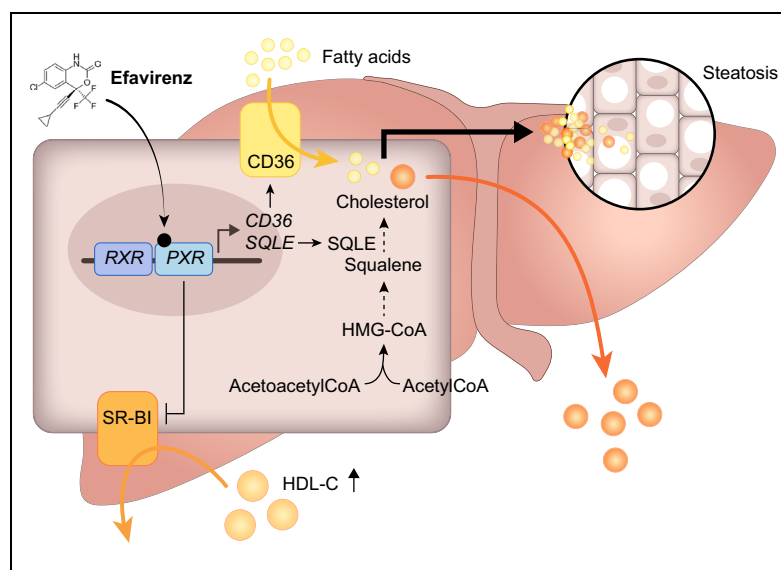


Non-nucleoside reverse transcriptase inhibitor efavirenz activates PXR to induce hypercholesterolemia and hepatic steatosis

Graphical abstract



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Lay summary

Efavirenz is widely prescribed for HIV-infected patients but has some side effects. It can increase lipid levels in patients' blood and liver. Here we show that efavirenz can activate a unique liver protein called PXR which mediates the adverse effects of efavirenz on lipid levels in mouse models.

Highlights

- Efavirenz is widely prescribed for patients with HIV.
- Efavirenz increases the risk of dyslipidemia and hepatic steatosis in patients.
- Efavirenz is a potent agonist of the nuclear receptor PXR.
- Efavirenz activates PXR to elicit the adverse effects on lipid homeostasis in mice.
- PXR regulates the expression of several key lipogenic genes in the liver.



Non-nucleoside reverse transcriptase inhibitor efavirenz activates PXR to induce hypercholesterolemia and hepatic steatosis

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Background & Aims: The most prescribed non-nucleoside reverse transcriptase inhibitor, efavirenz, has been associated with elevated risk of dyslipidemia and hepatic steatosis in HIV-infected patients but the underlying mechanisms remain elusive. Herein, we investigated the role of pregnane X receptor (PXR) in mediating the adverse effects of efavirenz on lipid homeostasis.

Methods: Cell-based reporter assays, primary cell culture, and multiple mouse models including conditional knockout and humanized mice were combined to study the impact of efavirenz on PXR activities and lipid homeostasis *in vitro* and *in vivo*. A novel liver-specific *Pxr* knockout mouse model was also generated to determine the contribution of hepatic PXR signaling to efavirenz-elicited dyslipidemia and hepatic steatosis.

Results: We found that efavirenz is a potent PXR-selective agonist that can efficiently activate PXR and induce its target gene expression *in vitro* and *in vivo*. Treatment with efavirenz-induced hypercholesterolemia and hepatic steatosis in mice but deficiency of hepatic PXR abolished these adverse effects. Interestingly, efavirenz-mediated PXR activation regulated the expression of several key hepatic lipogenic genes including fatty acid transporter CD36 and cholesterol biosynthesis enzyme squalene epoxidase (SQLE), leading to increased lipid uptake and cholesterol biosynthesis in hepatic cells. While CD36 is a known PXR target gene, we identified a DR-2-type of PXR-response element in the SQLE promoter and established SQLE as a direct transcriptional target of PXR. Since PXR exhibits considerable differences in its pharmacology across species, we also confirmed these findings in PXR-humanized mice and human primary hepatocytes.

Conclusions: The widely prescribed antiretroviral drug efavirenz induces hypercholesterolemia and hepatic steatosis by activating PXR signaling. Activation of PXR should be taken into consideration for patients undergoing long-term treatment with PXR agonistic antiretroviral drugs.

Lay summary: Efavirenz is widely prescribed for HIV-infected patients but has some side effects. It can increase lipid levels in patients' blood and liver. Here we show that efavirenz can activate a unique liver protein called PXR which mediates the adverse effects of efavirenz on lipid levels in mouse models.

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Introduction

As the average lifespan of HIV-infected patients receiving antiretroviral therapy (ART) lengthens, morbidity and mortality from cardiovascular disease (CVD) pose considerable challenges.^{1–4} Not only does HIV infection elevate the risk of CVD, but the use of antiretroviral (ARV) drugs has also been associated with dyslipidemia and increased risk of CVD, thereby exacerbating a serious health challenge for long-term survivors.^{1–2,4} Current optimal ART options consist of a combination of several drug classes including protease inhibitors (PIs), nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitor (NNRTIs), and integrase strand transfer inhibitors (INSTIs, also known as integrase inhibitors).^{2,3} Although several first-generation PIs such as amprenavir and ritonavir with documented dyslipidemic properties have been used on a limited basis, many currently recommended first-line ARV drugs have also been associated with dyslipidemia and increased CVD risk.^{2–6} For example, the NNRTI efavirenz, one of the most prescribed antiretroviral drugs worldwide, has been consistently associated with dyslipidemia including increased total, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol levels in multiple clinical studies.^{2,5–7} Despite the strong evidence linking certain ARV drugs with dyslipidemia and CVD risk, the underlying mechanisms responsible for the adverse effects of ARV drugs remain elusive.

Alongside others, we have previously identified several HIV PIs, including amprenavir and ritonavir, as potent agonists of pregnane X receptor (PXR); also known as steroid and xenobiotic receptor, or NR1I2).^{8,9} PXR is a nuclear receptor activated by numerous endogenous hormones, dietary steroids, pharmaceutical agents, and xenobiotic chemicals.^{10–12} PXR functions as a xenobiotic sensor that induces the expression of genes required for xenobiotic metabolism in the liver and intestine.^{11,12} It has recently been demonstrated that PXR signaling also plays an important role in lipid homeostasis.^{9,12–15} PXR signaling has

Keywords: HIV; Antiretroviral drugs; Dyslipidemia; Hepatic steatosis; Pregnane X receptor; Squalene epoxidase.

Received 11 September 2018; received in revised form 17 December 2018; accepted 23 December 2018; available online 21 January 2019

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also been implicated in contributing to ARV drugs' dyslipidemic effects. For example, treatment with ritonavir, a potent PXR activator,⁸ caused hyperlipidemia and was associated with increased risk of CVD in patients with HIV.¹⁶ We also demonstrated that another PI, amprenavir, can activate PXR to elevate plasma cholesterol levels in mice.⁹

In the present study, we tested currently recommended ARV drugs, and identified several widely prescribed ARV drugs such as efavirenz as potent PXR agonists. Interestingly, treatment with efavirenz-stimulated hypercholesterolemia and hepatic steatosis in mice in a PXR-dependent manner. Efavirenz-mediated PXR activation altered the expression of several key hepatic lipogenic genes including fatty acid transporter CD36 and cholesterol biosynthesis enzyme squalene epoxidase (SQLE), leading to increased lipid uptake and cholesterol synthesis in hepatic cells.

Materials and methods

Animals

C57BL/6 wild-type (WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The PXR-humanized (hPXR-mPxr^{-/-}) and Pxr-null (mPxr^{-/-}) mice on C57BL/6 background have been described before.^{17,18} The hPXR-mPxr^{-/-} and mPxr^{-/-} mice used in this study were littermates which had the same background (mPxr null alleles) except for one allele of hPXR-mPxr^{-/-} mice carrying the human PXR gene. To investigate the tissue-specific role of PXR in xenobiotic and lipid metabolism, we have obtained mouse embryonic stem cell clones containing a conditional Pxr-flox allele from International Knockout Mouse Consortium (EUMMCR, EPD0141_1_G04) and generated mice carrying Pxr-flox alleles (Pxr^{F/F}) on a C57BL/6 background. Pxr^{F/F} mice were further crossed with Albumin-Cre transgenic mice (The Jackson Laboratory) to generate hepatocyte-specific PXR-deficient mice (termed as Pxr^{ΔHep}). The Pxr^{F/F} and Pxr^{ΔHep} mice used in this study were also littermates and Pxr^{ΔHep} mice carried heterozygous knock-in for Albumin-Cre. All animals were housed in a pathogen-free environment with a light-dark cycle, under a protocol approved by the University of Kentucky Institutional Animal Care and Use Committee. For the treatment, 8-week-old male mice were fed a semisynthetic low-fat AIN76 diet containing 0.02% cholesterol (Research Diet; New Brunswick, NJ)^{9,13} and treated by oral gavage with vehicle (corn oil), 25, 50, or 100 mg/kg body weight efavirenz or emtricitabine daily for 1 week. On the day of euthanasia, mice were fasted for 6 h following the dark cycle (feeding cycle) and blood and tissues were then collected as previously described.^{9,13}

Statistical analysis

All data are presented as the mean ± SEM. Individual pairwise comparisons were analyzed by 2-sample, 2-tailed Student's *t* test unless otherwise noted, with *p* < 0.05 was regarded as significant. One-way analysis of variance (ANOVA) was used when multiple comparisons were made, followed by Dunnett's *t* test for multiple comparisons to a control. Two-way ANOVA was used when multiple comparisons were made followed by a Bonferroni multiple comparisons test. N numbers are listed in figure legends.

For further details regarding other materials and methods, please refer to the [CTAT table](#) and [supplementary information](#).

Results

Currently recommended ARV drugs including efavirenz are potent PXR agonists

We first tested currently recommended ARV drugs from commonly used drug classes including NNRTI, NRTI, PI, and INSTI by transfections assays (Fig. 1A and B). Since PXR exhibits considerable differences in its pharmacology across species,¹¹ the potent PXR ligands pregnenolone 16 α -carbonitrile (PCN) and rifampicin (RIF) were used as the positive control for mouse (m) and human (h) PXR, respectively. We found that several widely prescribed ARV drugs, including NNRTI efavirenz and PIs darunavir and lopinavir can potentially activate both human and mouse PXR (Fig. 1A and B). Rilpivirine and lopinavir can also affect PXR activity but they are relatively weak agonists for PXR. By contrast, the NRTIs emtricitabine, lamivudine, and tenofovir, as well as the INSTI raltegravir had no effects on either mouse or human PXR activities. Efavirenz is one of the most prescribed ARV drugs for treatment of HIV infection worldwide and dose-response analysis showed that efavirenz can activate hPXR at concentrations in the low μ M range, with an EC50 of 4.7 μ M, which is comparable to potent PXR agonist RIF (Fig. 1C).

Efavirenz is a PXR-selective agonist that modulates the interactions between PXR and co-regulators

We next tested the ability of efavirenz to activate a panel of other nuclear receptors (Fig. 1D). Efavirenz can activate PXR but was unable to activate any of the other nuclear receptors such as liver X receptor (LXR) and peroxisome proliferator-activated receptor (PPAR). The NRTI emtricitabine did not affect the activities of PXR or the other nuclear receptors. Nuclear receptor co-regulators play key roles in nuclear receptor signaling. We found that efavirenz but not emtricitabine promoted the specific interactions between PXR and the co-activators steroid receptor coactivator-1 (SRC-1) and PPAR binding protein (PBP) (Fig. 1E). Unliganded PXR interacted with PXR co-repressors including nuclear receptor co-repressor (NCoR) and silencing mediator of retinoid and thyroid hormone (SMRT) (Fig. 1F), and efavirenz disrupted this interaction as did the potent human PXR ligand RIF. By contrast, emtricitabine did not affect PXR-co-repressor interactions. Taken together, binding of efavirenz to PXR inhibits PXR-co-repressor interaction and promotes PXR-co-activator recruitment, thereby inducing PXR transcriptional activation in a concentration-dependent manner.

Efavirenz stimulates PXR target gene expression *in vitro* and *in vivo*

Using mouse primary hepatocytes and human hepatoma HepaRG cells,⁹ we found that efavirenz induced the expression of bona fide PXR target genes in these hepatic cells (Fig. S1). As expected, treatment with emtricitabine had no effects on PXR target gene expression in those cells. To investigate the effects of efavirenz on PXR activity *in vivo*, WT mice were treated with vehicle control, 25, 50 or 100 mg/kg body weight of efavirenz daily by oral gavage for 1 week. Consistent with *in vitro* results, efavirenz treatment resulted in significantly increased hepatic expression of PXR target genes in a dose-dependent manner (Fig. 2A).

Efavirenz induces hypercholesterolemia and hepatic steatosis in WT mice

Efavirenz treatment has also been associated with increased hepatic steatosis and dyslipidemia in humans.^{5-7,19,20} We found that efavirenz treatment did not affect body weight but induced

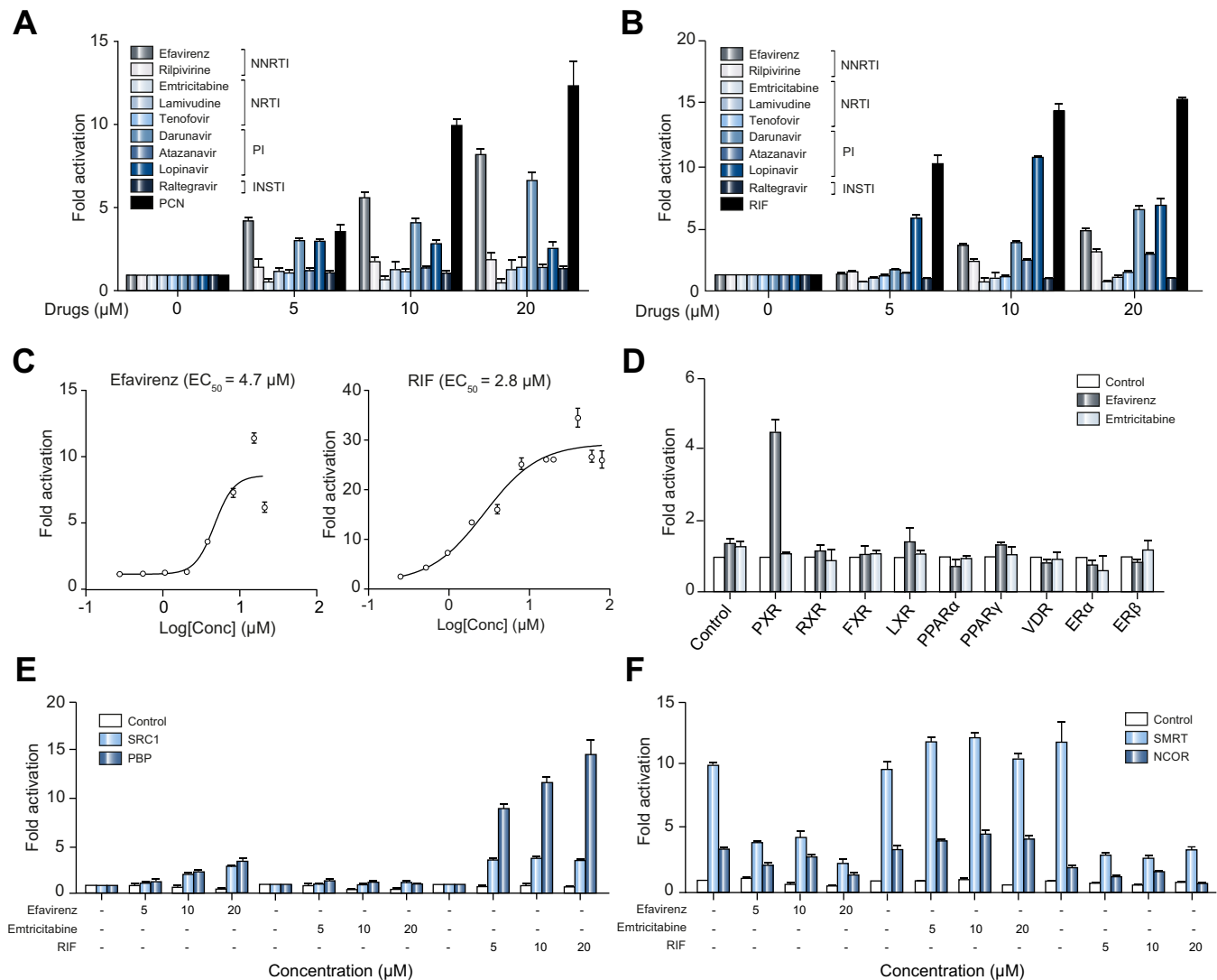


Fig. 1. Non-nucleoside reverse transcriptase inhibitor efavirenz is a potent PXR-selective agonist. (A and B) HepG2 cells were transfected with (A) full-length mPxr together with a mPxr reporter ((*Cyp3a2*)₃-luc) or (B) full-length hPXR together with hPXR reporter (*CYP3A4*-luc) and CMX-β-galactosidase control plasmid. Cells were then treated with DMSO control, ARV drugs, and PCN (mPxr ligand) or RIF (hPxr ligand) at the indicated concentrations for 24 h. (C) HepG2 cells were transfected with hPXR and *CYP3A4*-luc reporter together with CMX-β-galactosidase plasmid. Cells were then treated with efavirenz or RIF at the indicated concentrations for 24 h. (D) HepG2 cells were transfected with a GAL4 reporter and a series of GAL4 plasmids in which the GAL4 DNA-binding domain is linked to the indicated nuclear receptor ligand-binding domain. Cells were treated with DMSO control or 10 μM efavirenz or emtricitabine for 24 h. (E and F) HepG2 cells were transfected with a GAL4 reporter, VP16-hPXR vector, and expression vector for GAL4 DBD or GAL4 DBD linked to the receptor interaction domains of PXR co-activators (GAL4-SRC1 or GAL4-PBP) (E) or PXR co-repressors (GAL4-SMRT or GAL4-NCoR) (F). Cells were treated with DMSO control, efavirenz, emtricitabine, or RIF at the indicated concentrations for 24 h. Data are shown as fold induction of normalized luciferase activity compared with DMSO treatment and represent the mean of triplicate experiments. ARV, antiretroviral; DBD, DNA binding domain; INSTI, integrase strand transfer inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside/nucleotide reverse transcriptase inhibitor; PCN, pregnenolone 16α-carbonitrile; PI, protease inhibitors; RIF, rifampicin.

hepatomegaly (Fig. 2B) and hepatic steatosis in mice (Fig. 2C). Consistently, hepatic cholesterol and triglyceride contents were significantly increased in efavirenz-treated mice in a dose-dependent manner (Fig. 2D). WT mice treated with efavirenz also had increased total cholesterol levels in the plasma but unchanged triglycerides compared with control WT mice (Fig. 2E). Thus, efavirenz treatment can induce hypercholesterolemia and hepatic steatosis in WT mice.

Generation of liver-specific PXR knockout mice

PXR is expressed at high levels in the liver, an organ central for maintaining whole-body lipid homeostasis. To define the

liver-specific role of PXR in xenobiotic and lipid metabolism, we have successfully generated mice carrying *Pxr*-floxed alleles (*Pxr*^{F/F}) (Fig. S2). *Pxr*^{F/F} mice were further crossed with Albumin-Cre transgenic mice to generate hepatocyte-specific PXR-deficient mice (termed *Pxr*^{ΔHep}) (Fig. S2A). PCR analysis of genomic DNA indicated that the recombination was specific to the liver of *Pxr*^{ΔHep} mice (Fig. S2B). As expected, the mRNA levels and protein levels of PXR were significantly decreased in liver but not in other major tissues of *Pxr*^{ΔHep} mice compared to *Pxr*^{F/F} mice (Fig. 3A and B), which demonstrated the specific and efficient *Pxr* deletion in the liver of *Pxr*^{ΔHep} mice.

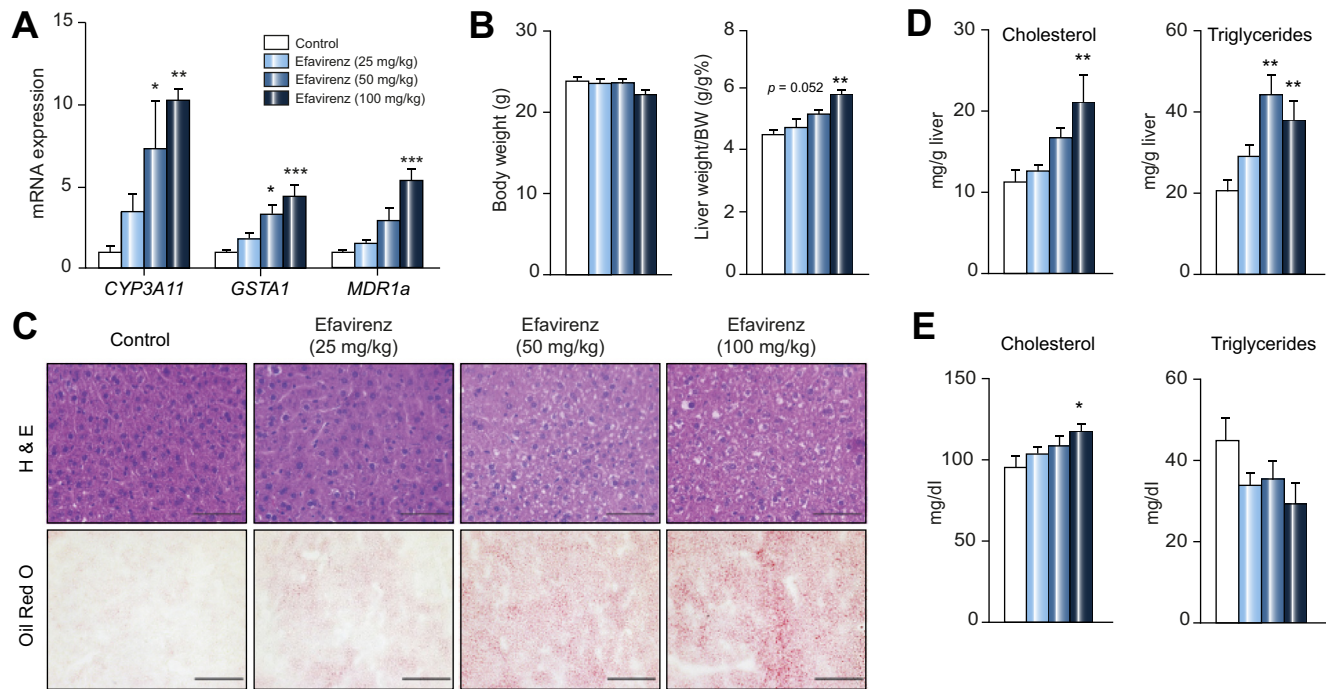


Fig. 2. Exposure to efavirenz induces hypercholesterolemia and liver steatosis in WT mice. Eight-week-old male WT mice were treated with vehicle, 25, 50, or 100 mg/kg/day of efavirenz by oral gavage for 1 week. (A) qPCR analysis of the hepatic expression of PXR target genes. (B) Body weight and the ratio of liver weight to body weight. (C) Hematoxylin and eosin (top, bar = 50 μ m) and Oil red O (bottom, bar = 100 μ m) stained liver sections. (D) Hepatic total cholesterol and triglyceride levels. (E) Plasma total cholesterol and triglyceride levels. Significance was determined by 1-way ANOVA (A, B, D, and E). (n = 5–6, * p < 0.05, ** p < 0.01, and *** p < 0.001 compared to control group). qPCR, quantitative PCR; WT, wild-type. (This figure appears in colour on the web.)

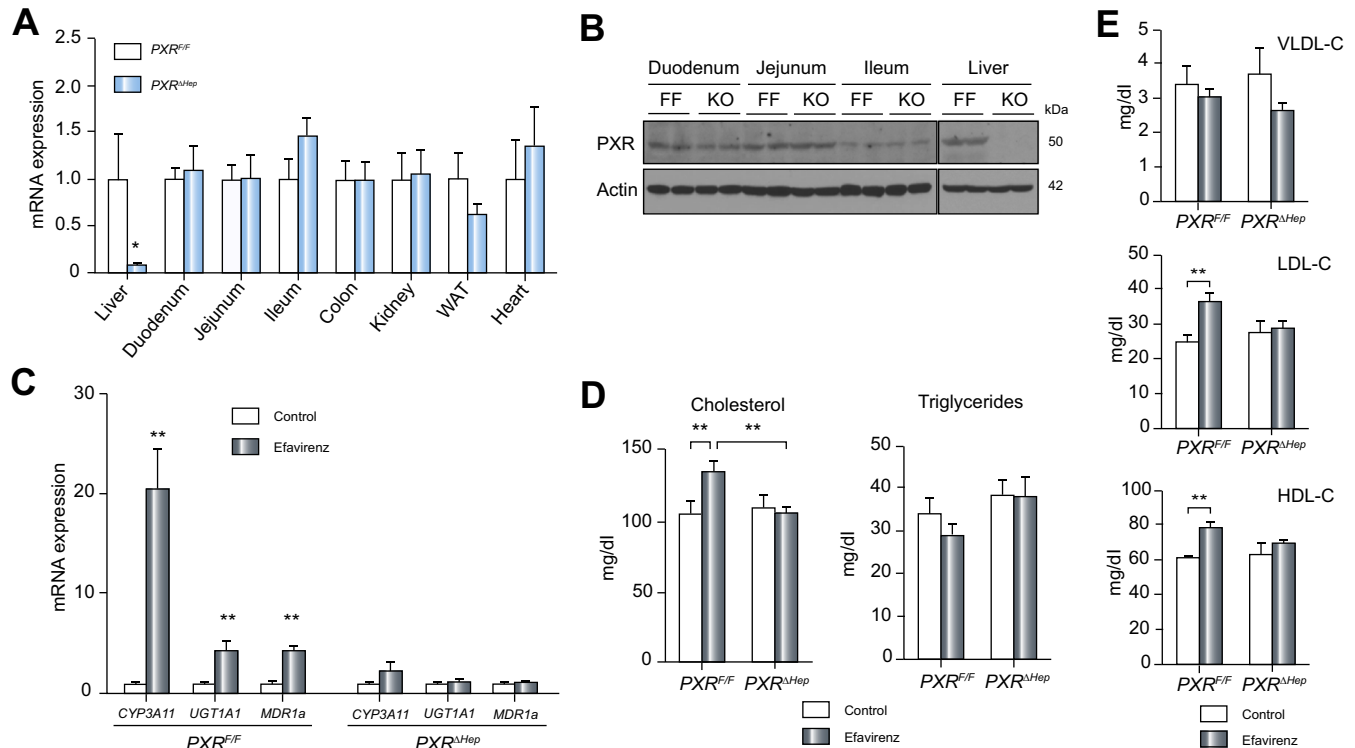


Fig. 3. Efavirenz elicits hypercholesterolemia in *Pxr*^{F/F} mice but not in *Pxr*^{ΔHep} mice. (A) qPCR analysis of *Pxr* mRNA levels in major tissues of *Pxr*^{F/F} and *Pxr*^{ΔHep} mice (n = 4–6, * p < 0.05). (B) Western blot analysis of PXR proteins in the liver and intestine of *Pxr*^{F/F} and *Pxr*^{ΔHep} mice. (C–F) Eight-week-old male *Pxr*^{F/F} and *Pxr*^{ΔHep} littermates were treated with vehicle control or 100 mg/kg/day of efavirenz by oral gavage for 1 week. Hepatic gene expression was analyzed by qPCR (C). Plasma total cholesterol and triglyceride levels were measured by standard methods (D). Lipoprotein fractions (VLDL, LDL, and HDL) were isolated and the cholesterol levels of each fraction were measured (E). Significance was determined by Student's *t* test (A and C) or 2-way ANOVA (D and E). (n = 5–7, ** p < 0.01). HDL, high density lipoprotein; LDL, low-density lipoprotein; qPCR, quantitative PCR; VLDL, very low-density lipoprotein.

Efavirenz stimulates hypercholesterolemia and hepatic steatosis through hepatic PXR signaling

To determine whether efavirenz induces dyslipidemia and hepatic steatosis through hepatic PXR signaling, $Pxr^{F/F}$ and $Pxr^{\Delta Hep}$ littermates were treated with efavirenz (100 mg/kg/day) or vehicle control for 1 week. The 100 mg/kg/day dose was selected based on previously studies which suggest that this dose is a mouse equivalent dose to those used clinically in humans (600 mg/day) when considering the interspecies scaling factor between mice and humans (12.3:1).²¹ As expected, efavirenz treatment also stimulated the expression of PXR target genes such as *Cyp311* in the liver of $Pxr^{F/F}$ mice but not in $Pxr^{\Delta Hep}$ mice (Fig. 3C). Similar to WT mice, efavirenz treatment did not affect fasting plasma triglyceride levels but significantly increased total cholesterol levels in $Pxr^{F/F}$ mice (Fig. 3D). Lipoprotein fraction analysis showed that efavirenz treatment significantly increased both LDL and HDL cholesterol levels but did not affect very low-density lipoprotein cholesterol levels in $Pxr^{F/F}$ mice (Fig. 3E). Interestingly, deficiency of hepatic PXR abolished the impact of efavirenz treatment on plasma total and lipoprotein cholesterol levels in $Pxr^{\Delta Hep}$ mice, suggesting that hepatic PXR signaling is required for efavirenz-elicited hypercholesterolemia. $Pxr^{F/F}$ mice treated with efavirenz also had liver enlargement and increased hepatic steatosis (Fig. 4A and B). However, short-term efavirenz treatment did not significantly affect liver fibrosis assessed by trichrome staining (Fig. 4B). Hepatic cholesterol and triglyceride levels were also significantly elevated in efavirenz-treated $Pxr^{F/F}$ mice (Fig. 4C). Deficiency of PXR abolished the impact of efavirenz on hepatic steatosis in $Pxr^{\Delta Hep}$ mice (Fig. 4B and C). Consistent with increased hepatic steatosis, efavirenz also increased plasma alanine aminotransferase and aspartate aminotransferase levels in a PXR-dependent manner (Fig. 4D).

In addition to efavirenz, $Pxr^{F/F}$ and $Pxr^{\Delta Hep}$ mice were also treated with emtricitabine, another widely prescribed ARV drug that does not activate PXR (Fig. 1). As expected, emtricitabine did not affect hepatic PXR target gene expression in both $Pxr^{F/F}$ and $Pxr^{\Delta Hep}$ mice (Fig. S3A). Plasma lipid levels and hepatic lipid accumulation were not affected by emtricitabine treatment in either $Pxr^{F/F}$ or $Pxr^{\Delta Hep}$ mice (Fig. S3B–F). Taken together, these results suggest that adverse effects of efavirenz on plasma cholesterol levels and hepatic steatosis are mediated through hepatic PXR signaling in mice.

Efavirenz-mediated PXR activation alters the key hepatic lipogenic gene expression in mice

We next analyzed the expression of key hepatic genes mediating lipid homeostasis to elucidate potential molecular mechanisms for the adverse effects of efavirenz. While efavirenz-mediated PXR activation did not affect the expression of several well-studied lipogenic genes including *Hmgcr*, *Srebp1c*, *Srebp2*, *Dgat3*, and *Fas* (Fig. 5A and S4A), exposure to efavirenz led to altered expression of key lipid transporters *Sr-bi* (*Scarb1*) and *Cd36*, and cholesterol synthesizing enzyme *Sqle* (Fig. 5A). In addition, efavirenz treatment also induced a couple of genes involved in β -oxidation, *Acox1* and *Cpt2* in the liver of $Pxr^{F/F}$ but not $Pxr^{\Delta Hep}$ mice (Fig. S4A). We also measured the expression of several hepatic inflammatory genes and found that *Mcp-1* expression was also elevated in efavirenz-treated $Pxr^{F/F}$ mice (Fig. S4B). It is possible that increased β -oxidation and inflammatory gene expression was due to the increased hepatic steatosis in these mice.

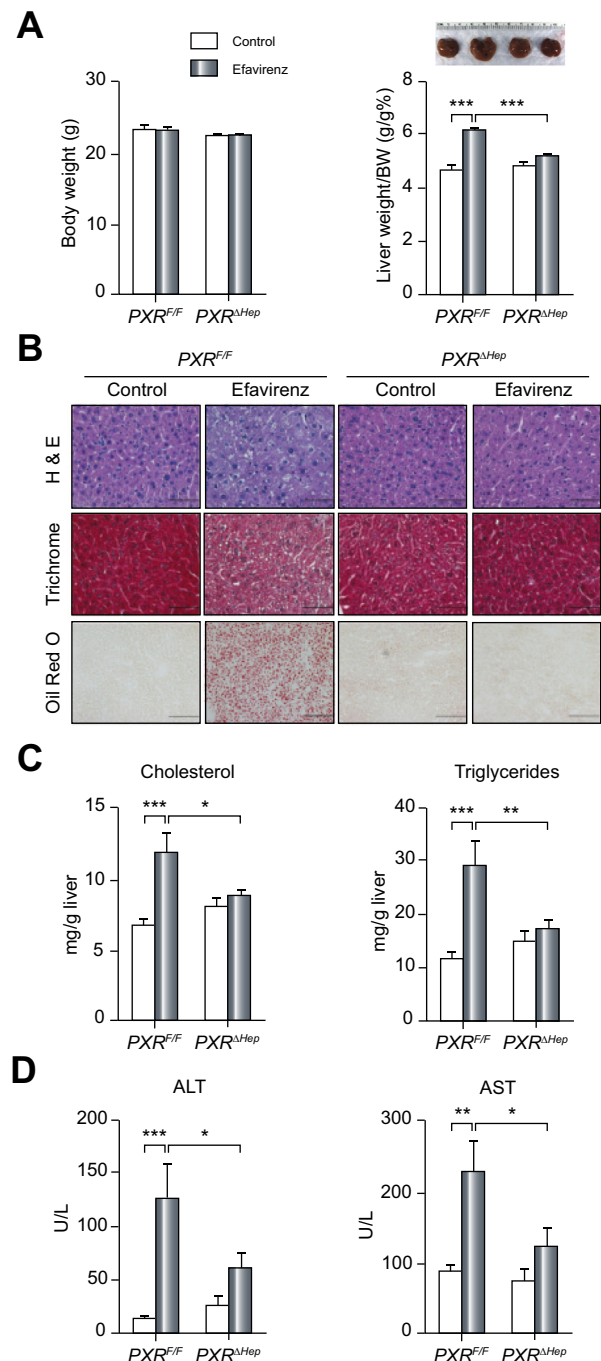


Fig. 4. Efavirenz treatment leads to increased hepatic steatosis in $Pxr^{F/F}$ mice but not in $Pxr^{\Delta Hep}$ mice. Eight-week-old male $Pxr^{F/F}$ and $Pxr^{\Delta Hep}$ littermates were treated with vehicle control or 100 mg/kg/day of efavirenz for 1 week. (A) Body weight, the ratio of liver weight to body weight, and representative liver image ($n = 6-7$, *** $p < 0.001$). (B) Representative hematoxylin and eosin (bar = 50 μ m), Trichrome (bar = 50 μ m), and Oil red O (bottom, bar = 100 μ m) stained liver sections. (C and D) Hepatic total cholesterol and triglyceride levels (C) and plasma ALT and AST levels (D) ($n = 7$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). Significance was determined by 2-way ANOVA (A, C, and D). ALT, alanine aminotransferase; AST, aspartate aminotransferase. (This figure appears in colour on the web.)

SR-BI is a major receptor for HDL uptake in the liver,²² and decreased expression or loss-of-function of SR-BI has been associated with elevated plasma HDL levels in animals and humans.^{22,23} PXR signaling has been previously shown to

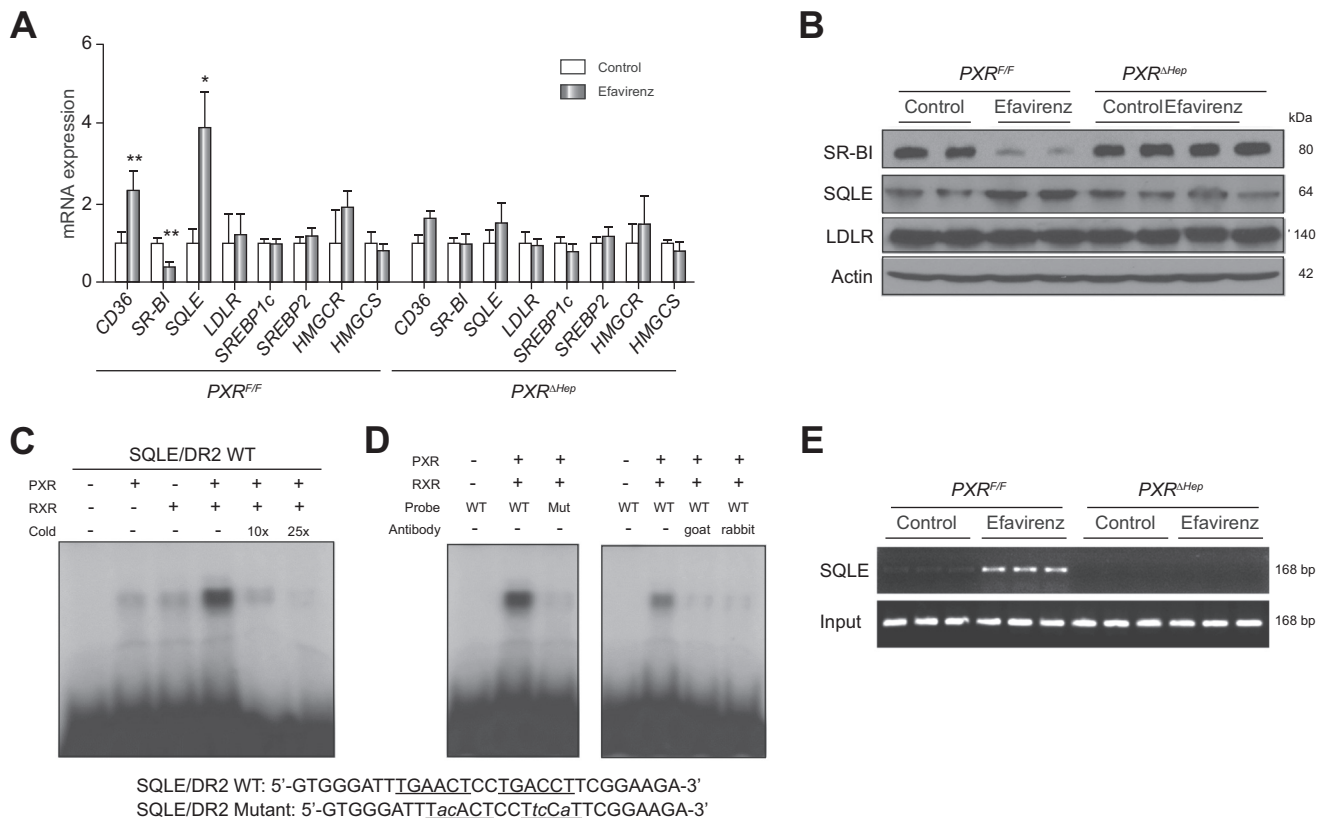


Fig. 5. Squalene epoxidase is a direct transcriptional target of PXR. (A and B) Eight-week-old male *Pxr^{F/F}* and *Pxr^{ΔHep}* littermates were treated with vehicle control or 100 mg/kg/day of efavirenz for 1 week. (A) Hepatic gene expression was analyzed by qPCR ($n = 5-7$, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). (B) Western blot analysis of hepatic SR-BI, SQLE, and LDLR proteins in control or efavirenz-treated mice. (C) *In vitro* translated human PXR and RXR, as indicated, were incubated with [³²P]-labeled SQLE/DR-2 probes for EMSA analysis. (D) PXR/RXR proteins were incubated with [³²P]-labeled WT or mutated SQLE/DR-2 probes for EMSA analysis (left panel). PXR/RXR proteins were incubated with two different anti-PXR antibodies, goat anti-PXR or rabbit anti-PXR antibodies for 1 h prior to the addition of the WT [³²P]-labeled SQLE/DR-2 probes for EMSA analysis (right panel). (E) Primary hepatocytes isolated from *Pxr^{F/F}* and *Pxr^{ΔHep}* mice were treated with control or 20 μ M efavirenz for 24 h. ChIP analysis was then performed to determine the recruitment of PXR onto the SQLE promoter. Significance was determined by Student's *t* test (A). ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; qPCR, quantitative PCR; WT, wild-type.

downregulate hepatic expression of SR-BI *in vitro* and *in vivo*.^{14,24} Consistently, efavirenz treatment decreased hepatic SR-BI mRNA levels and protein levels in *Pxr^{F/F}* but not *Pxr^{ΔHep}* mice (Fig. 5A and B). It is likely that PXR-mediated downregulation of SR-BI expression contributed to the increased plasma HDL levels in efavirenz-treated mice. In addition to SR-BI, efavirenz significantly increased hepatic expression of fatty acid transporter CD36 in *Pxr^{F/F}* but not *Pxr^{ΔHep}* mice (Fig. 5A and B). CD36 is a direct transcriptional target of PXR that contributes significantly to PXR ligand-mediated hepatic steatosis.¹⁵ Thus, the adverse effects of efavirenz on hepatic steatosis may be mediated, at least in part, through PXR-CD36 axis in mice.

Squalene epoxidase is a direct transcriptional target of PXR

The role of PXR in regulating hepatic SR-BI and CD36 expression has previously been demonstrated.^{15,24} However, the impact of PXR activation on SQLE expression has not been reported. Efavirenz-mediated PXR activation increased both mRNA and protein levels of SQLE in liver (Fig. 5A and B). While the role of HMGCR in cholesterol homeostasis has been extensively studied by many groups, little is known about the transcriptional regulation of SQLE which is another rate-limiting enzyme involved in the first oxidative step in cholesterol biosynthesis.^{25,26} We then analyzed the promoter of SQLE gene and

identified a DR-2-type (direct repeat spaced by two nucleotides) of nuclear receptor response element (TGAACCTCTGACCT), similar to DR-2 element found in other PXR target genes.¹⁰ Electrophoretic mobility shift assay (EMSA) confirmed that PXR and RXR heterodimer can bind to this DR-2 element. The binding of SQLE/DR-2 by PXR-RXR was specific as excessive cold probe decreased PXR-RXR binding to this element (Fig. 5C). In addition, mutations of the DR-2 element can abolish the binding of PXR-RXR dimers to the mutant DR-2 site (Fig. 5D). Two different anti-PXR antibodies also disrupted the protein-DNA complex (Fig. 5D), suggesting that PXR is a component of the protein complex that binds to the SQLE/DR2 element. Next, chromatin immunoprecipitation (ChIP) assays demonstrated that efavirenz treatment can increase the recruitment of PXR onto the *Sqle* promoter region containing DR-2 element in primary hepatocytes isolated from *Pxr^{F/F}* mice, but deficiency of PXR abolished these effects (Fig. 5E). Taken together, these results demonstrated that *Sqle* is a direct transcriptional target of PXR.

Efavirenz-mediated PXR activation stimulates fatty acid uptake and cholesterol synthesis in hepatic cells

To further investigate the impact of efavirenz-mediated PXR activation on hepatic lipogenesis, we isolated primary

hepatocytes from $Pxr^{F/F}$ and $Pxr^{\Delta Hep}$ mice. Consistent with *in vivo* results, treatment with efavirenz and the known PXR ligand PCN altered the expression of bona fide PXR target genes (Fig. S5) and key lipogenic genes including *Cd36*, *Sqle*, and *Sr-bi* in control primary hepatocytes but not in PXR-deficient hepatocytes (Fig. 6A). By contrast, the NRTI emtricitabine did not affect the expression of any of those genes in control or PXR-deficient hepatocytes.

CD36 plays an important role in regulating fatty acid uptake¹⁵ and we then performed fatty acid uptake assays using [³H]-palmitic acid to determine the effects of efavirenz on hepatocyte fatty acid uptake. Indeed, activation of PXR by efavirenz or PCN significantly increased [³H]-palmitic acid uptake by control hepatocytes and deficiency of PXR abolished PXR ligand-mediated fatty acid uptake in hepatocytes of $Pxr^{\Delta Hep}$ mice (Fig. 6B). By contrast, emtricitabine treatment did not affect fatty acid uptake by either control or PXR-deficient hepatocytes (Fig. 6B).

Since SQLE is a rate-limiting enzyme in cholesterol biosynthesis, we also performed cholesterol synthesis assays to determine whether activation of PXR can directly affect cholesterol synthesis in hepatocytes. Consistently, efavirenz and PCN treat-

ment significantly increased [³H]-acetate incorporation into [³H]-cholesterol in control hepatocytes but did not affect cholesterol biosynthesis in PXR-deficient hepatocytes (Fig. 6C). As expected, emtricitabine did not affect cholesterol biosynthesis in control or PXR-deficient hepatocytes. Collectively, these results confirmed that efavirenz-mediated PXR activation can stimulate fatty acid uptake and cholesterol synthesis in hepatic cells.

Efavirenz induces dyslipidemia and hepatic lipid accumulation in PXR-humanized mice

PXR exhibits considerable differences in its pharmacology and its ligand-binding domain is divergent across species.^{10,11} We next evaluated the effects of efavirenz on hepatic lipogenesis and plasma lipid levels in PXR-humanized ($hPXR-mPxr^{-/-}$) mice that express the human PXR gene in place of mouse *Pxr* gene.^{17,18} Efavirenz treatment also significantly increased the hepatic expression of known PXR target genes and newly identified *Sqle* in $hPXR-mPxr^{-/-}$ but not $mPxr^{-/-}$ mice (Fig. 7A). By contrast, efavirenz-stimulated PXR target gene induction was abolished in the livers of $mPxr^{-/-}$ littermates. Consistent with results obtained from WT and $Pxr^{F/F}$ mice, exposure to efavirenz

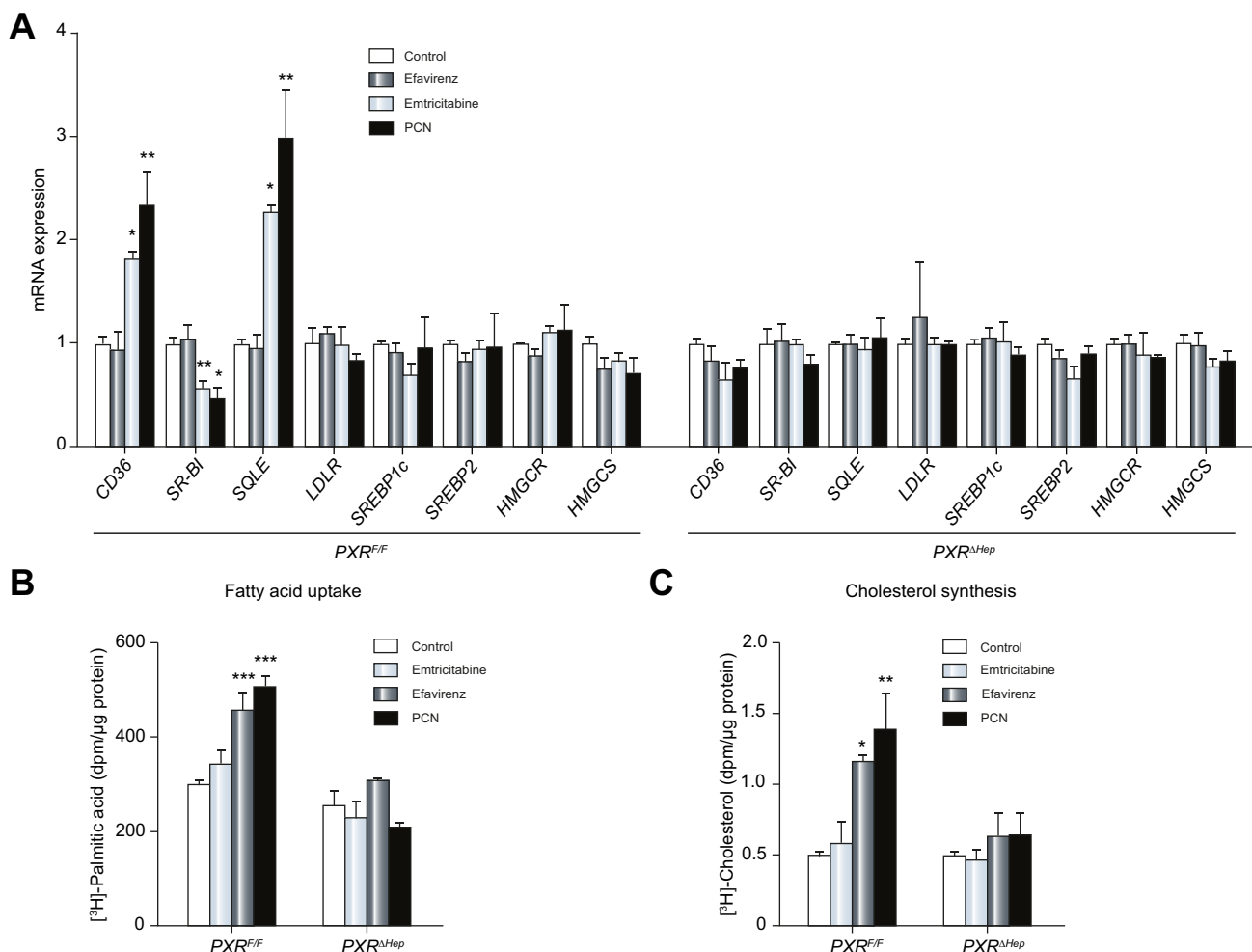


Fig. 6. Efavirenz-mediated PXR activation stimulates fatty acid uptake and cholesterol synthesis in hepatocytes. Primary hepatocytes isolated from $Pxr^{F/F}$ or $Pxr^{\Delta Hep}$ mice were treated with 20 μ M emtricitabine, efavirenz, or PCN for 24 h. (A) qPCR analysis of lipogenic gene expression. (B) Fatty acid uptake assays were performed using [³H]-palmitic acid. (C) Cholesterol synthesis assays were performed using [³H]-acetate. Significance was determined by 1-way ANOVA (A–C). (n = 3, **p < 0.01 and ***p < 0.001 compared to control group). PCN, pregnenolone 16 α -carbonitrile; qPCR, quantitative PCR.

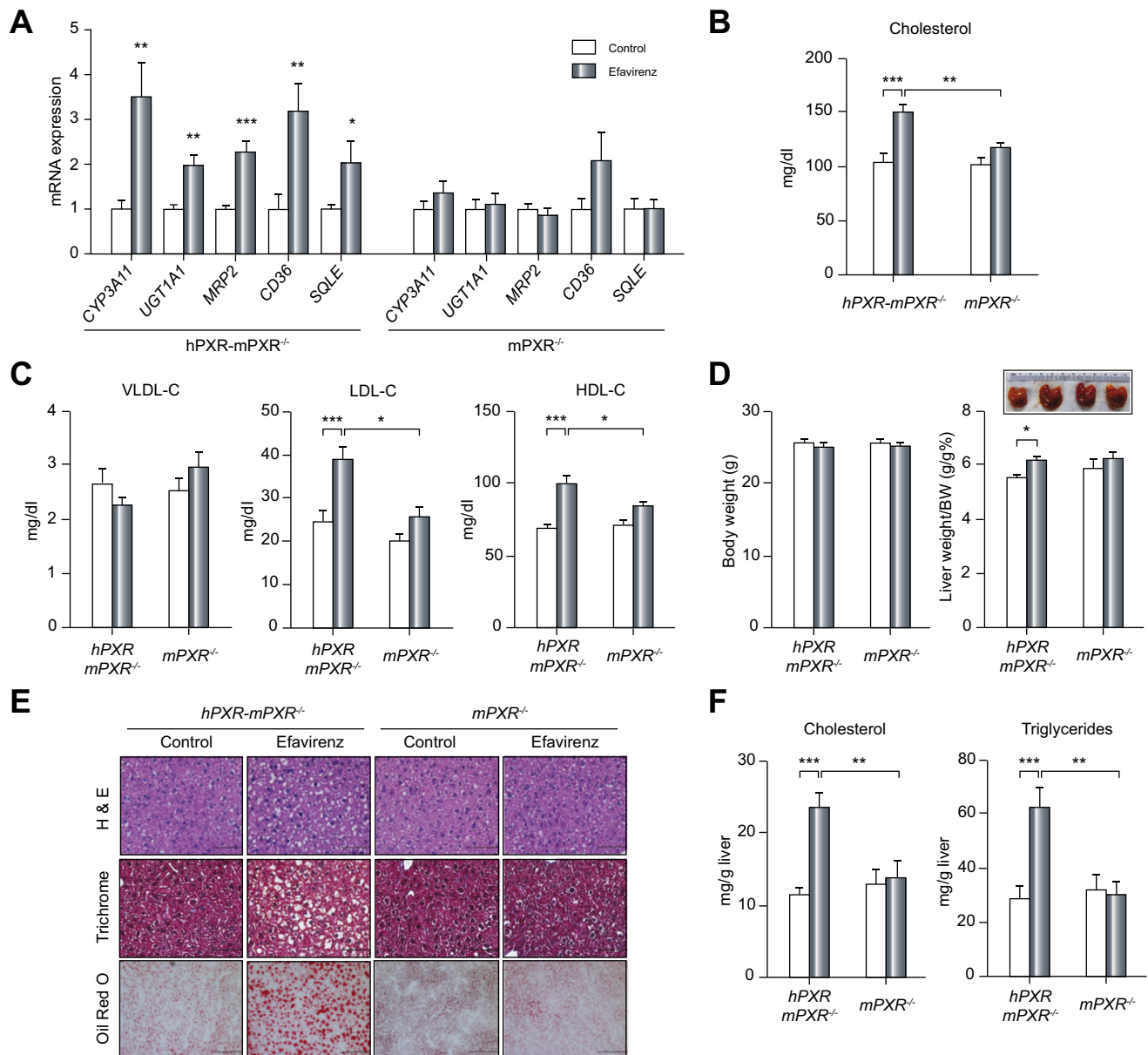


Fig. 7. Efavirenz induces hypercholesterolemia and hepatic steatosis in PXR-humanized mice. Eight-week-old male hPXR-mPxr^{-/-} and mPxr^{-/-} littermates were treated with vehicle control or 100 mg/kg/day of efavirenz for 1 week. (A) qPCR analysis of hepatic gene expression. (B and C) Plasma total cholesterol (B) and VLDL, LDL, and HDL cholesterol levels (C). (D) Body weight, the ratio of liver weight to body weight, and representative liver images. (E) Representative H&E (bar = 50 μ m), Trichrome (bar = 50 μ m), and Oil red O (bar = 100 μ m) stained liver sections. (F) Hepatic total cholesterol and triglyceride levels. Significance was determined by Student's *t* test (A) or 2-way ANOVA (B-D, and F). (n = 5–7, **p* < 0.05, ***p* < 0.01 and ****p* < 0.001). HDL, high density lipoprotein; LDL, low-density lipoprotein; qPCR, quantitative PCR; VLDL, very low-density lipoprotein. (This figure appears in colour on the web.)

also led to elevated plasma total, LDL, and HDL cholesterol levels; hepatomegaly; and hepatic steatosis and lipid accumulation in hPXR-mPxr^{-/-} mice but not in mPxr^{-/-} mice (Fig. 7B–F).

Efavirenz-mediated PXR activation regulates lipogenic gene expression in primary hepatocytes from PXR-humanized mice and humans

In addition to *in vivo* studies, we also performed *in vitro* assays using primary hepatocytes of hPXR-mPxr^{-/-} and mPxr^{-/-} mice (Fig. 8A–C). In addition to regulating known PXR target genes, both efavirenz and RIF increased *Sqle* and *Cd36* expression and suppressed *Sr-bi* expression in hepatocytes isolated from

hPXR-mPxr^{-/-} mice but not in PXR-deficient hepatocytes (Fig. 8A). As expected, emtricitabine did not affect any of those genes in the hepatocytes of those mice (Fig. 8A). Further, efavirenz and RIF, but not emtricitabine, significantly increased both fatty acid uptake and cholesterol biosynthesis in hepatocytes isolated from hPXR-mPxr^{-/-} mice, and deficiency of PXR abolished these effects in hepatocytes of mPxr^{-/-} mice (Fig. 8B and C).

Lastly, human primary hepatocytes were cultured and treated with efavirenz at different doses. Efavirenz treatment also resulted in increased *Sqle* and *Cd36* but decreased *Sr-bi* expression in a dose-dependent manner (Fig. 8D). Immunoblotting

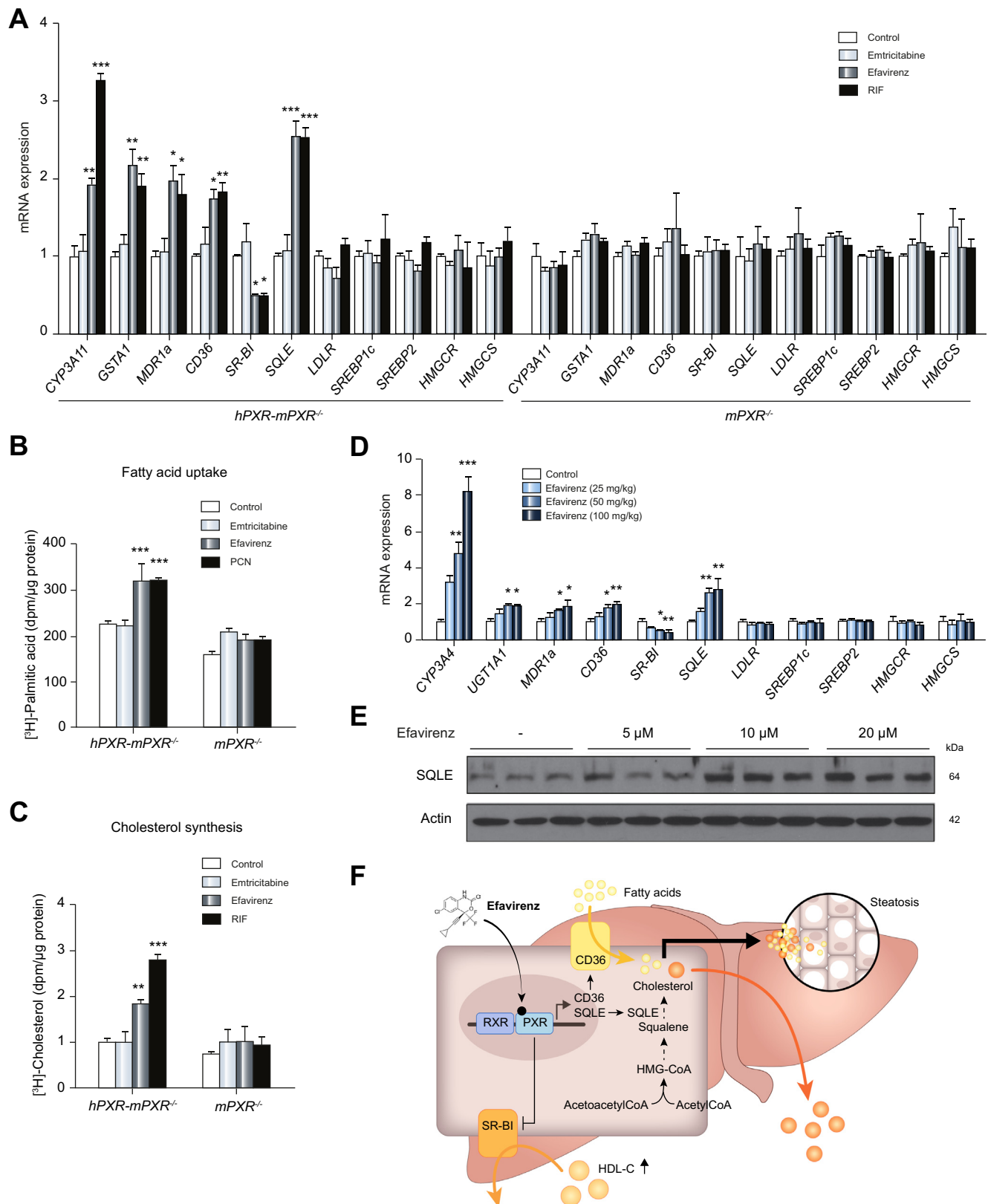


Fig. 8. Efavirenz-mediated PXR activation regulates lipogenic gene expression in primary hepatocytes from PXR-humanized mice and humans. (A–C) Primary hepatocytes isolated from *hPXR-mPXR^{-/-}* or *mPXR^{-/-}* mice were treated with 20 μM emtricitabine, efavirenz, or RIF for 24 h. qPCR analysis (A), fatty acid uptake assays using [³H]-palmitic acid (B), cholesterol synthesis assays using [³H]-acetate (C). (n = 3, *p < 0.05, **p < 0.01 and ***p < 0.001). (D and E) Human primary hepatocytes were treated with efavirenz at indicated concentrations for 24 h. qPCR analysis of lipogenic genes (D) and Western blot analysis of SQLE proteins (E) in control or efavirenz-treated hepatocytes (n = 3, *p < 0.05, **p < 0.01 and ***p < 0.001). (F) Schematic representation of the mechanism through which efavirenz-mediated PXR activation elicits hypercholesterolemia and hepatic steatosis. Significance was determined by 1-way ANOVA (A–D), qPCR, quantitative PCR; RIF, rifampicin. (This figure appears in colour on the web.)

results also confirmed elevated SQLE protein levels in efavirenz-treated human primary hepatocytes (Fig. 8E). Therefore, efavirenz-mediated PXR activation may have a clinically relevant impact on hepatic lipogenic pathways in humans.

Discussion

Despite significant reductions in the morbidity and mortality associated with HIV infection over the past few decades, HIV-infected patients treated with effective ART have an elevated risk of dyslipidemia and CVD.^{1–4} In the current study, we identified currently recommended ARV drugs including efavirenz as potent PXR agonists. Short-term exposure to efavirenz significantly increased plasma cholesterol levels and elicited hepatic steatosis in mice in a hepatic PXR-dependent manner. Interestingly, PXR can regulate the expression of several key hepatic lipogenic genes including *CD36*, *SR-BI*, and *SQLE* which play important roles in hepatic lipid uptake and cholesterol biosynthesis (Fig. 8F). While current ART uses a combination of several drug classes, we found that only PIs and NNRTIs including efavirenz, darunavir, and lopinavir can activate PXR by transfection assays. By contrast, none of the NRTIs or INSTI we tested activated PXR. Interestingly, all of these PXR agonistic drugs have been associated with dyslipidemia in HIV-infected patients.^{2,5–6} For example, clinical studies have demonstrated that both efavirenz and lopinavir can cause hyperlipidemia in HIV-infected patients, and the combination of efavirenz and lopinavir has more profound effects on elevating lipid levels in patients.^{2,6} On the contrary, several NRTIs including emtricitabine and tenofovir that do not activate PXR, confer a low risk of dyslipidemia.^{27–29} Consistently, we found that efavirenz but not emtricitabine was able to induce hypercholesterolemia and hepatic steatosis in mice, and deficiency of PXR abolished efavirenz-elicited adverse effects. These results strongly suggest the involvement of PXR in certain ARV drug-associated dyslipidemia.

Previous studies have demonstrated that activation of PXR by various ligands can affect the expression of hepatic lipogenic genes including *CD36*, *SR-BI*, *S14*, and *LPIN1*.^{11,12,14,15,24,30} For example, *CD36*, an important fatty acid transporter in the liver, has been shown to be a direct target gene of PXR.¹⁵ In the current study, efavirenz-mediated PXR activation stimulated *CD36* expression and promoted fatty acid uptake by hepatocytes. Therefore, the increased *CD36* expression likely contributed significantly to the efavirenz-elicited hepatic steatosis *in vivo*. Efavirenz treatment also suppressed the hepatic expression of another scavenger receptor, *SR-BI* which is a major receptor mediating selective uptake of HDL. PXR can suppress hepatic *SR-BI* expression^{14,24} and decreased *SR-BI* expression has been well-established to increase plasma HDL levels in animal models.²² Although HDL cholesterol has been considered to have beneficial effects on CVD, *SR-BI* loss-of-function mutations significantly elevated plasma HDL levels in humans, but paradoxically increased their risk of CVD, which is likely due to the impaired reverse cholesterol transport.²³ Therefore, the efavirenz-induced HDL levels may also have deleterious instead of beneficial effects on CVD in HIV-infected patients.

In addition to altered *CD36* and *SR-BI* expression, efavirenz-mediated PXR activation also stimulated the expression of *SQLE*, a member of flavoprotein monooxygenase family. *SQLE* catalyzes the epoxidation of squalene to generate 2,3-oxidosqualene, which is the first oxidative step in cholesterol

biosynthesis.²⁵ Squalene epoxidation is a rate-limiting step and also an important regulatory point in cholesterol synthesis. Thus, in addition to *HMGCR*, *SQLE* has been considered a promising target for hypercholesterolemia treatment.²⁵ We then identified a PXR binding site in the *Sqle* promoter, and both EMSA and ChIP assays confirmed *Sqle* as a direct transcriptional target of PXR. Consistent with the known function of *SQLE* in cholesterol biosynthesis, efavirenz-mediated PXR activation can indeed elevate cholesterol synthesis in cultured primary hepatocytes.

In summary, we have identified several widely-used ARV drugs including the most prescribed NNRTI efavirenz as potent agonists of PXR. Efavirenz can efficiently activate PXR and stimulate its target gene expression *in vitro* and *in vivo*. Interestingly, efavirenz treatment led to hypercholesterolemia and hepatic steatosis in mice, and deficiency of hepatic PXR abolished those adverse effects. Efavirenz-mediated PXR activation altered the hepatic expression of several key genes including *CD36*, *SR-BI*, and *SQLE* that mediate lipid uptake and cholesterol synthesis in the liver. These findings provide mechanistic insights and new understandings of ART-associated dyslipidemia and CVD risk, and activation of PXR should be taken into consideration for patients undergoing long-term treatment with ARV drugs.

Financial support

This work was supported by NIH grants, R01HL123358, R01ES023470, R01HL131925, and R21ES022745 (to C.Z.). R.N.H. was supported by a predoctoral fellowship from PhRMA Foundation. The authors also acknowledge the core services (supported by NIH grant P30GM127211).

Conflict of interest

The authors have no conflict of interest to declare.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

C.Z. conceptualized and designed the research. T.G. and Z.M. performed most of the experiments and analyzed the data with the help from Y.S., R.N.H., S.-H.P., S.W., and R.N.G. C.Z., T.G., Z.M., Y.S., S.W., and R.N.G. wrote the manuscript.

Acknowledgement

We thank Dr. Frank Gonzalez at National Cancer Institute for the PXR-humanized mice and Dr. Chingwen Yang at Rockefeller University for the help with PXR-flox mouse generation.

Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhep.2018.12.038>.

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Author names in bold designate shared co-first authorship

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