT) primers. qPCR was performed using the ABI Prism 7900 Sequence Detector System (Applied Biosystems, Foster City, CA) with the following primer and probe sets purchased from Applied Biosystems (Foster City, CA):

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CYP2C8 (Hs00258314_m1), CYP2C9 (Hs00426397_m1), CYP2C19 (Hs00426380_m1), ACOX1 (Hs01074241_m1), CPT1A (Hs00912671_m1), PANK1 (Hs00332073_s1) and GAPDH (Hs03929097_g1). Each cDNA (100 ng) was mixed with 1X Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA). mRNA levels were normalized with GAPDH as the endogenous control. To analyze miRNA expression, TaqMan MicroRNA assays (Applied Biosystems) were used to quantify levels of mature miRNAs following the manufacturer's instructions. Briefly, total RNA including small RNA isolated using miRNeasy mini kit was reverse-transcribed using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystem) and expression of mature miRNA-107 and miRNA-103 was quantitated using the individual TaqMan® MicroRNA Assays. RNU44 was used as the endogenous control to normalize miRNA expression. The primers/probes for qPCR were purchased from Applied Biosystems as follows: Hsa-miR107 (000443), Hsa-miR103 (000439) and RNU44 (001094). All qPCR experiments were performed in triplicate with cDNA samples from independent samples, as described previously.

Preparation of microsomes

Cultured human primary hepatocytes in six well plates were obtained from Triangle Research Labs (TRL, Research Triangle Park, NC). The information of the human donors is shown in Table 2. Hepatocytes were cultured in hepatocyte maintenance media containing supplements (MM250) according to instructions provided by TRL. Immunoblotting experiments were performed in microsomes to detect CYP2C proteins before and after treatment with BF or DMSO controls. Microsomes were prepared from cultured human hepatocytes as previously described (Makia et al., 2014). Briefly, Cells were suspended in ice-cold buffer (0.1 M potassium phosphate, pH 7.4 containing 0.25M sucrose and 1 mM EDTA) and homogenized using a Potter-

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Elvehjem homogenizer. The 10,000 x g supernatant was subjected to ultracentrifugation for 2 h at 112,000 x g using a TLA-55 rotor (Beckman Coulter, Palo Alto, CA). The microsomal pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.4 containing 2% glycerol and 1 mM EDTA, and stored at -80˚C.

Western blotting

Total cell and nuclear extracts were prepared from HepG2 cells as described previously (Makia et al., 2012). The extracts were separated on 4-20% SDS-PAGE gels and transferred onto nitrocellulose membranes. Membranes were probed with antibodies against GAPDH (1:5000) as the endogenous control or 1:1000 dilutions of all other primary antibodies. Horseradish peroxidase-conjugated goat anti-rabbit or -mouse secondary (1:10,000) antibodies were used and the proteins were visualized using SuperSignal West Pico or Dura Western blotting detection system (Thermo Scientific, Rockford, IL). Microsomal proteins were separated using a Protean II xi Cell (BioRad), and Western blots were performed with the following rabbit antibodies: 1590 (raised to recombinant purified CYP2C9 expressed in *E coli* which recognizes CYP2C9>CYP2C19>>CYP2C8), 1592 (raised to recombinant purified CYP2C9 expressed in E coli which recognizes (CYP2C9>>CYP2C19 and CYP2C8) and 1937 (a specific anti-CYP2C8 peptide antibody which recognizes only CYP2C8) (Zhang et al., 2012). The following CYP2C standards were also used: human liver microsomes (Gentest Corp., Woburn, MA), and recombinant yeast CYP2C9, CYP2C8 and CYP2C19 proteins.

Transcription Factor Binding Sites Analysis

The Genomatrix MatInspector software was used to analyze the human *CYP2C8* (-3077/+1) promoter for putative PPAR response elements (PPRE). The canonical PPRE is a direct repeat 1 (DR-1) with sequence AGGTCAAAGGTCA.

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Site-Directed Mutagenesis

A QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to mutate the DR1-B site located at positions -2109 of the human *CYP2C8* promoter according to the manufacturer's instructions. The primers for mutagenesis of the DR1-B site at position -2109 bp to generate the luciferase reporter constructs, 2C8-3k/DR1BM and 2C8-2.5k/DR1BM were as follows, (forward, 5'- CAGCAAATTACTACTTCGGTTTGCGGTGGATAAAGGGTTCA -3' and reverse, 5'- TGAACCCTTTATCCACCGCAAACCGAAGTAGTAATTTGCTG -3'). The mutated sites are underlined. The mutation of the DR1-B site was confirmed by sequencing to eliminate spurious mutations.

Transfection of HepG2 Cells and Luciferase Reporter Assays

The human hepatocellular carcinoma cell line, HepG2 (HB8065, American Type Culture Collection, Rockville, MD) was maintained in Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (HyClone, Logan UT), 1 mM sodium pyruvate, 2 mM glutamine and penicillin-streptomycin (Life Technologies, Carlsbad, CA). Cells were seeded in 24-well plates and transfected with 200 ng/well of luciferase reporter construct and 20 ng of renilla luciferase plasmid (pRL-TK) using Lipofectamine 2000. The cells were co-transfected with pcDNA3.1 (control vector), pcDNA3-hPPARα (100 ng), pSG5-mPPARα (100 ng) and pcDNA-Flag-hPPARγ (100 ng) plasmids. Twenty four hours after transfection cells were treated with BF (0.5 mM), Wy14643 (50 μM) or rosiglitazone (5 μM) for 24 h. The cells were resuspended in passive lysis buffer (Promega, Madison, WI) and luciferase activity was assayed with a Dual-Glo luciferase reporter assay system. The data were expressed relative to renilla luciferase activity to normalize for transfection efficiency. Transfection experiments were performed in triplicate and repeated at least twice for confirmation.

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In vitro Transcription/Translation of PPARα and RXRα proteins

Human PPARα and RXRα proteins were synthesized *in vitro* from 1 μg of pcDNA3-hPPARα or pGEM-3T-RXRα plasmids using a TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI) in the presence of unlabeled methionine following the manufacturer's instructions. Additional controls containing the TNT Quick master mix with no template DNA were also performed. The proteins were separated on a 4-20% Tris-Glycine polyacrylamide gel and verified by Western blotting using antibodies against PPARα and RXRα.

Electrophoretic Mobility Shift Assays (EMSA)

EMSAs with the *in vitro* transcribed proteins were performed essentially as described (Makia et al, 2014) by incubating 2 μl of the *in vitro* translated PPARα and RXRα proteins with labeled double stranded oligonucleotides containing PPRE control and various DR1. The sequences of the complementary oligonucleotides were as follows: PPRE control (forward: 5'-CAGGGGACCAGGACAAAGGTCACGTTCGGGA-3'' and reverse: 5'-TCCCGAACGTGACCTTTGTCCTGGTCCCCTG -3'). DR1-A (forward: 5'-ACCCTATGTGAACTTCGAACTTTGGTTGATG-3'and reverse: 5'-CATCAACCAAAGTTCGAAGTTCACATAGGGT -3'). DR1-B (Forward: 5'-AATTACTACTTCCCTTTGCCCTGGATAAAGG-3' and reverse: 5'-CCTTTATCCAGGGCAAAGGGAAGTAGTAATT-3'). DR1BMut (Forward: 5'-AATTACTACTTCGGTTTGCGGTGG ATAAAGG -3' and reverse: 5'-CCTTTATCCACCGCAAACCGAAGTAGTAATT-3'). DR1-C (Forward: TAAAACCAAACACGTCTGACCCACATTTTAC-3' and reverse: 5'-GTAAAATGTGGGTCAGACGTGTTTGGTTTTA-3'. DR1-D (Forward: 5'-TAAAAAGAAAGGTCAAGGCAGGAGCCTCAGC-3'and reverse: 5'-

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GCTGAGGCTCCTGCCTTGACCTTTCTTTTTA-3'). The complementary oligonucleotides (1 pmol/μl) were annealed by incubating at 95˚C for 5 min with annealing buffer (10 mM Tris-HCl, 1 mM EDTA and 50 mM NaCl, pH 8.0). Double-stranded oligonucleotides were labeled with [γ-^{32}P]ATP using a T4 polynucleotide kinase kit following the supplier's instructions (Promega, Madison, WI). Unincorporated nucleotides were removed by chromatography on microspin G-25 columns (GE Healthcare, Piscataway, NJ). Protein-DNA complexes were formed by incubating 5 μg of nuclear proteins and 10^6 cpm of ^{32}P-labeled oligonucleotide probe for 30 min at room temperature in a total volume of 20 μl with binding buffer (10 mM Tris-HCl, 1 mM EDTA, 1 mM $MgCl_2$, 5% glycerol, 1 mM DTT and 50 ng/μl poly(dI-dC). Binding specificity was assessed by addition of 100-fold excess of unlabeled double stranded oligonucleotides. For super shift analyses, antibodies (4 μg) were added to the binding reactions after the initial 20 min incubation and incubation was continued for 2 h at 4°C. Loading buffer was added to the reactions and the 10 μl of the binding reactions were resolved by electrophoresis on 5% polyacrylamide gels using 0.5X TBE buffer (50 mM Tris, pH 8.3, 50 mM sodium borate and 1 mM EDTA) at 200 V for 2 h. The gel was transferred to Whatman 3MM filter paper, dried and exposed to film overnight at -80˚C.

Chromatin Immunoprecipitation Assay (ChIP)

ChIP assays were performed as described previously (Makia et al., 2012) using the MAGnify Chromatin-Immunoprecipitation System (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol with minor modifications. Briefly, human primary hepatocytes in 10 cm dishes treated with either DMSO (control) or BF (0.5 mM) were fixed in 1% formaldehyde at room temperature for 10 min to cross-link the nuclear proteins to DNA, and the reaction was stopped by incubation with 125 mM glycine for 10 min. Cells were resuspended in

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lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 0.5% NP-40) containing 1X complete protease inhibitor (Roche Diagnostics, Indianapolis, IN) and incubated on ice for 30 min. The nuclei were harvested and solubilized in buffer containing 50 mM Tris-Cl, pH 8.0, 1% SDS, 10 mM EDTA, and 0.5 mM phenylmethanesulfonyl fluoride with 1X complete protease inhibitor. The homogenate was sonicated on ice at 40% setting (Branson sonicator, North Olmsted, OH) to shear the chromosomal DNA into fragments of ~200 to 500 bp in size. Immunoprecipitation of the sonicated DNA fragments was performed overnight at 4˚C with 8 µg antibodies against IgG (negative control), PPARα, RXRα or RNA Pol II conjugated to Dynabeads protein A/G. The cross-linked protein-DNA complexes were uncrosslinked in the presence of proteinase K and the purified DNA was analyzed by PCR using PCR SuperMix High Fidelity (Life Technologies, Carlsbad, CA) with primers spanning DR1-B (position -2109), negative control region (position -1500 in the promoter of *CYP2C8*, the PPRE of human HMGCR (positive control for PPARa and RXRα) and α-actin (negative control for all antibodies). The sequences of the primers were as follows: 2C8-DR1B (Forward: 5’-ATTGCTCTAAAGAGAGAAAG-3’and reverse: 5'- AAT TCT AGC ACC AGT TGA GT -3'). 2C8 negative control (Forward: 5’-AGGAGTAGGACAAAAGAACA-3’and reverse: 5'-TAAGACAGCTGTGAGCTTGC-3'). Human HMGCR promoter (Forward: 5’-ACGCTGATTTGGGTCTATGG-3’ and reverse: 5’-GTGTAAATGGCTCCGGTCAC-3’). Human α-actin coding region (Forward: 5’-CTTCTGCCCTCCGCAGCTGA-3’ and reverse: 5’-GTGAATGCCCGCCGACTCCA-3’). PCR products were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining. For qPCR analysis, the purified DNA (10 µl) immunoprecipitated with antibodies against PPARα and IgG were mixed with 1X Power SYBR green PCR Master Mix (Applied Biosystems, Foster

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City, CA) containing 290 nM of the forward and reverse primers. All PCR results were normalized to input control and presented as fold enrichment over IgG.

Statistical Analysis:

All error bars indicate the mean ± SEM. Data were analyzed with Student's *t*-tests or one-way analysis of variance (ANOVA) followed by Tukey's test using Sigma Stat software (Sigma Stat 3.5, SPSS, San Jose, CA). Differences were considered to be statistically significant at a *P*-level of 0.05.

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Results

BF induces PANK1/miR107 expression in primary human hepatocytes. MicroRNA 107 is located in intron 5 of PANK1 gene (Fig. 1A). Previous studies showed that the *PANK1* gene is a target of PPARα in HepG2 cells (Ramaswamy et al., 2004). We examined whether miR107 expression is also induced by activation of PPARα in primary human hepatocytes at 24 h and 48 h after BF (0.5mM) treatment. BF induced miR107 expression while miR103 expression was not increased (Fig. 1B). Consistent with possible coregulation of the PANK1 gene and miR107 expression, we also observed BF concurrently induced expression of PANK1 (pri-miR107) (Fig. 1C). Treatment of HepG2 cells with BF also resulted in 1.5 fold increase in miR107 expression. Ectopic expression of PPARα in HepG2 cells and treatment with BF resulted in 3-fold increase in miR107 expression (Fig. 1D).

BF induces CYP2C8 expression in primary human hepatocytes but had minimal effects on CYP2C9 or CYP2C19 expression. BF (a PPARα ligand/agonist) had only a marginal effect on expression of two other *CYP2C* genes (CYP2C9 and CYP2C19 mRNA) (Figs. 2A and 2B). ACOX1 and CPT1A are positive controls for PPARα activation. We examined the effect of varying concentrations of BF on the expression of PANK1 and *CYP2C* genes at 48 h. There was a concentration dependent increase in CYP2C8 mRNA to a maximum of 3.5 fold increase at 0.5 mM (Fig. 2C) and a 2.5-fold increase at 0.75 mM. PANK1 mRNA was induced ~2.5-fold at 0.125 mM BF which remained constant through a concentration of 0.75 mM BF. CYP2C9 and CYP2C19 mRNA were increased only slightly (1.5 fold) at 0.75 mM BF. Treatment of primary hepatocytes with BF also induced expression of CYP2C8 protein (~6.5-fold) after 48 h but did not appear to induce CYP2C9 or CYP2C19 protein (Fig. 3A and 3B).

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***CYP2C8* promoter luciferase activity is transactivated by ectopic expression of PPARα and treatment with BF in HepG2 cells.** We examined whether *CYP2C8* transcription is induced by PPARα activation in HepG2 cells. HepG2 cells were co-transfected with a 2C*8*-3k luciferase reporter construct (containing -2966 to +1 of the *CYP2C8* promoter upstream in a luciferase reporter) and vector control with human or mouse PPARα expression plasmids. Cells were treated with either DMSO control or BF (0.5 mM) for 24 h. We observed ~16- and ~10-fold increases in *CYP2C8* promoter luciferase activity after transfection with human and mouse PPARα, respectively and treatment with BF (Fig. 4A). *CYP2C8* promoter activity was increased ~12-fold by activation of human PPARα using a more specific PPARα ligand, 50 μM WY14643 (Fig. 4B) and ~18-fold after treatment with rosiglitazone and cotransfection with PPARγ (5 μM) (Fig. 4C). In contrast, neither *CYP2C9* (*2C9*-3k) nor *CYP2C19* (*2C19*-2.7k) promoter was induced by treatment of ectopically expressed human PPARα with BF (Fig. 4D). As a control for PPARα transcriptional activation, the PPRE-X3-TK luciferase construct that contains three copies of the rat ACOX1-PPRE upstream of the TK promoter was transactivated by activation of human (120-fold) and mouse (80-fold) PPARα in HepG2 cells (Fig. 4E).

Functional characterization of direct repeats 1 (DR-1) in the *CYP2C8* promoter in HepG2 cells. Promoter sequence analysis of the *CYP2C8* gene promoter revealed five putative PPARα response element (PPRE) sites located at positions -2772 (DR1-A), -2109 (DR1-B), -2039 (DR1-C), -1501 (DR1-D) and -152 (DR1-E) relative to the translation start site. The sequences of the DR1 and their 5' flanking region, and the strand orientation are shown in Table 1. The consensus sequence of the PPRE (AGGTCAAAGGTCA) is also shown. Fig. 5A is a schematic diagram of the *CYP2C8* promoter showing the position of the various DR1. HepG2 cells were co-transfected with various deletion luciferase constructs and the human PPARα

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expression plasmid. Cells were treated with either DMSO control or BF for 24 h. We observed about 20-fold increase in *CYP2C8* luciferase activity with the *2C8*-3k construct. Similar levels of *CYP2C8* luciferase activity were observed with the *2C8*-3k/-8.9-8.5 (containing the upstream CAR/PXR site) and *2C8*-2.5k constructs. No significant effect on *CYP2C8* luciferase activity was observed with the *2C8*-2k, 2C8-1.5k, *2C8*-500 and *2C8*-300. This indicates that DR1-B rather than DR1-A might be essential for transactivation of the *CYP2C8* promoter activity by PPARα. We then used the *2C8*-3k/ΔBgl II constructs generated by digestion and ligation of Bgl II restriction sites at positions -2342 and -698. This construct contains the DR1-A but not DR1-B site. When HepG2 cells were transfected with this construct no significant difference in *CYP2C8* luciferase activity was observed, suggesting again that the DR1-A site might not be important for the increased *CYP2C8* promoter activation by PPARα. Site directed mutagenesis was used to investigate the relative importance DR1-B in the transactivation of the *CYP2C8* promoter by PPARα activation with BF. Mutation of the DR1-B in the 2C8-3k or 2C8-2.5k constructs completely abolished *CYP2C8* reporter activation by BF (Fig. 5B). These results confirm that DR1-B is required for maximal transactivation of the *CYP2C8* luciferase promoter construct by PPARα.

EMSA analysis of CYP2C8 DR1 and PPRE control using *in vitro* translated PPARα or RXRα proteins. Human PPARα and RXRα synthesized using a TNT Quick Coupled Transcription/Translation System in the presence of unlabeled methionine were verified by Western blot using antibodies to human PPARα and RXRα (Supplemental Fig. S1). EMSA was performed to determine whether PPARα and RXRα bind to the various DR1 of *CYP2C8* and PPRE control (rat ACOX1). The sequences of the oligonucleotide used for the EMSA are shown in Fig. 6A. ^{32}P-labeled double-stranded oligonucleotides containing *CYP2C8* DR1-A, DR1-B,

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DR1-C, DR1-D, DR1-BM (mutation of GG to CC in DR1-B) and a positive control for PPARα binding (PPRE from rat ACOX1 gene) were incubated for 30 min with in vitro transcribed/translated RXRα or both hPPARα and RXRα proteins at 4℃. We observed formation of a DNA-protein complex with the labeled PPRE consensus and DR1-B oligonucleotides when incubated with both PPARα and RXRα. No complex was formed with incubation of DR1-A, DR1-C or DR1-D oligonucleotides with PPARα and RXRα (Fig. 6A). The DNA-protein complex with the DR1-B and PPRE were effectively inhibited by addition of excess unlabeled oligonucleotide (cold), but not excess unlabeled mutant oligonucleotide indicating specificity of binding to the DR1B (Fig. 6B). Supershift experiments showed that the protein complexes formed with the *CYP2C8* DR1-B and PPRE control oligonucleotides were shifted by antibodies against PPARα and RXRα but not with IgG. These results indicate specific binding of PPARα and RXRα to the DR1-B site at -2109 of the *CYP2C8* promoter.

ChIP analysis of transcription factor binding to the DR1-B in PHH before and after treatment with BF. ChIP experiments were utilized to assess *in vivo* recruitment of PPARα and RXRα to the DR1-B before and after treatment of primary human hepatocytes with BF. Chromatin was prepared from cultured human hepatocytes treated DMSO control or BF for 48 h. The chromatin was sheared by sonication into fragments approximately 200-500 bp in size and immunoprecipitated with IgG, PPARα, RXRα or RNA Pol II (positive control for transcriptionally active genes) antibodies conjugated to dynabeads. Conventional PCR was performed with primers spanning the *CYP2C8* DR1-B site, *CYP2C8* promoter region that does not contain DR1 (negative control), the PPRE of human HMGCR promoter (positive control for PPARα and RXRα binding), human α-actin coding region (a negative control for all antibodies). The positions of the primers used the ChIP analyses are shown in Fig. 7A. As seen in Fig. 7B,

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we observed enhanced recruitment of PPARα, RXRα and Pol II to the DR1-B and HMGCR after treatment with BF compared to DMSO control treatment. These factors were not recruited to the *CYP2C8* negative control site or α-actin promoter. SYBR green quantitative PCR was performed using primers flanking the *CYP2C8* DR1-B, a *CYP2C8* promoter region that does not contain DR1 (negative control), the PPRE of human HMGCR and α-actin with chromatin extracted from control or BF treated primary human hepatocytes immunoprecipitated with antibodies specific to PPARα or IgG. Again we observed enhanced enrichment of PPARα to the DR1-B and HMGCR after treatment with BF compared to DMSO control treatment but not the 2C8 negative control site or α-actin. Consistent with the EMSA, these results indicate the in vivo binding of PPARα to the *CYP2C8* DR1-B site.

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Discussion

In the present study, we show for the first time that *CYP2C8* but not *CYP2C9* or *CYP2C19* is transcriptionally upregulated by PPARα activation in primary human hepatocytes. We demonstrate that this induction is independent of activation of CAR/PXR or changes in miR107 by hypolipidemic drugs via PPARα activation. Until recently, the microsomal fatty acid ω-hydroxylases CYP members, the CYP4 family, were the only known PPARα regulated CYP genes (Aldridge et al., 1995). A recent study investigated the possible involvement of PPARα in the regulation of drug biotransformation genes using gene silencing experiments in primary human hepatocytes (Thomas et al., 2013). Their data showed that CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP3A4 and CYP7A1 but not CYP2C9, CYP2C19, CYP2D6 or CYP2E1 were selectively regulated by PPARα agonists such as WY14643 or fibrates in primary human hepatocytes (Thomas et al., 2013). These data are consistent with other previous studies using mouse and human hepatocytes (Prueksaritanont et al., 2005; Rakhshandehroo et al., 2009). These studies imply an involvement of PPARα in modulating the constitutive and xenobiotic-induced expression of *CYP2C8* gene. However, except for CYP3A4 and CYP1A1, no other studies have formally addressed direct regulation of other CYP genes by PPARα (Seree et al., 2004; Thomas et al., 2013; Villard et al., 2011). It is now known that CYP1A1 is directly regulated by PPARα via two PPRE within its proximal promoter (Seree et al., 2004;Villard et al., 2011). Our data are consistent with these previous studies showing induction of CYP2C8 but not CYP2C9 or CYP2C19 by PPARα ligand/agonists in primary human hepatocytes. Moreover, for the first time, we identified a PPRE (DR1-B) at position -2109 bp in the promoter of *CYP2C8* and showed that in vitro translated PPARα binds to this site in the presence of RXRα. We also observed enhanced recruitment of PPARα and RXRα to the DR1-B site but not a negative

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control region in *CYP2C8* promoter by bezafibrate. Apart from CYPs, PPARα has also been shown to directly regulate other drug biotransformation enzymes including UDP-glucuronosyltransferases (Barbier et al., 2003a; Barbier et al., 2003b), sulfotransferase (Fang et al., 2005) and transporters (Ghonem et al., 2014; Hoque et al., 2012; Moffit et al., 2006).

CYP2C8 gene is regulated post-transcriptionally by miRNA107 and miRNA103 in primary human hepatocytes (Zhang et al., 2012). miR107 had little or no effect on CYP2C8 mRNA levels but decreases CYP2C8, CYP2C9 and CYP2C19 proteins in primary human hepatocytes. miR107 is transcribed from the intron of *PANK1* gene, which catalyzes the rate limiting step of CoA biosynthesis and is involved in the regulation of acetyl-CoA levels, cell stress, insulin sensitivity and lipid metabolism (Trajkovski et al., 2011; Wilfred et al., 2007). Previous studies demonstrate that PANK1 and miR107 are coregulated by p53 in different cellular systems through a p53-binding site on *PANK1* promoter (Bohlig et al., 2011; Yamakuchi et al., 2010). *PANK1* transcription is regulated by PPARα in HepG2 cells leading to increased CoA levels in cells (Ramaswamy et al., 2004). The induction of *PANK1* gene by PPARα was mediated by the PPREs located within its proximal promoter (Ramaswamy et al., 2004). We showed that miR107 was induced by PPARα activation using BF in primary human hepatocytes while miR103 expression was not upregulated. Consistent with the hypothesis of coregulation between PANK1 gene and miR107 expression by PPARα, miR107 and *PANK1* gene expression were induced by activated PPARα in primary human hepatocytes and HepG2 cells. But contrary to the expectation that *CYP2C* genes might be post-transcriptionally decreased by miR107 up-regulation after BF treatment, CYP2C8 protein expression was significantly increased in primary human hepatocytes by BF treatment. However, neither CYP2C9 nor CYP2C19 proteins were induced by activation of PPARα. Therefore, we hypothesize that the regulation of CYP2C8 by

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PPARα is transcriptional rather than indirectly by enhancing miR107 expression in human liver. If increases in miR107 affect CYP2C8 transcription, the effects are not substantial enough to override the direct induction by PPARα. *CYP2C8* luciferase activity was induced by activated ectopically expressed human and mouse PPARα in HepG2 cells. Interestingly, *CYP2C8* promoter activity was also induced by the CYP2C8 substrate rosiglitazone, and the induction was dependent on human PPARγ activation. Previous studies showed that fibrates and other hypolipdemic drugs regulate gene expression by activation of the nuclear receptor CAR and PXR (Aouabdi et al., 2006; Prueksaritanont et al., 2005). However, the absence of transactivation of *CYP2C9* and *CYP2C19* promoter luciferase activity by BF which are known CAR/PXR-regulated genes indicates that the regulation of *CYP2C8* gene is independent of CAR/PXR. Furthermore, there was no significant difference in the transactivation of *CYP2C8* luciferase by activated PPARα between the *CYP2C8* luciferase construct that contains (2C8-3k/-8.9-8.5) or lacks (2C8-3k) a distal CAR/PXR-binding site located at position -8.8 kb. This CAR/PXR-binding site was shown to be essential for induction of *CYP2C8* reporter by rifampicin (PXR agonist) and CITCO (CAR ligand) in primary human hepatocytes (Ferguson et al., 2005).

A unique feature of the *CYP2C8* promoter compared to other CYP2C subfamily members is that no CAR/PXR-responsive elements were identified within the proximal 3 kb of the *CYP2C8* promoter but rather one element at -8.8kb (Ferguson et al., 2005). Thus, the observed induction of *CYP2C8* luciferase reporter activity by BF is likely not due to activation of CAR/PXR. Promoter sequence analysis of *CYP2C8* gene promoter revealed five putative PPARα response element (PPRE) sites within the proximal 3 kb region relative to the translation start site located at positions -2772 (DR1-A), -2109 (DR1-B), -2039 (DR1-C), -1501 (DR1-D) and -

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152 (DR1-E). PPARα regulates gene expression by binding to PPRE on target gene promoters. The consensus sequence of the PPRE is AGGTCAAAGGTCA which consists of a direct repeat of AGGTCA half-sites with one base-pair spacing (DR1) (Kliewer et al., 1992; Wahli and Michalik, 2012). However, imperfect half-sites of the canonical DR1 have been identified in PPARα regulated genes such as CYP4A1 (AGGGTAAAGTTCA), VLDL receptor (AGGTCAGATGGCA), cyclic AMP response element binding (AGGTCAAAGGACA), the mouse hypoxia-inducible lipid droplet-associated (AGGGGAAAGGTCA) and the rat ACOX (AGGACAAAGGTCA) (Gao et al., 2014; Juge-Aubry et al., 1997; Mattijssen et al., 2014; Roy et al., 2013). It is widely accepted that the sequence of the 5'-flanking region (6 or 7 bp segment) in addition to the consensus DR1 sequence is important for binding of PPAR to various PPREs (Chandra et al., 2013; Juge-Aubry et al., 1997; Palmer et al., 1995). This is due to the fact that the hinge region of PPARα and γ recognizes an additional six base-pair segments located upstream to the DR1 core element. The half site of the CYP2C8 DR1-C site at position -2039 was previously shown to bind RORα and RORγ and important for transcriptional regulation of *CYP2C8* by ROR in human liver and other tissues such as colon and intestine (Chen et al., 2009). Both the HNF4α homodimer and the PPAR/RXRα heterodimer are known to recognize the same DR1 sequence (Chandra et al., 2013; Pineda Torra et al., 2002). An in vitro binding assay showed that the DR1-E is an HNF4α-binding site located within the *CYP2C8* basal promoter (Ferguson et al., 2005). This site was shown to be necessary for constitutive activation of *CYP2C8* reporter by co-transfected HNF4α in Hela cells and primary human hepatocytes (Rana et al., 2010). We observed about 20-fold increase in *CYP2C8* luciferase activity with the *2C8*-3k and *2C8*-2.5k constructs. *CYP2C8* promoter contains several potential PPREs but site directed mutagenesis and deletion analyses demonstrate that DR1-B at -2109 bp with sequence

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AGGGCAAAGGGAA was solely required for transactivation of the *CYP2C8* luciferase by PPARα activation. The DR1-B sequence is absent in the promoter of *CYP2C9* and *CYP2C19* which might explain the specific regulation of CYP2C8 by PPARα. Since both PPARα and PPARγ are known to bind identical PPREs, we anticipate that the DR1-B site is also essential for PPARγ-mediated regulation of CYP2C8 by rosiglitazone.

In conclusion, we showed for the first time that *CYP2C8* is directly transcriptionally regulated by activated PPARα in human primary hepatocytes. PPARα dependent regulation of *CYP2C8* is mediated by DR1-B site at position -2109 bp in *CYP2C8* promoter. This regulation is specific for CYP2C8 but not CYP2C9 or CYP2C19. Because CYP2C8 is involved in metabolism of fatty acids, it is reasonable to suggest that, like CYP4A, induction of endogenous CYP2C8 by PPARα may serve a role in the oxidative metabolism of arachidonic acid to EETs. EETs have been shown to have potent vasodilatory and anti-inflammatory functions (Wray and Bishop-Bailey, 2008) . Because CYP2C8 also has been detected in endothelial cells and arteries, oxidation of arachidonic acid to produce 11,12- and 14,15-EETs may play vasodilatory and anti-inflammatory roles (Delozier et al., 2007; Wray and Bishop-Bailey, 2008). This study also indicates the possibility of previously underrepresented drug-drug interaction due to up-regulation of CYP2C8 by hypolipidemic (fibrates, WY14643) and antidiabetic (rosiglitazone) drugs. The discovery that *CYP2C8* is transactivated by PPARα and PPARγ in hepatocytes suggests the possibility that the clearance of CYP2C8 substrates might be modulated by ligands of PPARα/γ.

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Authorship contributions*:*

Participated in research design: Makia, Goldstein

Conducted experiments: Makia

Contributed new reagents or analytic tools:

Performed data analysis: Makia, Goldstein

Wrote or contributed to the writing of the manuscript: Makia, Goldstein

References

Aldridge TC, Tugwood JD and Green S (1995) Identification and characterization of DNA elements implicated in the regulation of CYP4A1 transcription. *The Biochemical journal* **306 (Pt 2)**: 473-479.

Aouabdi S, Gibson G and Plant N (2006) Transcriptional regulation of the PXR gene: identification and characterization of a functional peroxisome proliferator-activated receptor alpha binding site within the proximal promoter of PXR. *Drug metabolism and disposition: the biological fate of chemicals* **34**(1): 138-144.

Barbier O, Duran-Sandoval D, Pineda-Torra I, Kosykh V, Fruchart JC and Staels B (2003a) Peroxisome proliferator-activated receptor alpha induces hepatic expression of the human bile acid glucuronidating UDP-glucuronosyltransferase 2B4 enzyme. *The Journal of biological chemistry* **278**(35): 32852-32860.

Barbier O, Villeneuve L, Bocher V, Fontaine C, Torra IP, Duhem C, Kosykh V, Fruchart JC, Guillemette C and Staels B (2003b) The UDP-glucuronosyltransferase 1A9 enzyme is a peroxisome proliferator-activated receptor alpha and gamma target gene. *The Journal of biological chemistry* **278**(16): 13975-13983.

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**(2): 281-297.

Bohlig L, Friedrich M and Engeland K (2011) p53 activates the PANK1/miRNA-107 gene leading to downregulation of CDK6 and p130 cell cycle proteins. *Nucleic acids research* **39**(2): 440-453.

Chandra V, Huang P, Potluri N, Wu D, Kim Y and Rastinejad F (2013) Multidomain integration in the structure of the HNF-4alpha nuclear receptor complex. *Nature* **495**(7441): 394-398.

Chen Y, Coulter S, Jetten AM and Goldstein JA (2009) Identification of human CYP2C8 as a retinoid-related orphan nuclear receptor target gene. *The Journal of pharmacology and experimental therapeutics* **329**(1): 192-201.

Chen Y and Goldstein JA (2009) The transcriptional regulation of the human CYP2C genes. *Current drug metabolism* **10**(6): 567-578.

Delozier TC, Kissling GE, Coulter SJ, Dai D, Foley JF, Bradbury JA, Murphy E, Steenbergen C, Zeldin DC and Goldstein JA (2007) Detection of human CYP2C8, CYP2C9, and CYP2J2 in cardiovascular tissues. *Drug metabolism and disposition: the biological fate of chemicals* **35**(4): 682-688.

Ding X and Kaminsky LS (2003) Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. *Annual review of pharmacology and toxicology* **43**: 149-173.

Fang HL, Strom SC, Cai H, Falany CN, Kocarek TA and Runge-Morris M (2005) Regulation of human hepatic hydroxysteroid sulfotransferase gene expression by the peroxisome proliferator-activated receptor alpha transcription factor. *Molecular pharmacology* **67**(4): 1257-1267.

Ferguson SS, Chen Y, LeCluyse EL, Negishi M and Goldstein JA (2005) Human CYP2C8 is transcriptionally regulated by the nuclear receptors constitutive androstane receptor, pregnane X receptor, glucocorticoid receptor, and hepatic nuclear factor 4alpha. *Molecular pharmacology* **68**(3): 747-757.

Fisslthaler B, Popp R, Kiss L, Potente M, Harder DR, Fleming I and Busse R (1999) Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Nature* **401**(6752): 493-497.

Gao Y, Shen W, Lu B, Zhang Q, Hu Y and Chen Y (2014) Upregulation of hepatic VLDLR via PPARalpha is required for the triglyceride-lowering effect of fenofibrate. *Journal of lipid research* **55**(8): 1622-1633.

Garcia-Martin E, Pizarro RM, Martinez C, Gutierrez-Martin Y, Perez G, Jover R and Agundez JA (2006) Acquired resistance to the anticancer drug paclitaxel is associated with induction of cytochrome P450 2C8. *Pharmacogenomics* **7**(4): 575-585.

Gerbal-Chaloin S, Pascussi JM, Pichard-Garcia L, Daujat M, Waechter F, Fabre JM, Carrere N and Maurel P (2001) Induction of CYP2C genes in human hepatocytes in primary culture. *Drug metabolism and disposition: the biological fate of chemicals* **29**(3): 242-251.

Ghonem NS, Ananthanarayanan M, Soroka CJ and Boyer JL (2014) Peroxisome proliferator-activated receptor alpha activates human multidrug resistance transporter 3/ATP-binding cassette protein subfamily B4 transcription and increases rat biliary phosphatidylcholine secretion. *Hepatology (Baltimore, Md)* **59**(3): 1030-1042.

Gulick T, Cresci S, Caira T, Moore DD and Kelly DP (1994) The peroxisome proliferator-activated receptor regulates mitochondrial fatty acid oxidative enzyme gene expression. *Proceedings of the National Academy of Sciences of the United States of America* **91**(23): 11012-11016.

Hoque MT, Robillard KR and Bendayan R (2012) Regulation of breast cancer resistant protein by peroxisome proliferator-activated receptor alpha in human brain microvessel endothelial cells. *Molecular pharmacology* **81**(4): 598-609.

Hsu MH, Savas U, Griffin KJ and Johnson EF (2007) Human cytochrome p450 family 4 enzymes: function, genetic variation and regulation. *Drug metabolism reviews* **39**(2-3): 515-538.

Juge-Aubry C, Pernin A, Favez T, Burger AG, Wahli W, Meier CA and Desvergne B (1997) DNA binding properties of peroxisome proliferator-activated receptor subtypes on various natural peroxisome proliferator response elements. Importance of the 5'-flanking region. *The Journal of biological chemistry* **272**(40): 25252-25259.

Kliewer SA, Umesono K, Noonan DJ, Heyman RA and Evans RM (1992) Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. *Nature* **358**(6389): 771-774.

Klose TS, Blaisdell JA and Goldstein JA (1999) Gene structure of CYP2C8 and extrahepatic distribution of the human CYP2Cs. *Journal of biochemical and molecular toxicology* **13**(6): 289-295.

Lai XS, Yang LP, Li XT, Liu JP, Zhou ZW and Zhou SF (2009) Human CYP2C8: structure, substrate specificity, inhibitor selectivity, inducers and polymorphisms. *Current drug metabolism* **10**(9): 1009-1047.

Lalloyer F and Staels B (2010) Fibrates, glitazones, and peroxisome proliferator-activated receptors. *Arteriosclerosis, thrombosis, and vascular biology* **30**(5): 894-899.

Makia NL, Amunom I, Falkner KC, Conklin DJ, Surapureddi S, Goldstein JA and Prough RA (2012) Activator protein-1 regulation of murine aldehyde dehydrogenase 1a1. *Molecular pharmacology* **82**(4): 601-613.

Makia NL, Surapureddi S, Monostory K, Prough RA and Goldstein JA (2014) Regulation of human CYP2C9 expression by electrophilic stress involves activator protein 1 activation and DNA looping. *Molecular pharmacology* **86**(2): 125-137.

Mattijssen F, Georgiadi A, Andasarie T, Szalowska E, Zota A, Krones-Herzig A, Heier C, Ratman D, De Bosscher K, Qi L, Zechner R, Herzig S and Kersten S (2014) Hypoxia-inducible lipid droplet-associated (HILPDA) is a novel peroxisome proliferator-activated receptor (PPAR) target involved in hepatic triglyceride secretion. *The Journal of biological chemistry* **289**(28): 19279-19293.

Moffit JS, Aleksunes LM, Maher JM, Scheffer GL, Klaassen CD and Manautou JE (2006) Induction of hepatic transporters multidrug resistance-associated proteins (Mrp) 3 and 4 by clofibrate is regulated by peroxisome proliferator-activated receptor alpha. *The Journal of pharmacology and experimental therapeutics* **317**(2): 537-545.

Palmer CN, Hsu MH, Griffin HJ and Johnson EF (1995) Novel sequence determinants in peroxisome proliferator signaling. *The Journal of biological chemistry* **270**(27): 16114-16121.

Pineda Torra I, Jamshidi Y, Flavell DM, Fruchart JC and Staels B (2002) Characterization of the human PPARalpha promoter: identification of a functional nuclear receptor response element. *Molecular endocrinology (Baltimore, Md)* **16**(5): 1013-1028.

Prueksaritanont T, Richards KM, Qiu Y, Strong-Basalyga K, Miller A, Li C, Eisenhandler R and Carlini EJ (2005) Comparative effects of fibrates on drug metabolizing enzymes in human hepatocytes. *Pharmaceutical research* **22**(1): 71-78.

Rakhshandehroo M, Hooiveld G, Muller M and Kersten S (2009) Comparative analysis of gene regulation by the transcription factor PPARalpha between mouse and human. *PloS one* **4**(8): e6796.

Ramaswamy G, Karim MA, Murti KG and Jackowski S (2004) PPARalpha controls the intracellular coenzyme A concentration via regulation of PANK1alpha gene expression. *Journal of lipid research* **45**(1): 17-31.

Rana R, Chen Y, Ferguson SS, Kissling GE, Surapureddi S and Goldstein JA (2010) Hepatocyte nuclear factor 4{alpha} regulates rifampicin-mediated induction of CYP2C genes in primary cultures of human hepatocytes. *Drug metabolism and disposition: the biological fate of chemicals* **38**(4): 591-599.

Raucy JL, Mueller L, Duan K, Allen SW, Strom S and Lasker JM (2002) Expression and induction of CYP2C P450 enzymes in primary cultures of human hepatocytes. *The Journal of pharmacology and experimental therapeutics* **302**(2): 475-482.

Roy A, Jana M, Corbett GT, Ramaswamy S, Kordower JH, Gonzalez FJ and Pahan K (2013) Regulation of cyclic AMP response element binding and hippocampal plasticity-related genes by peroxisome proliferator-activated receptor alpha. *Cell reports* **4**(4): 724-737.

Schoonjans K, Staels B and Auwerx J (1996) The peroxisome proliferator activated receptors (PPARS) and their effects on lipid metabolism and adipocyte differentiation. *Biochimica et biophysica acta* **1302**(2): 93-109.

Seree E, Villard PH, Pascussi JM, Pineau T, Maurel P, Nguyen QB, Fallone F, Martin PM, Champion S, Lacarelle B, Savouret JF and Barra Y (2004) Evidence for a new human CYP1A1 regulation pathway involving PPAR-alpha and 2 PPRE sites. *Gastroenterology* **127**(5): 1436-1445.

Synold TW, Dussault I and Forman BM (2001) The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux. *Nature medicine* **7**(5): 584-590.

Thomas M, Burk O, Klumpp B, Kandel BA, Damm G, Weiss TS, Klein K, Schwab M and Zanger UM (2013) Direct transcriptional regulation of human hepatic cytochrome P450 3A4 (CYP3A4) by peroxisome proliferator-activated receptor alpha (PPARalpha). *Molecular pharmacology* **83**(3): 709-718.

Totah RA and Rettie AE (2005) Cytochrome P450 2C8: substrates, inhibitors, pharmacogenetics, and clinical relevance. *Clinical pharmacology and therapeutics* **77**(5): 341-352.

Trajkovski M, Hausser J, Soutschek J, Bhat B, Akin A, Zavolan M, Heim MH and Stoffel M (2011) MicroRNAs 103 and 107 regulate insulin sensitivity. *Nature* **474**(7353): 649-653.

Villard PH, Barlesi F, Armand M, Dao TM, Pascussi JM, Fouchier F, Champion S, Dufour C, Ginies C, Khalil A, Amiot MJ, Barra Y and Seree E (2011) CYP1A1 induction in the colon by serum: involvement of the PPARalpha pathway and evidence for a new specific human PPREalpha site. *PloS one* **6**(1): e14629.

Wahli W and Michalik L (2012) PPARs at the crossroads of lipid signaling and inflammation. *Trends in endocrinology and metabolism: TEM* **23**(7): 351-363.

Waxman DJ (1999) P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR, and PPAR. *Arch Biochem Biophys* **369**(1): 11-23.

Wilfred BR, Wang WX and Nelson PT (2007) Energizing miRNA research: a review of the role of miRNAs in lipid metabolism, with a prediction that miR-103/107 regulates human metabolic pathways. *Molecular genetics and metabolism* **91**(3): 209-217.

Wray J and Bishop-Bailey D (2008) Epoxygenases and peroxisome proliferator-activated receptors in mammalian vascular biology. *Experimental physiology* **93**(1): 148-154.

Yamakuchi M, Lotterman CD, Bao C, Hruban RH, Karim B, Mendell JT, Huso D and Lowenstein CJ (2010) P53-induced microRNA-107 inhibits HIF-1 and tumor angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **107**(14): 6334-6339.

Zeldin DC (2001) Epoxygenase pathways of arachidonic acid metabolism. *The Journal of biological chemistry* **276**(39): 36059-36062.

Zhang SY, Surapureddi S, Coulter S, Ferguson SS and Goldstein JA (2012) Human CYP2C8 is post-transcriptionally regulated by microRNAs 103 and 107 in human liver. *Molecular pharmacology* **82**(3): 529-540.

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Footnotes

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Figure Legends

Fig.1. miR-107 expression is induced by BF in human primary hepatocytes and HepG2 cells. (A) Human PANK1 gene structure (adapted from (Yamakuchi et al., 2010). miR-107 is intronic to PANK1 gene and is co-regulated with PANK1 gene. The gene encoding miR-107 is shown in black. PANK1 gene untranslated and translated exons are shown in white and gray, respectively. (B) miR-107 but not miR-103 expression is induced by BF in primary human hepatocytes. Human primary hepatocytes were treated with BF (0.5 mM) for 24 h or 48 h and miRNA expression was measured by quantitative PCR normalized to RNU44. Data represent human hepatocytes isolated from at least three different donors as shown in table 2. Each donor sample was analyzed in triplicates and data indicate mean ± SEM from at least three different donors. ***, $p < 0.001$, significantly different compared with DMSO control (Student's *t*-test). (C) PANK1/pri-miR107 expression is upregulated by exposure of human primary hepatocytes with BF at 24 h and 48 h. Data represent human hepatocytes isolated from four different donors as shown in table 2. Each donor sample was analyzed in triplicates and data indicate means ± SEM from at least three different donor hepatocyte preparations. **, $p < 0.01$, significantly different compared with DMSO control; ***, $p < 0.001$, significantly different compared with DMSO control (Student's *t*-test); (D). miR-107 is induced by BF in HepG2 cells and the induction is depended on activation of PPARα. ***, $p < 0.001$, significantly different compared with vector control (One-way ANOVA followed by Tukey's test).

Fig.2. Human CYP2C8 mRNA levels were induced by BF in primary human hepatocytes. A, B and C represent human hepatocytes isolated from at least three different donors as shown in table 2. CYP2C8, CYP2C9 and CYP2C19 mRNA expression in cultured human hepatocytes

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following treatment with 0.5 mM BF for 24 h (A) and (B) 48 h were analyzed by quantitative PCR. CYP2C8 mRNA was significantly increased 24 and 48 h after BF ($p<0.001$) compared to minimal increases * ($p<0.05$) in CYP2C9 and CYP2C19 mRNA 48 h after BF compared with DMSO control. CPT1A and ACOX, known PPARα target genes were dramatically increased by BF (***, $p < 0.001$) compared with DMSO control (Student's *t*-test). (C) Human primary hepatocytes were treated with 0.125 mM, 0.25 mM, 0.5 mM and 0.75 mM BF for 48 h. Total RNA was extracted and gene expression examined by quantitative PCR. Each donor sample was analyzed in triplicates and data indicate mean ± SEM of at least three different donor preparations. *, $p < 0.05$, significantly different compared with DMSO control; **, $p < 0.01$, significantly different compared with DMSO control; ***, $p < 0.001$, significantly different compared with DMSO control (One-way ANOVA followed by Tukey's test).

Fig.3. CYP2C8 but not CYP2C9 protein levels were induced by BF in human primary hepatocytes. Human primary hepatocytes isolated from between three to five different donors were treated with either DMSO control or 0.5 mM BF for 48 h. Microsomes prepared from DMSO control or BF-treated cells were analyzed by Western blot for CYP2C proteins. The figure is a representative blot. HLM: Human liver microsomes (Gentest). The following recombinant yeast CYP2C protein controls were used: 2C9 (CYP2C9), 2C8 (CYP2C8) and 2C19 (CYP2C19). 1590 is an anti-CYP2C antibody while 1937 is a specific antibody for a CYP2C8 peptide which does not cross-react with other human CYP2C proteins. The specificity of the antibodies is presented in Materials and Methods. (B) Densitometry analysis of Western blot using Image J software. Data indicate mean ± SEM calculations of a minimum of three

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independent donor preparations. ***, $p < 0.001$, significantly different compared with DMSO control (Students *t*-test).

Fig.4. *CYP2C8* promoter luciferase activity is transactivated by ectopic expression of PPARα and treatment with BF in HepG2 cells. (A) Activation of ectopic expressed PPARα by BF transcriptionally activates *CYP2C8* luciferase activity. HepG2 cells were co-transfected with 2C8-3k luciferase reporter construct (spanning -2966 to +1 of the *CYP2C8* promoter) together with control vector (pcDNA3.1), human PPARα (hPPARα) or mouse PPARα (mPPARα) expression plasmids as indicated. Transfected cells were subsequently exposed to either DMSO (0.1%) or 0.5 mM BF. (B) Transfected cells were treated with either DMSO (0.1%) or 50 μM WY14,643. Cells were harvested and luciferase activity was normalized to renilla luciferase activity. Results were expressed as fold induction from at least three independent experiments compared with that of vector control. (C) HepG2 cells were co-transfected with *2C8*-3k luc together with control vector (pcDNA3.1) or human PPARγ (hPPARγ) expression plasmids. Transfected cells were treated with either DMSO (0.1%) or 5 μM rosiglitazone (D) HepG2 cells were co-transfected with *2C8*-3k, *2C9*-3k or *2C19*-2.7k luciferase reporter constructs together with either control vector (pcDNA3.1) or human PPARα (hPPARα) expression plasmids. Transfected cells were treated with either DMSO (0.1%) or 0.5 mM BF for 24 h (E) HepG2 cells were transfected with PPRE-X3-TK luciferase constructs (contains three copies of ACOX1-PPRE upstream of TK gene promoter) and either control vector (pcDNA3.1), human (hPPARα) or mouse PPARα expression plasmids. Transfected cells were treated with either DMSO (0.1%) or 0.5 mM BF for 24 h. The PPRE-X3-TK is a positive control for PPARα transcription regulation. Luciferase activity was normalized to renilla luciferase and results were expressed as

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fold induction from at least three independent experiments compared with that of control transfected cells. *, $p < 0.05$, significantly different compared with pcDNA3/DMSO treated cells; **, $p < 0.01$, significantly different compared with pcDNA3/DMSO treated cells ***, $p < 0.001$, significantly different compared with pcDNA3/DMSO treated cells (One-way ANOVA followed by Tukey's test); †, $p < 0.05$, significantly different compared with PPARα/DMSO treated cells (One-way ANOVA followed by Tukey's test).

Fig. 5. PPARα mediated regulation of C*YP2C8* expression is mediated by a PPRE (DR1-B) located at -2109 bp upstream of the translation start site. (A) Computer analysis of the *CYP2C8* promoter showed five putative PPRE sites located at -2772 (DR1-A), -2109 (DR1-B), -2039 (DR1-C), -1501 (DR1-D) and -152 (DR1-E) bp relative to the translation start site. (B) HepG2 cells were transiently transfected with various luciferase constructs containing indicated sequences of *CYP2C8* promoter co-transfected with hPPARα expression plasmids. Twenty four hours after transfection, cells were treated with either DMSO (0.1%) or BF (0.5 mM) for 24 h. Cells were harvested and luciferase and renilla luciferase activity measurement. Results were expressed as fold induction compared to DMSO treated cells. ***, $p < 0.001$, significantly different compared with DMSO-treated cells (One-way ANOVA followed by Tukey's test).

Fig. 6. Specific binding of PPARα to the DR1-B at -2109 bp of the *CYP2C8* promoter by gel shift assay. (A) Sequences of the oligonucleotides used for the binding assays. Putative and known PPREs are written in red text and the DR1 half sites are underlined. GG in DR1B half sites were mutated to CC to generate DR1BM. (A) ^{32}P-labeled double-stranded oligonucleotides containing *CYP2C8* DR1-A, DR1-B, DR1-C, DR1-D and a positive control for PPARα binding

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(PPRE from rat ACOX1 gene) were incubated for 30 min with in vitro transcribed/translated RXRα or hPPARα and RXRα proteins at 4°C (B) ^{32}P-labeled double-stranded oligonucleotides containing *CYP2C8* DR1-B and the known PPRE (rat ACOX1) were incubated for 30 min with in vitro translated hPPARα and RXRα at 4C. In competition experiments, a 100-fold excess of unlabeled double-stranded oligonucleotide inhibited formation of the complex. Supershift experiments were then performed by incubating the binding reactions with 4 μg of antibodies to PPARα, RXRα or IgG for 2 h at 4˚C.

Fig. 7. ChIP analysis showed in vivo binding of PPARα and RXRα to the *CYP2C8* DR1-B sites. (A) CYP2C8 promoter with position of primers used for ChIP PCR analysis. (B) Chromatin was prepared from cultured primary human hepatocytes treated with DMSO control or BF (0.5 mM) for 48 h, sheared, immunoprecipitated with IgG (negative control), PPARα, RXRα or RNA Pol II (positive control for transcriptional active genes) antibodies conjugated to Dynabeads protein A/G. PCR was performed with primers spanning the *CYP2C8* DR1-B, *CYP2C8* promoter region that does not contain DR1 (negative control), the PPRE of human HMGCR promoter (positive control for PPARα and RXRα binding), human α-actin coding region (a negative control for all antibodies). (C) Chromatin extracted from control and BF treated primary human hepatocytes were immunoprecipitated with antibodies specific to PPARα and IgG. SYBR green qPCR was performed using primers flanking the *CYP2C8* DR1-B, the *CYP2C8* promoter region that does not contain DR1 (negative control), the PPRE of human HMGCR and α-actin. Data represent human hepatocytes isolated from at least three different donors as shown in table 2. Each treatment was analyzed in triplicates and data indicate mean ± SEM from at least three different donor preparations. ***, $p < 0.001$, significantly different compared with DMSO

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control (Students *t*-test).

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Table 1. Direct repeat 1 (DR1) identified in the *CYP2C8* promoter

Designation	Position	5'-flank region	Sequence (Strand)
DR1-A	-2772	CAACCAA	AGTTCG A AGTTCA (-)
DR1-B	-2109	TTTATCC	AGGGCA A AGGGAA (-)
DR1-C	-2039	AAAATGT	GGGTCA G ACGTGT (+)
DR1-D	-1501	GAACTGA	TAGTCA A TGGTCA (-)
DR1-E	-152	TATCCAT	GGGCCA A AGTCCA (-)
Consensus		CAAAA/TCT	AGGTCA A AGGTCA

DR1, direct repeat with distance of one base

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Table 2. Clinical histories of human donors

Donor	Age (year)	Sex	Cause of death	Medication	Smoking/Alcohol
HUM4078	24	Male	Stroke	None reported	No/No
HUM4083	46	Female	Stroke	None reported	No/Social
HUM4089	36	Male	Stab wound	None reported	Quit 1 year ago /No
HUM4096	34	Female		None	No/Occasional
HUM4097	53	Female		None	No/Occasional

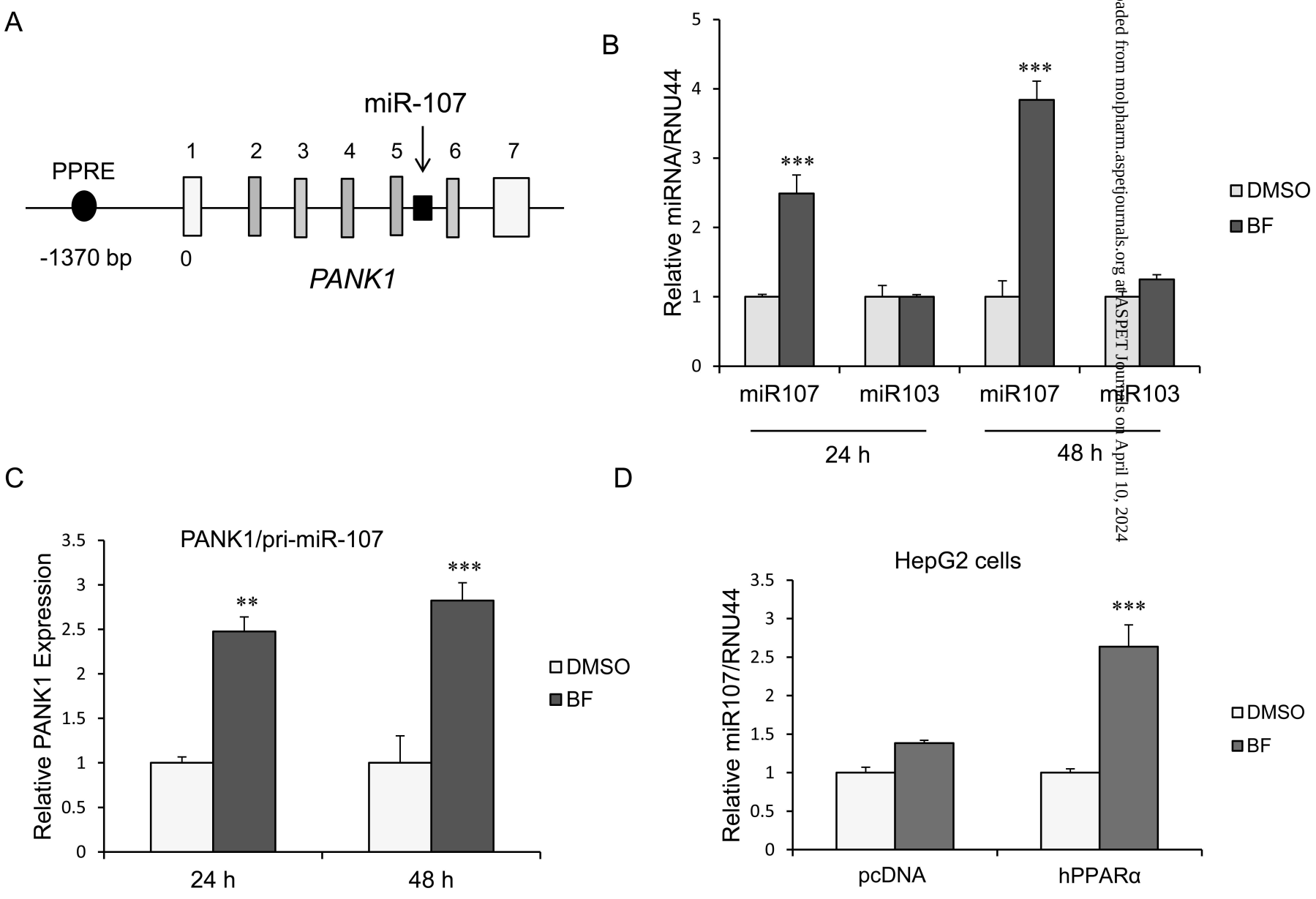


Figure 1

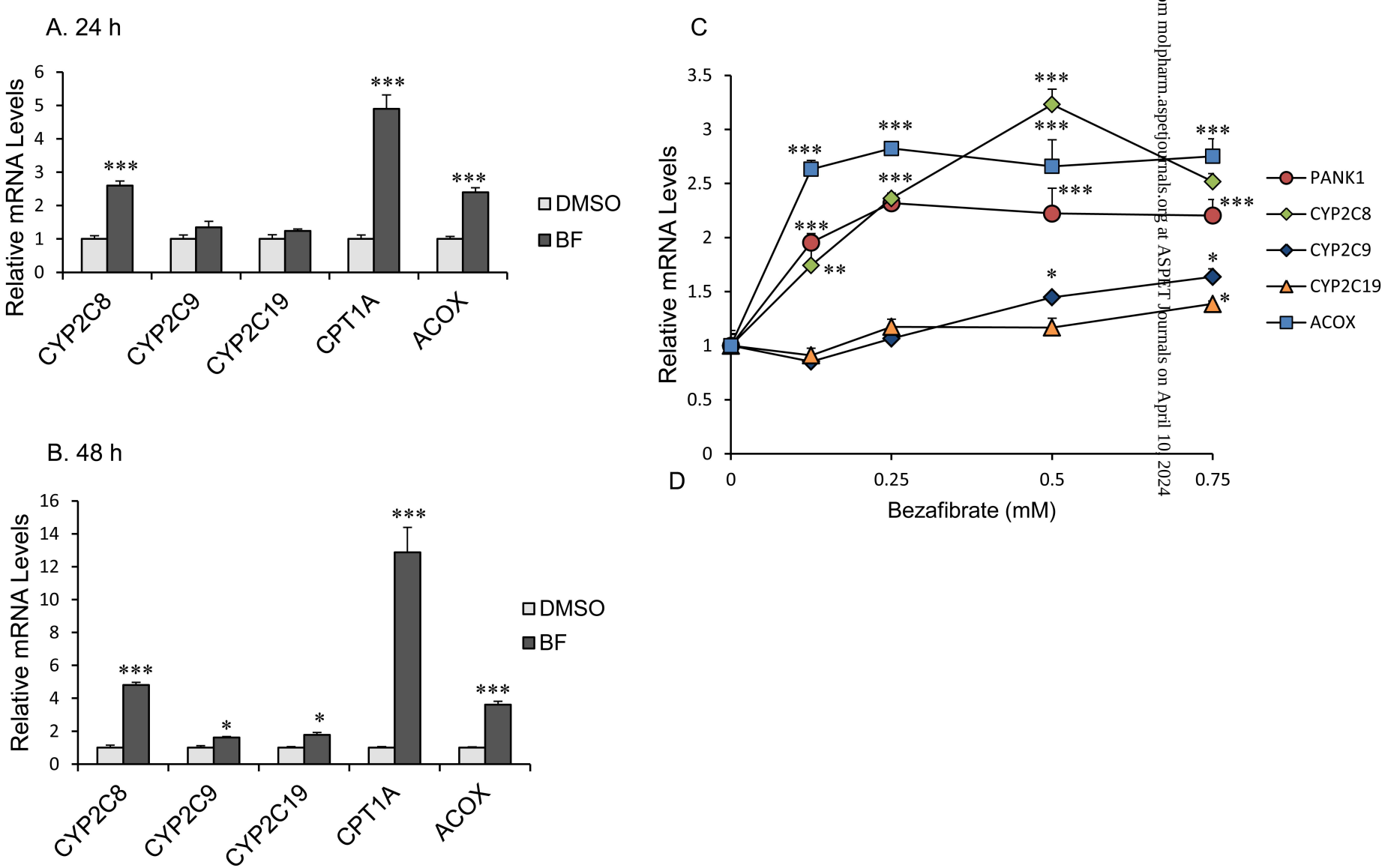


Figure 2

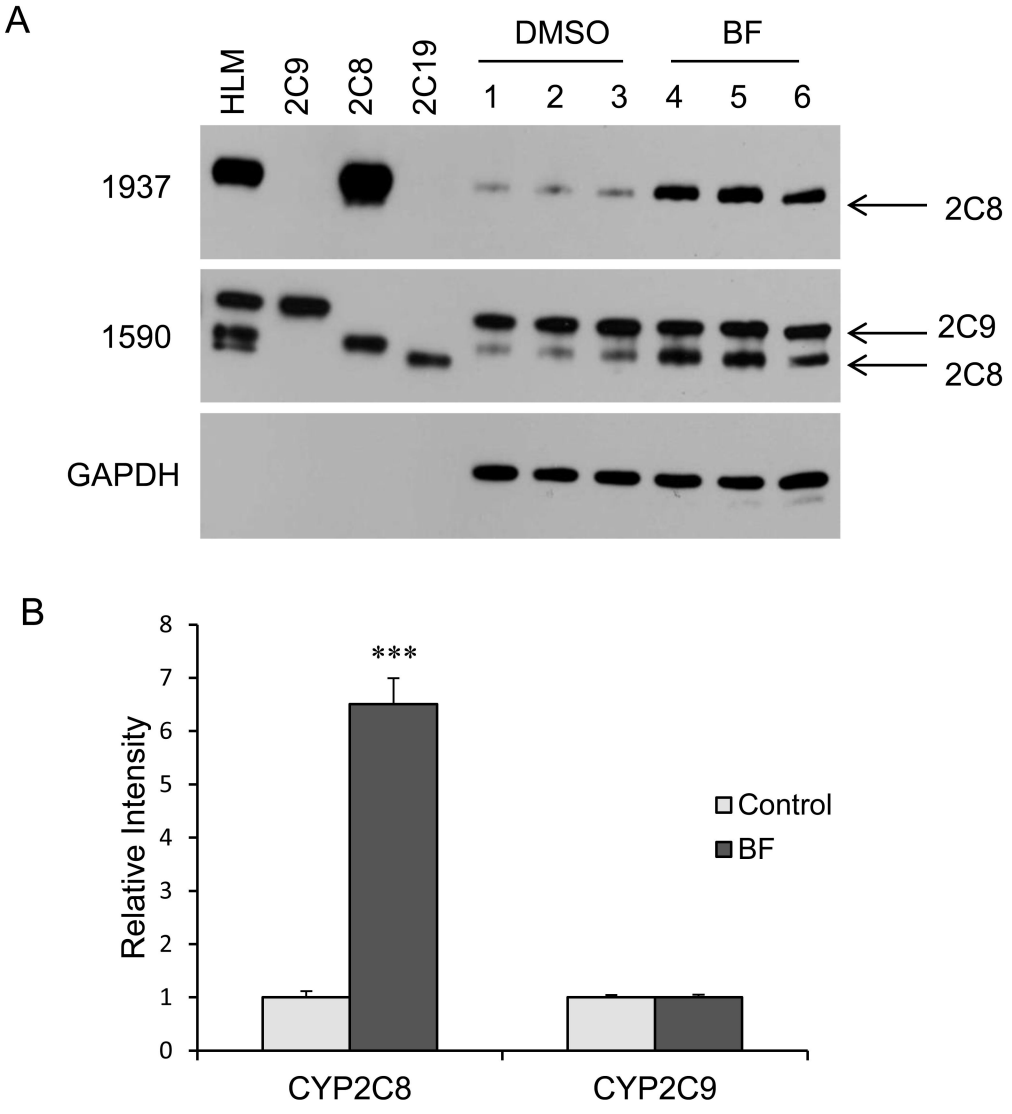
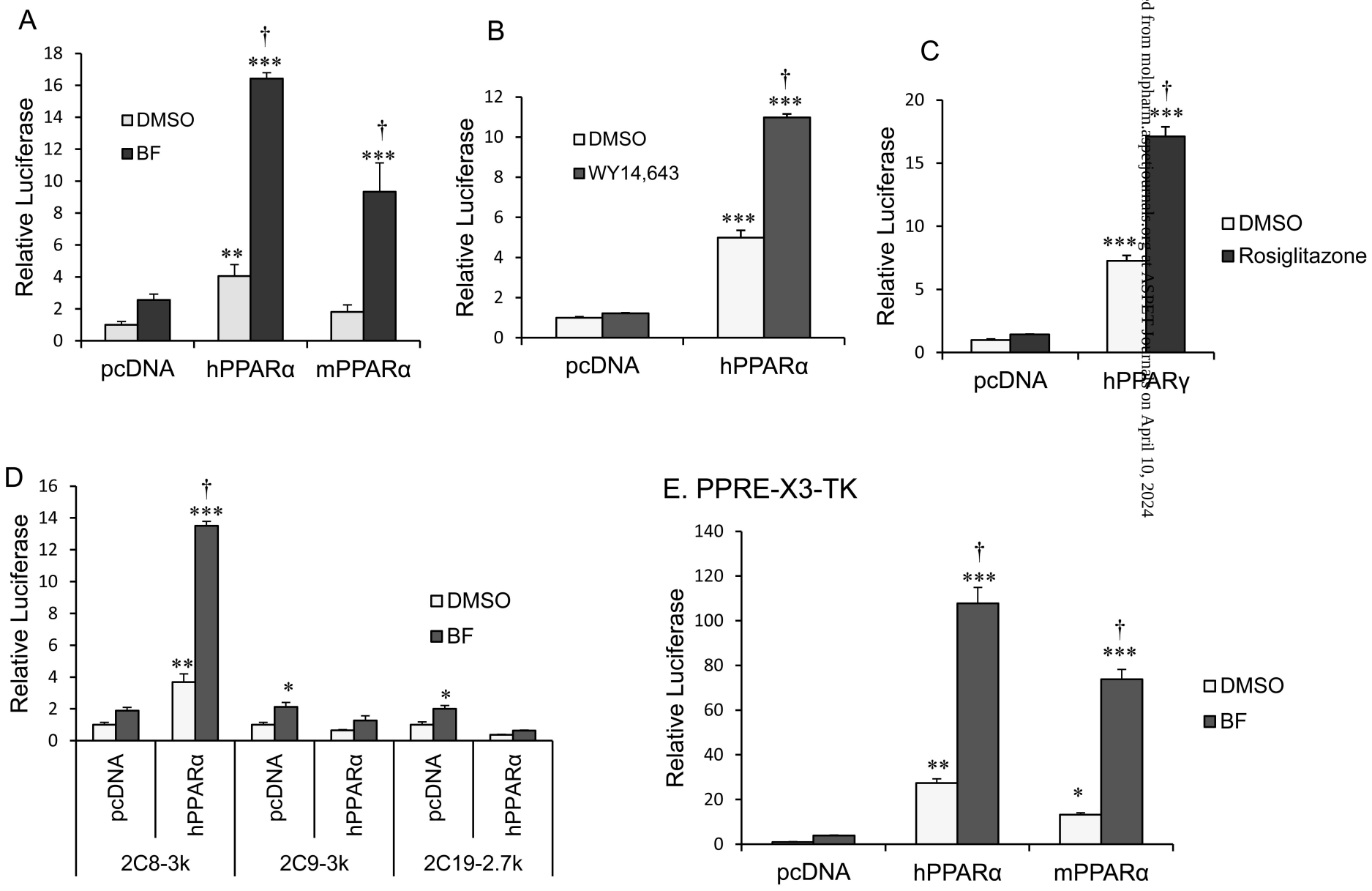


Figure 3



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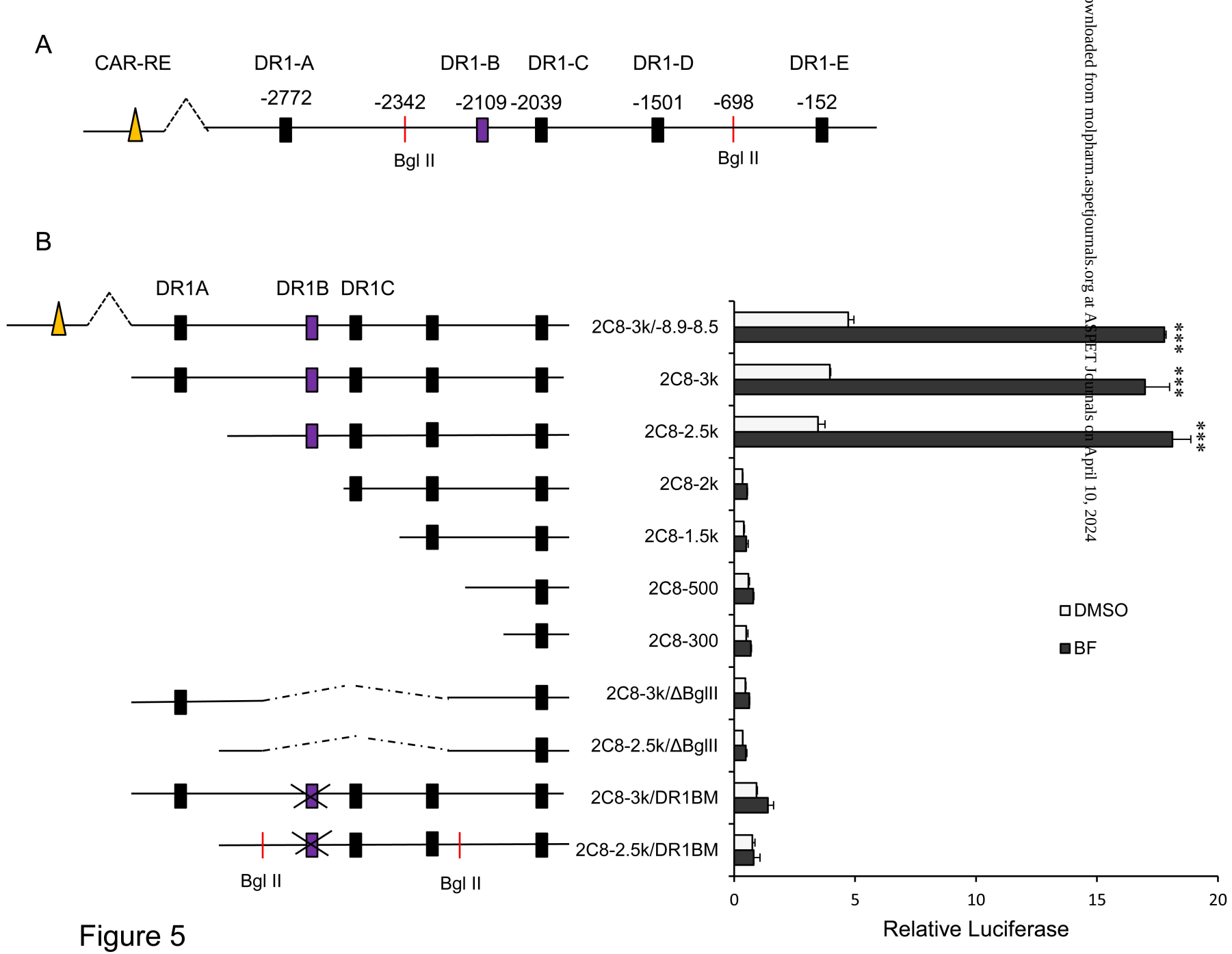


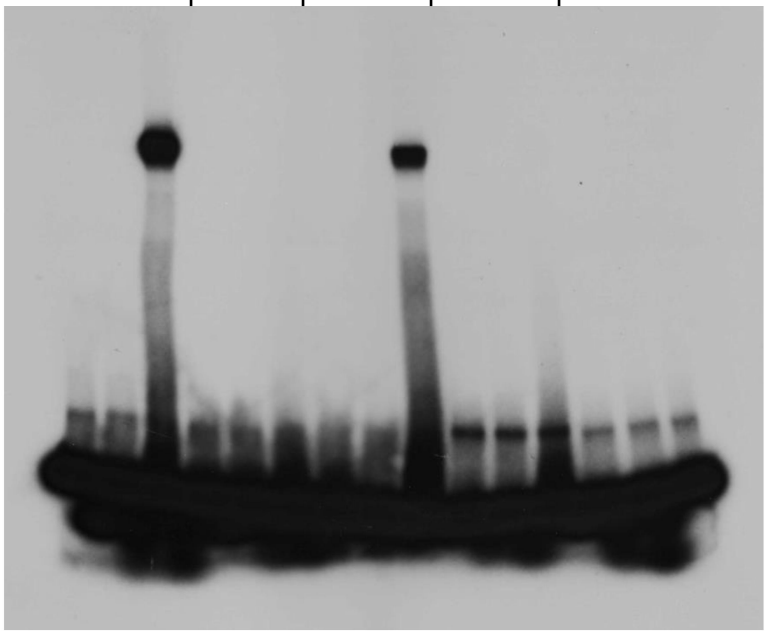
Figure 5

A

DR1-A: 5'-CATCAACCAAAGTTCGAAGTTCACATAGGGT -3‘
DR1-B: 5'- CCTTTATCCAGGGCAAAGGGAAGTAGTAATT-3‘
DR1BM: 5'- CCTTTATCCACCGCAAACCGAAGTAGTAATT-3
DR1-C: 5'- GTAAAATGTGGGTCAGACGTGTTTGGTTTTA-3‘
DR1-D: 5’- TAAAAAGAAAGGTCAAGGCAGGAGCCTCAGC-3’
PPRE (ACOX): 5’-CAGGGGACCAGGACAAAGGTCACGTTCGGGA-3’

B

Probe	PPRE			DR1-A			DR1-B			DR1-C			DR1-D		
PPARα	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+
RXRα	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+



C

	PPRE						DR1-B						
PPARα	-	+	+	+	+	+	-	+	+	+	+	+	+
RXRα	-	+	+	+	+	+	-	+	+	+	+	+	+
Cold PPRE	-	-	+	-	-	-	-	-	-	-	-	-	-
Cold DR1-B	-	-	-	-	-	-	-	-	+	-	-	-	-
Cold DR1BM	-	-	-	-	-	-	-	-	-	+	-	-	-
anti-PPARα	-	-	-	+	-	-	-	-	-	-	+	-	-
anti-RXRα	-	-	-	-	+	-	-	-	-	-	-	+	-
anti-IgG	-	-	-	-	-	+	-	-	-	-	-	-	+

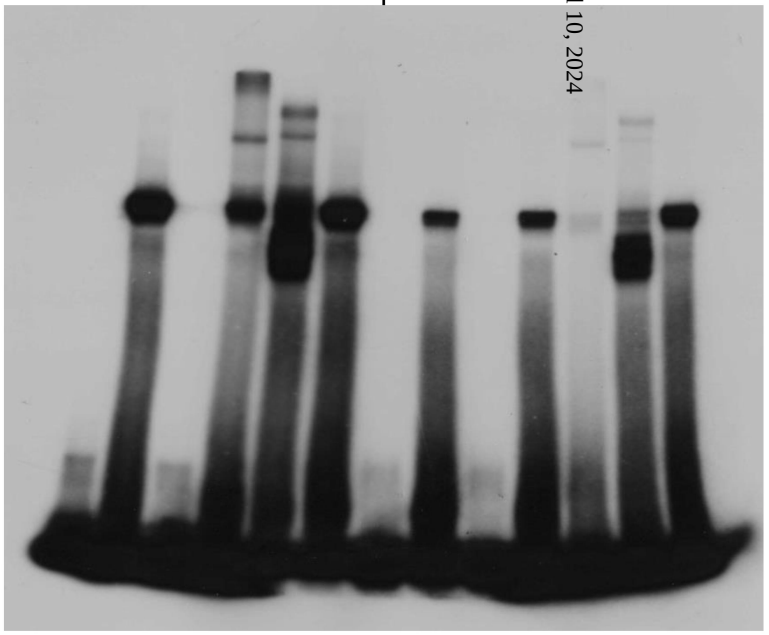


Figure 6

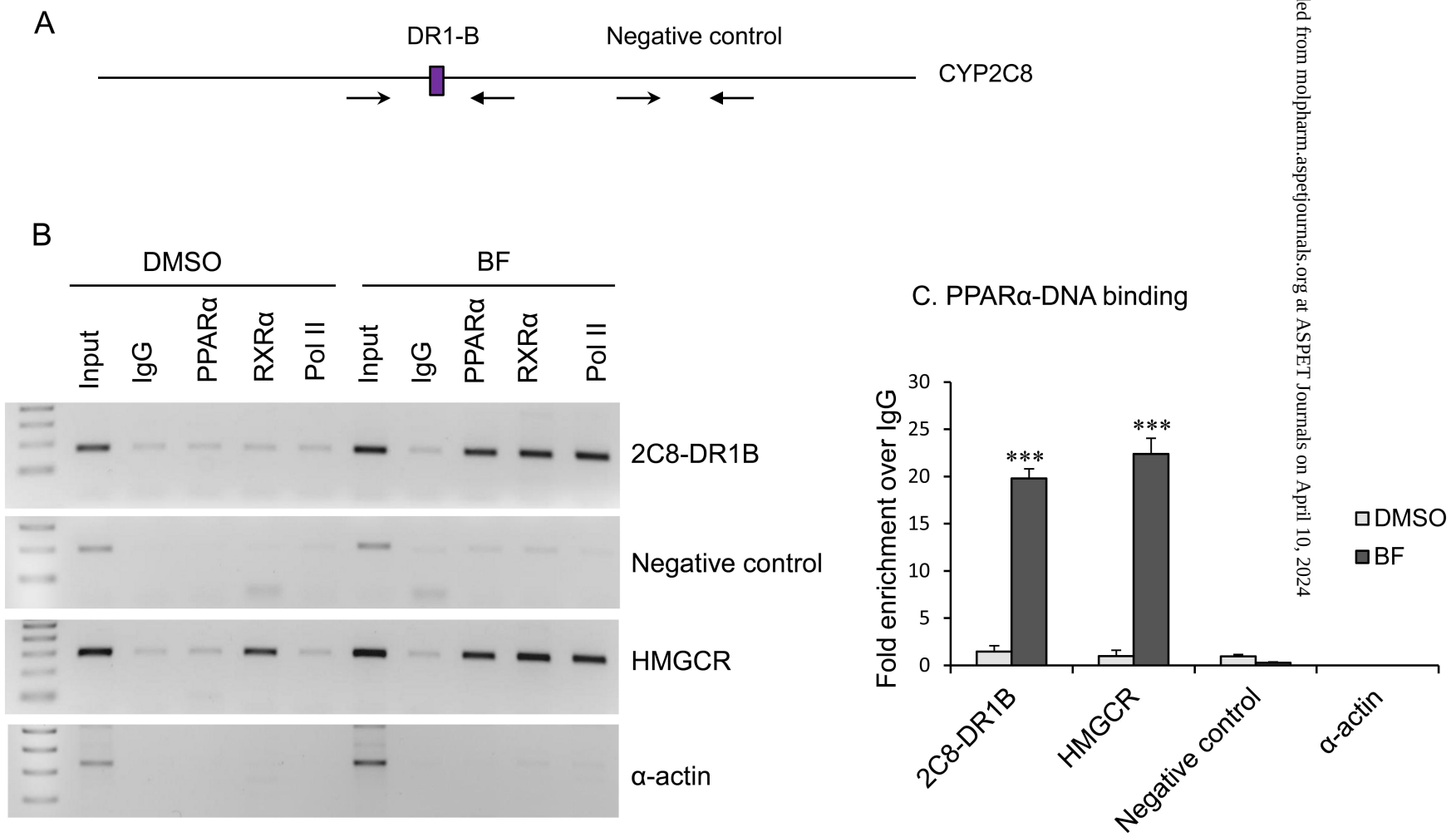


Figure 7