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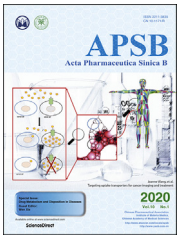

ORIGINAL ARTICLE

The 3′-untranslated region contributes to the pregnane X receptor (PXR) expression down-regulation by PXR ligands and up-regulation by glucocorticoids

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Abstract Pregnane X receptor (PXR) is the major regulator of xenobiotic metabolism. PXR itself is controlled by various signaling molecules including glucocorticoids. Moreover, negative feed-back regulation has been proposed at the transcriptional level. We examined the involvement of the 3′-untranslated region (3′-UTR) of *NR1I2* mRNA and microRNAs in PXR- and glucocorticoid receptor (GR)-mediated regulation of *NR1I2* gene expression. PXR ligands were found to significantly down-regulate *NR1I2* mRNA expression in a set of 14 human hepatocyte cultures. Similarly, PXR was downregulated by PCN in the C57/BL6 mice liver. In mechanistic studies with the full-length 3′-

Abbreviations: 3′-UTR, 3′-untranslated region; CAR, constitutive androstane receptor; CYP3A4, cytochrome P450 3A4; DEX, dexamethasone; DMEs, drug metabolizing enzymes; DMSO, dimethyl sulfoxide; ER, estrogen receptor; Gluc, Gaussia luciferase; GRα, glucocorticoid receptor α; LBD, ligand binding domain; miRNA, microRNA; MRE, miRNA-response element; NR, nuclear receptor; PB, phenobarbital; PCN, pregnenolone 16α-carbonitrile; PHHs, primary human hepatocytes; PPARα, peroxisome proliferator-activated receptor α; PXR, pregnane X receptor; Rif, rifampicin; RXRα, retinoid X receptor α; SEAP, secreted alkaline phosphatase.

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UTR cloned into luciferase reporter or expression vectors, we showed that the 3′-UTR reduces PXR expression. From the miRNAs tested, miR-18a-5p inhibited both *NR1I2* expression and *CYP3A4* gene induction. Importantly, we observed significant upregulation of miR-18a-5p expression 6 h after treatment with the PXR ligand rifampicin, which indicates a putative mechanism underlying *NR1I2* negative feed-back regulation in hepatic cells. Additionally, glucocorticoids upregulated *NR1I2* expression not only through the promoter region but also *via* 3′-UTR regulation, which likely involves downregulation of miR-18a-5p. We conclude that miR-18a-5p is involved in the down-regulation of *NR1I2* expression by its ligands and in the upregulation of *NR1I2* mRNA expression by glucocorticoids in hepatic cells.

1. Introduction

Pregnane X receptor (PXR, encoded by *NR1I2* gene) is a member of the nuclear receptor (NR) superfamily, which includes the steroid, retinoid, and thyroid hormone receptors. PXR is the major xenobiotic receptor that coordinately regulates transcription of key phase I and II biotransformation enzymes, as well as some drug transporters, to detoxify and eliminate xenobiotics and endotoxins from the body. As a ligand-activated nuclear receptor, PXR triggers the transcription of various target genes, such as *CYP3A4* and *CYP2C9*, conjugation enzymes, such as *UGT1A1*, and transporters, such as *ABCB1*[1–3]. In some cases, however, activation of PXR has been associated with downregulation of gene expression, as exemplified by the *OCT1* gene[4,5]. In addition to its canonical xenobiotic sensing function, PXR has many other cellular and physiological roles and has been implicated in glucose, lipid and bile acid metabolism, energy homeostasis, inflammatory response, cell proliferation, apoptosis, and cell migration[1,2]. PXR agonists include a broad spectrum of structurally diverse compounds, such as rifampicin, phenobarbital, SR-12813, and hyperforin, due to the large and considerably flexible ligand-binding domain (LBD) cavity of PXR[3].

Although tremendous effort has been spent studying the function of PXR in both exogenous and endogenous metabolism, less is known about the exact mechanisms that are responsible for its regulation. *NR1I2* expression and transcriptional activity are coordinately regulated at the transcriptional, post-transcriptional, and posttranslational levels, and numerous signaling cascades have been implicated in modulating PXR activity[6,7]. As a consequence of this multifactorial regulation, human hepatic *NR1I2* mRNA is substantially variable in its expression[8].

Recent *in silico* predictions identified a number of potential binding sites for other NRs within the human *NR1I2* gene proximal promoter (∼2.2 kb) suggesting hormonal or endobiotic regulation of PXR. Forced overexpression of estrogen receptor (ER), glucocorticoid receptor α (GRα), and peroxisome proliferator-activated receptor α (PPARα) exert positive effects on *NR1I2* transcription, but PXR and constitutive androstane receptor (CAR) have negative effects on PXR expression in hepatic cells[7,9]. However, no putative binding sites for either PXR or CAR have been identified within the *NR1I2* promoter by detailed bioinformatic analysis[9]. Glucocorticoids have been shown to regulate hepatic *NR1I2* expression, and the crosstalk between PXR and GRα has been reported in detail in primary human hepatocytes with dexamethasone at submicromolar concentrations[10,11]. Additionally, positive transactivation of *NR1I2* by HNF4α through the direct repeat 1 (DR1) element located in the −88 to −76 bp region or by PPARα *via* a binding site located in the −1514 to −1321 bp region has been described[9,12]. Nevertheless, *NR1I2* mRNA expression does not correlate with PXR protein levels in the liver, which is an organ where *NR1I2* is abundantly expressed; this suggests that PXR is subject to post-transcriptional regulation[13].

MicroRNAs (miRNAs) are small noncoding RNAs approximately 22 nt in length that are considered the principal post-transcriptional regulators[14]. To date, more than 2600 mature human miRNAs have been annotated in the miRBase repository (v. 22.1, October 2018)[15]. Primarily but not exclusively, miRNAs bind to responsive elements within the 3′-untranslated region (3′-UTR) of a target gene mRNA, resulting in degradation of the mRNA or translational repression. Interestingly, individual mRNAs can be targeted by many diverse miRNAs, leading to fine-tuning of gene expression. In contrast, specific miRNAs can simultaneously control the expression of many target mRNAs[14,16]. It is currently supposed that most human genes are under the control of miRNAs. Indeed, a growing body of evidence has also confirmed that miRNAs play a significant role in the regulation of genes involved in xenobiotic metabolism. In addition, drugs may regulate their pharmacokinetics and response *via* miRNAs, which is a topic that has been reviewed elsewhere[17–19]. In line with this assumption, it has been demonstrated that miRNAs may interfere with *NR1I2* expression, as suggested for miR-18a-5p[20], miR-148a[13], miR-30c-1-3p[21], miR-34a-5p[8,22], miR-449a[22], and miR-140-3p[23]. Importantly, negative regulation of *NR1I2* expression *via* miR-18a-5p by PXR ligands has been shown recently in LS180 cells[20].

Since ligand-activated NRs are subjects of positive and negative regulation[24] and keeping in mind the complexity of transcriptional and posttranscriptional regulation of *NR1I2* expression, we hypothesized for the first time in this work that miRNAs may be involved in a *NR1I2* autoregulatory feedback loop. To test this hypothesis, we analyzed changes in *NR1I2* expression after PXR activation, employing both primary human hepatocytes and mice. Furthermore, we evaluated the mechanisms underlying such changes with a focus on transcriptional and posttranscriptional regulation. Subsequently, we examined the impact of activated GR on posttranscriptional stabilization of *NR1I2* transcripts. By

employing various constructs with the 3′-UTR of *NR1I2* mRNA, we deciphered a key role for miRNA-18a-5p in PXR-mediated and GR-induced regulation of PXR expression through the full-length 3′-UTR of the *NR1I2* gene.

2. Materials and methods

2.1. Chemicals

Rifampicin (a prototypical ligand of the human PXR, Rif), SR-12813 (a human PXR ligand), and phenobarbital (PB) were purchased from Sigma−Aldrich (St. Louis, MO, USA). Dexamethasone (DEX) (a prototypical ligand of GR), RU486 (mifepristone, GR antagonist), DMSO (dimethyl sulfoxide), pregnenolone 16α-carbonitrile (PCN, an agonist of mouse PXR), hydrocortisone acetate, corticosterone, triamcinolone acetonide, and 6α-methylprednisolone were purchased from Sigma−Aldrich. Stock solutions (1000×) were prepared in DMSO prior to dilution in cell culture medium. The final concentration of DMSO in the media did not exceed 0.2% (*v*/*v*) in any experiments. SPA70, an PXR inhibitor, has been purchased from abcr GmbH Im (Karlsruhe, Germany)[25].

miRCURY LNA microRNA mimics cel-miR-39-3p (mimic control, 479902-001), hsa-miR-18a-5p (471717-001), hsa-miR-148a-3p (339173 YM00472598-ADA), hsa-miR-34a-5p (339173 YM00473212-ADA), hsa-miR-449a (339173 YM00473262-ADA), and miRCURY LNA inhibitors, such as negative control A (inhibitor control, 199006-001), hsa-miR-18a-5p (4100723-001), and hsa-miR-34a-5p (339121 YI04100982-ADA), were obtained from Exiqon (now part of QIAGEN, Germantown, MD, USA).

2.2. Plasmids

The p3A4-luc plasmid bears a distal XREM (−7836/−7208) and a basal promoter sequence (prPXRE, −362/+53) from the *CYP3A4* gene promoter region. The pER6-3A4-tat-luc plasmid carries three copies of the proximal *CYP3A4* promoter ER6 response element cloned into the *NheI* and *BglII* sites of the pGL4.23 (Promega, Madison, WI, USA) upstream of the SV40 minimal promoter.

The pmiRGLO Dual-Luciferase miRNA target expression vector (Promega) is designed for an insertion miRNA target site downstream of the firefly luciferase gene. The pmiRGLO-UTR reporter plasmid, containing the 3′-UTR of the *NR1I2* gene sequence (1−1273 nt downstream the coding region), was prepared by cloning the 3′-UTR sequence into *XhoI* and *SalI* restriction sites of the pmiRGLO vector. The pmiRGLO-18a-5p-Compl construct was generated by insertion of a complementary sequence for miR-18a-5p (5′-ctatctgcactagatgcacctta-3′) into *XhoI* and *SalI* restriction sites of the pmiRGLO vector. The pmiRGLO-MRE-1041-1064 (5′-aagaaccatttacatgcacctta-3′), pmiRGLO-MRE-1041-1064-REV, and pmiRGLO-MRE-69-91 (5′-gccaagacagatggacactgcca-3′) constructs contain either a putative responsive element for miR-18a-5p (1041-1064 nt from the stop codon), a reverse sequence of the putative responsive element for miR-18a-5p, or a putative shared responsive sequence of the 3′-UTR of the *NR1I2* gene for miR-34a-5p and miR-449a (69-91 nt from the stop codon) in the pmiRGLO vector. All constructed vectors were sequence verified.

pMIR-luc-control (LR-1000) and pMIR-luc-34a-5p-Compl (LR-0014) constructs carrying a complementary binding site for miR-34a-5p were purchased from BioCat (Heidelberg, Germany).

pPXR1-Gluc or pPXR2-Gluc reporter vectors (HPRM45199-PG04 and HPRM23633-PG04) with secreted *Gaussia* luciferase (Gluc) were purchased from GeneCopoeia (Rockville, MD, USA) together with a luciferase detection kit. pPXR1-Gluc contains a sequence from −1375 to +238 and pPXR2-Gluc sequence from −1147 to +238 of the promoter region upstream of the human *NR1I2* gene. The sequence from −1375 to −1147 (228 nt) in pPXR1-Gluc, but not in pPXR2-Gluc, contains putative response elements for HNF4α, PPARα, Sp1 and NF-κB transcription factors[9] (see schematic in Fig. 3A). Both reporter constructs contain cDNA for secreted alkaline phosphatase (SEAP) as an internal control for transfection normalization.

The expression plasmid for human PXR receptor pSG5-hPXRΔATG (further abbreviated as pSG5-PXR) was kindly provided by Dr. S. Kliewer (University of Texas, Dallas, TX, USA). The constitutively active T248D mutant of PXR was described in our previous work[26]. The pSG5-PXR-UTR expression vector, including both open reading frames encoding human wild-type PXR and the 3′-UTR of the *NR1I2* gene mRNA, was constructed by insertion of the 3′-UTR region into the *BamHI* restriction site downstream of the cDNA for PXR. Orientation of the 3′-UTR insert in pSG5-PXR-UTR was verified by sequencing. The expression plasmid encoding human GRα (pSG5-hGRα) was a generous gift from Dr. J. Palvimo (University of Helsinki, Helsinki, Finland), and the pSG5-RXRα construct came from Dr. C. Carlberg (University of Kuopio, Kuopio, Finland). pRL-TK was acquired from Promega. The empty expression vector pSG5 was obtained from Agilent (Santa Clara, CA, USA).

2.3. Cell lines

Human hepatoblastoma-derived (HepG2), human hepatocellular carcinoma (Huh-7), and African green monkey kidney-derived (COS-1) cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC) and cultured in antibiotic-free Dulbecco's modified Eagle's medium (DMEM, 10569010, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, F2442, Sigma−Aldrich) at 37 °C in a humidified incubator under 5% CO_2.

2.4. Primary human hepatocytes

Primary human hepatocytes (PHHs) were provided by QPS Hepatic Biosciences (Barcelona, Spain) or were isolated, as described previously[27], from donor organs unsuitable for transplantation or from liver resections performed in adult patients for medical reasons unrelated to our research program (only noncancerous liver tissue was collected). Liver samples were obtained from the Biological Resource Centre of Montpellier University Hospital (CRB-CHUM; http://www.chu-montpellier.fr; Biobank ID: BB-0033-00031), and this study benefitted from the expertise of Dr. Jeanne Ramos (hepatogastroenterology sample collection) and Prof. Sylvain Lehmann (CRB-CHUM manager). Patient clinical characteristics are presented in Table 1. The procedures were approved by the French Ethics Committee, and written or oral consent was obtained from all patients or their families.

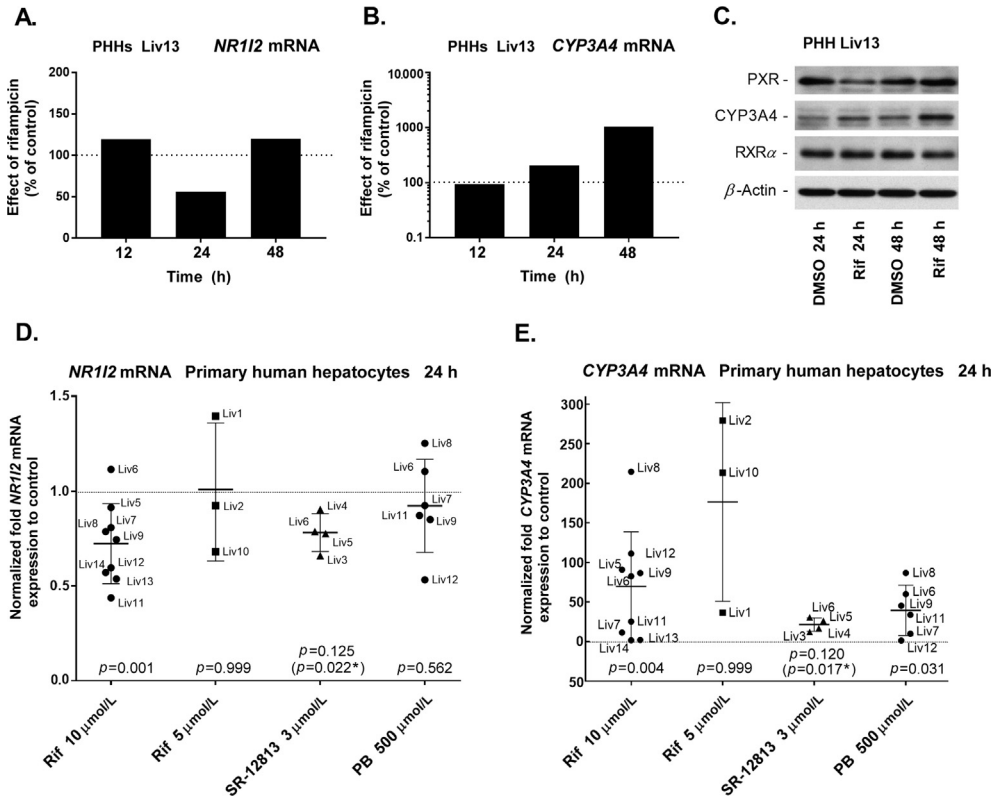

Figure 1 Ligand-dependent activation of PXR downregulates *NR1I2* expression in primary human hepatocytes. *NR1I2* mRNA (A) or *CYP3A4* mRNA (B) expression in primary human hepatocytes (donor Liv13) treated with vehicle (DMSO; 0.1%, *v/v*) or rifampicin (Rif, 10 µmol/L) for 12, 24, and 48 h, respectively. The expression data were normalized to the *RPLP0* reference gene, and data are expressed as relative change to corresponding control (DMSO-treated) experiments defined as 100%. (C) Western blotting analysis of PXR, CYP3A4 and RXRα expression in primary human hepatocytes (Liv13) treated with vehicle (DMSO; 0.1%) or Rif (10 µmol/L) for 24 or 48 h. β-Actin was used as a loading control. (D) *NR1I2* mRNA and (E) *CYP3A4* mRNA expression. Primary human hepatocytes (Liv1−13) and commercial (Liv14) human hepatocyte cultures were treated with vehicle (DMSO; 0.1%), Rif (10 or 5 µmol/L), SR-12813 (3 µmol/L) or phenobarbital (500 µmol/L) for 24 h. The data are presented as fold change in expression after PXR ligand treatment *versus* vehicle (DMSO)-treated controls defined as 1. Nonparametric Wilcoxon matched-pairs tests or paired *t* test were used to statistically compare paired expression data between control and PXR ligand-treated samples in human hepatocytes. $^{*}P < 0.05$, statistically significant effect of PXR ligand (paired *t* test, *P*-value in parentheses).

Primary human hepatocytes were seeded at 1.5×10^5 cells/cm^2 in collagen-coated plates and cultured in a 5% CO_2 humidified atmosphere at 37 °C in serum-free long-term (Lanford medium, LNF) or short-term (ISOM medium) culture medium[27] or hepatocyte growth medium (HGM: WME medium supplemented with 5 µg/mL insulin, 0.1 µmol/L hydrocortisone, 10 µg/mL transferrin, 250 µg/mL ascorbic acid, 3.75 mg/mL fatty acid-free bovine serum albumin, 2 mmol/L glutamine, penicillin and streptomycin). Primary human hepatocytes were incubated with rifampicin (Rif, 5 or 10 µmol/L), SR-12813 (3 µmol/L) or phenobarbital (500 µmol/L) for 24 or 48 h two days postseeding.

Information about the 13 donors of freshly isolated Primary human hepatocytes is shown. Details are included for each donor regarding age, sex, and pathology. Additionally, origin and cultivation medium are indicated for each individual culture of Primary human hepatocytes. Gender: M (male), F (female). ISOM medium[28]; HGM, hepatocyte growth medium; LNF, Lanford medium.

2.5. *Commercial human hepatocytes*

Commercial primary human hepatocytes (HEP220A0, Long-term human hepatocytes, batch Hep220980-MA96, a sample Liv14) were purchased from Biopredic International (Saint Gregoire, France). Prior to treatment, commercial Primary human hepatocytes were cultivated in a serum-free medium composed of basal hepatic cell medium (Biopredic, MIL600C, batch MIL600052) and additives for hepatocyte culture medium (Biopredic, ADD222C, batch ADD222039) overnight at 37 °C in a humidified incubator with 5% CO_2.

2.6. *Animal experiments*

Twelve-week-old male C57/BL6 mice (Velaz, Czech Republic) were housed in a temperature- and light-controlled facility with 12 h light−dark cycling. All mice were fed commercially available laboratory chow diet (Velaz) *ad libitum*. Mice were randomly divided into 12 groups consisting of 5 animals each and were all administered either a single i.p. injection of mouse PXR ligand pregnenolone 16α-carbonitrile (PCN, 50 mg/kg) dissolved in corn oil plus 30% DMSO or vehicle (olive oil plus 30% DMSO).

Individual groups of mice were then sacrificed by exsanguination under general anesthesia induced by isoflurane after 3, 6, 24, 48, 72, or 168 h postadministration of vehicle or PCN. Livers were immediately weighted, frozen in liquid nitrogen and subsequently stored at −80 °C until Western blot and RT-qPCR

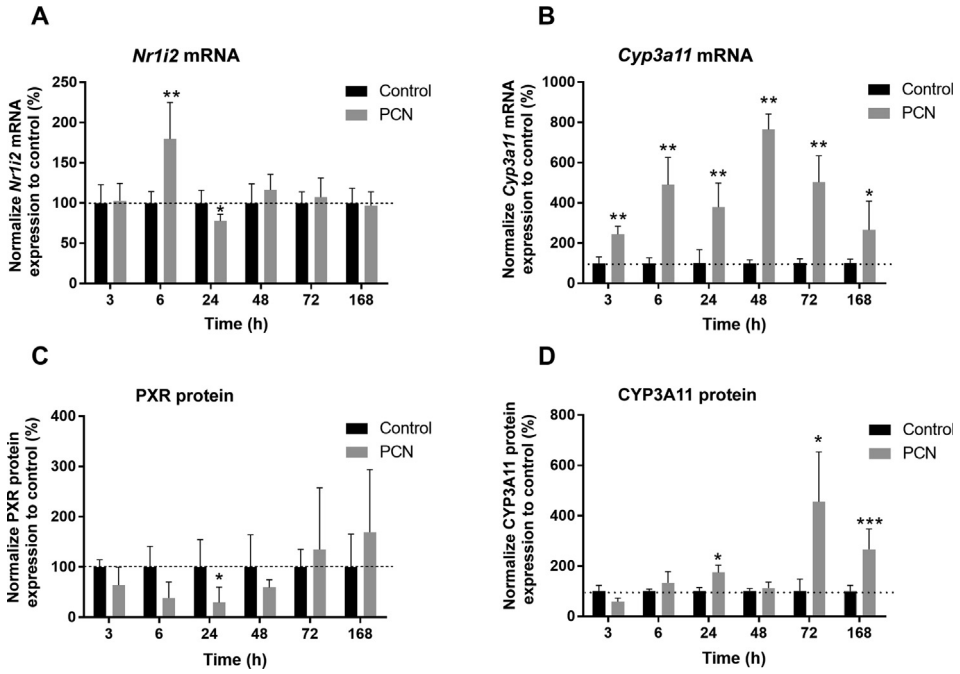

Figure 2 PCN-mediated activation of mouse PXR affects *Nr1i2* expression in mouse liver. Mouse PXR (A) or CYP3A11 (B) mRNA expression in mice that were administered a single i.p. injection of vehicle or PCN (50 mg/kg) and sacrificed after 3, 6, 24, 48, 72, or 168 h postapplication. In addition, liver sample homogenates were analyzed for mouse PXR (C) and CYP3A11 (D) protein expression employing Western blotting. *Gapdh* was used as a reference gene for RT-qPCR expression data normalization, and β-actin was used for Western blotting data normalization. The data are shown as the mean ± SD ($n = 5$ per group) and are expressed as change relative to vehicle-treated control samples defined as 100%. Mann−Whitney test was used to compare unpaired data in mice. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate a statistically significant effect of PCN treatment.

analysis. All animals received humane care in accordance with the EU Directive 2010/63/EU and guidelines for animal experiments set by the Institutional Animal Use and Care Committee of Charles University, Faculty of Medicine in Hradec Kralove, Czech Republic. The protocol for all experiments was approved by the same committee.

2.7. *Luciferase reporter gene assays*

All reporter gene assays were performed in HepG2, Huh-7 or COS-1 cells using Lipofectamine 3000 Reagent (Thermo Fisher Scientific) following the manufacturer's protocol, as described previously[4,29,30]. Briefly, cells were seeded into 48-well plates overnight and transfected with different luciferase reporter constructs either alone or in combination with appropriate expression plasmids, miRNA mimics or miRNA inhibitors at indicated concentrations. If necessary, an equivalent amount of an empty plasmid, such as pmiRGLO, pMIR-luc-control, or pSG5, was transfected into the cells to maintain equal quantities of DNA in respective controls. After stabilization, cells were, if needed, further treated with PXR or GR ligands at indicated times and concentrations. Compounds were diluted in Opti-MEM I Reduced Serum Medium (11058021, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5% FBS. Then, cells were lysed and measured for both firefly and *Renilla* luciferase activities by a plate reader, Synergy2 (BioTek, Winooski, VT, USA), employing a Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activities were normalized to *Renilla* luciferase activities to reduce variability in transfection efficiency. Since the pmiRGLO vector contains a *Renilla* luciferase coding sequence for transfection normalization, cotransfection with pRL-TK was performed only in experiments with different reporter vectors than those with pmiRGLO. In the indicated cases, measured firefly luciferase activities were double normalized according to the published protocol to eliminate the background effect of tested ligand or miRNA reagent on activity of the empty parent vector[31]. According to this procedure, the *Renilla*-normalized firefly luciferase activities obtained from different reporter vectors were further normalized to the corresponding data that were obtained under identical conditions with control (empty) reporter vectors. The results are presented as the relative change or fold change in normalized firefly luciferase activities to the control activities (defined as 100% or 1). The data are presented as the mean ± SD acquired from biological replicates performed in at least technical triplicates ($n \geq 3$).

2.8. *Dual secreted Gaussia luciferase and SEAP reporter assays*

HepG2 cells were seeded into 48-well plates and transiently transfected with a reporter gene construct (either pPXR1-Gluc or pPXR2-Gluc) together with expression vectors using Lipofectamine 3000 Reagent (Thermo Fisher Scientific, Waltham, MA, USA). The next day, the medium was changed based on the experimental setting to either conditioned or nonconditioned Opti-MEM I Reduced Serum Medium supplemented with 5% FBS. After 24 h, medium was analyzed for activities of both SEAP, with Ready-To-Glow Secreted Luciferase Reporter Systems (Clontech, Palo Alto, CA, USA), and *Gaussia* luciferase, with Secrete-Pair

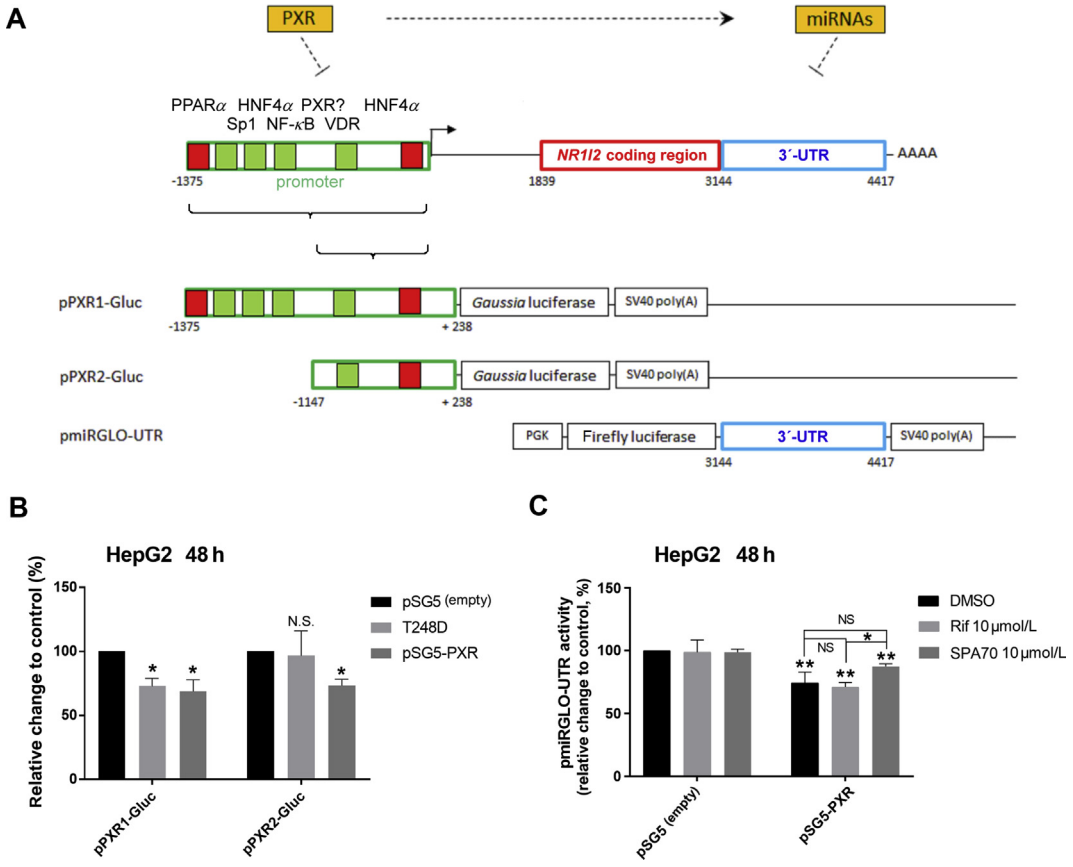

Figure 3 Implication of the *NR1I2* promoter region and 3′-UTR in PXR regulation. (A) Scheme depicting the *NR1I2* gene with its regulatory regions. Suggested or confirmed transcription binding sites within the promoter region are shown as green and red boxes, respectively[9,12]. A schematic overview of reporter plasmids alignment under the scheme. The numbers indicate nucleotide positions either in the promoter region or in mRNA of the *NR1I2* gene. (B) HepG2 cells were transiently transfected with either the pPXR1-Gluc or pPXR2-Gluc *Gaussia* luciferase reporter construct (150 ng/well) concomitantly with the pSG5-RXRα expression vector (50 ng) and with either pSG5-PXR, pSG5-T248D or pSG5 (empty) control vector (100 ng). The next day, the medium was changed and after 24 h, *Gaussia* luciferase and SEAP activities were assessed. The results are presented as relative change in SEAP-normalized *Gaussia* luciferase activities to control experiments cotransfected with empty pSG5 vector (100%). The data are shown as the mean ± SD ($n = 3$). (C) HepG2 cells were transiently transfected with either pmiRGLO-UTR or pmiRGLO (empty) vector (50 ng) together with either pSG5-PXR expression vector or pSG5 (empty) control vector (100 ng). The next day, cells were treated with rifampicin (Rif, 10 μmol/L), SPA70 (a PXR antagonist, 10 μmol/L) or vehicle (DMSO; 0.1%) for 24 h. Samples were subsequently analyzed by Dual-Luciferase Reporter Assay System. Firefly luciferase activities were normalized to *Renilla* luciferase activities and further normalized to the corresponding data obtained from experiments with the empty pmiRGLO vector. The data are shown as the mean ± SD ($n = 3$) and are expressed as relative change to DMSO-treated controls cotransfected with the empty pSG5 vector defined as 100%. $^{*}P < 0.05$ or indicates a statistically significant effect of PXR activation compared to empty pSG5 vector-transfected controls or to rifampicin-treated pSG-PXR cells; $^{**}P < 0.01$ indicates a statistically significant suppression of pmiRGLO-UTR vector activity; N.S. indicates a statistically insignificant effect.

Gaussia Luciferase Assay Kit (GeneCopoeia, Rockville, MD) and GL-S buffer for more stable activity. The *Gaussia* luciferase and SEAP signals were detected by the plate reader Synergy2 (Bio-Tek, Winooski, VT), and *Gaussia* luciferase activities were normalized to SEAP activities. The data are presented as the relative change in normalized *Gaussia* luciferase activities to control activities, which were defined as 100%. The data are presented as the mean ± SD ($n = 3$).

2.9. *RT-qPCR analysis*

HepG2 or Huh-7 cells seeded onto 12-well plates were transfected with expression plasmids and, if needed, further treated with PXR or GR ligands at indicated times and concentrations. Primary human hepatocytes (Liv14) were treated with Rif (10 μmol/L) for 24 h. RT-qPCR analyses (performed at the Faculty of Pharmacy, Charles University) were performed as described previously with gene specific assays for *CYP3A4* (hCYP3A4_Q2), hypoxanthine phosphoribosyltransferase (*HPRT*, 3033-F), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, 3058-F) and *NR1I2* (hNR1I2_Q8) (Generi Biotech, Hradec Kralove, Czech Republic)[4,29]. The results are presented as fold or relative change in gene expression compared to corresponding controls (defined as 1 or 100%). The data are presented as the mean ± SD ($n = 3$).

For mouse experiments (performed at the Medical Faculty, Charles University), total RNA from liver samples was isolated using TRI reagent (Sigma−Aldrich), converted into cDNA by High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher

Table 1 Donor information and culture conditions of isolated primary human hepatocytes.

Liver	Origin	Age (years)	Sex	Culture medium	Pathology
Liv1	QPS	61	M	ISOM	Cholangiocarcinoma
Liv2	Resection	52	F	HGM	Cyst adenoma
Liv3	QPS	66	F	ISOM	Colorectal cancer
Liv4	Resection	70	F	HGM	Cholangiocarcinoma
Liv5	Donor	73	M	HGM	Stroke
Liv6	Resection	76	M	ISOM, HGM	Metastasis from colon cancer
Liv7	Resection	70	M	ISOM	Metastasis from colon cancer
Liv8	Resection	46	F	ISOM	Cholangiocarcinoma
Liv9	Resection	64	M	ISOM	Hepatocellular carcinoma
Liv10	Resection	46	M	LNF	Angioma
Liv11	Resection	62	M	LNF	Metastasis from colon cancer
Liv12	Resection	70	M	LNF	Metastasis of GIST
Liv13	Resection	80	F	ISOM	Metastasis of colon cancer

Scientific, Waltham, MA, USA) and subsequently analyzed on a QuantStudio 7.0 PCR machine (Thermo Fisher Scientific) by using TaqMan Fast Universal PCR Master Mix and TaqMan Gene Expression Assays for detection of *Nr1i2* (Mm01344139_m1) or *Cyp3a11* (Mm00731567_m1) mRNA (Thermo Fisher Scientific). The results were calculated using threshold cycle values and normalized to *Gapdh* mRNA levels. The results are presented as relative change in gene expression compared to corresponding controls. The data are presented as the mean ± SD acquired from five biological samples per group.

RT-qPCR experiments (performed at INSERM) with Primary human hepatocytes were performed after total RNA extraction with TRIzol Reagent and reverse transcription of 500 ng total RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). qPCR was performed using the Roche SYBR Green reagent and a LightCycler 480 apparatus (Roche Diagnostics, Meylan, France) with the following program: one step at 95 °C for 10 min followed by 50 cycles of denaturation at 95 °C for 10 s, annealing at 65 °C for 15 s, and elongation at 72 °C for 15 s. Primer sequences are listed in Table 2. Relative quantification was performed delta-delta method and accounting for reaction efficiencies. C_t values of genes of interest were normalized to C_t values for the reference gene *RPLP0* (Liv3-13) or *GAPDH* (Liv1-2). The results are presented as relative change in gene expression compared to vehicle control (DMSO), which was defined as 1. The data are shown as the mean±SD obtained from biological samples. RTs were run in biological duplicates, and qPCR reactions were run in technical duplicates.

2.10. *Expression analysis of miRNAs*

HepG2 cells that were seeded into 12-well plates were transfected with miRNA inhibitor (50 ng)/mimic (25 ng) reagents or pSG5-PXR expression vector (400 ng) for 24 or 48 h. If needed, the cells were further treated with Rif (10 μmol/L) at the indicated times. Total RNA was isolated with spin column chromatography using a miRCURY RNA Isolation Kit (Cell & Plant; now part of QIAGEN, Germantown, MD, USA). Concentration and purity of total RNA were determined by a NanoDrop spectrophotometer (Thermo Fisher Scientific). Total RNA was reverse-transcribed to miR-18a-5p specific cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) at 16 °C for 30 min followed by incubation at 42 °C for 30 min with subsequent 85 °C for 5 min. cDNA samples were subsequently analyzed with TaqMan assays for miR-18a-5p (Assay ID 002422) and U6 snRNA (Assay ID 001973) using a TaqMan Fast Advanced Master Mix on the StepOnePlus Real-Time PCR System (all Thermo Fisher Scientific). The C_t values for miR-18a-5p were normalized to the C_t values of the reference gene *U6*. The results are presented as fold or relative change in gene expression compared to corresponding controls (defined as 1 or 100%). The data are shown as the mean±SD acquired from biological replicates performed in technical triplicates ($n = 3$).

2.11. *miRNA expression profiling*

Huh-7 cells were seeded into 12-well plates and transfected with pSG5-GRα (600 ng per well) overnight followed by treatment with DMSO (0.1%, *v/v*) or DEX (100 nmol/L) for 24 h. Cells were lysed in QIAzol Lysis Reagent (QIAGEN, Germantown, MD, USA), and total RNA, enriched for small RNAs, was isolated using the miRNeasy Mini Kit (QIAGEN, Germantown, MD, USA). MiRNA expression profiling of 754 miRNAs was performed using TaqMan Array Human MicroRNA Card Set v3.0 (Thermo Fisher Scientific). All procedures followed standard manufacturers' recommendations. Briefly, total RNA (100 ng) was reverse-transcribed into cDNA using TaqMan MicroRNA Reverse Transcription Kit together with Megaplex RT Primers (Thermo Fisher Scientific). cDNA samples were further analyzed on the 7900HT Real-Time PCR System (Thermo Fisher Scientific) using TaqMan Universal Master Mix II, no UNG (Thermo Fisher Scientific). Raw data were processed using SDS software version 2.4 (Thermo Fisher Scientific). The primary data were further processed using ExpressionSuite Software v1.1 (Thermo Fisher Scientific). The relative quantification was performed by delta-delta method assuming a PCR efficiency of 100%. *RNU48* was determined to be the most stable reference gene and was thus used for normalization of miRNA expression levels. The results are presented as relative change in miRNA expression compared to DMSO control of a representative experiment. Experiments have been repeated with the same results.

2.12. *Western blotting assays*

Western blotting experiments were performed using SDS-PAGE electrophoresis for total protein fractions of mouse liver samples as described elsewhere[32]. Primary antibodies used for detection were anti-PXR (PA5-41170) and anti-CYP3A11 (PA1-343, Thermo Fisher Scientific). Protein band intensity on PVDF membranes was quantified using Quantity One imaging software (Bio-Rad, Hercules, CA, USA). Protein expression was normalized to expression of β-actin (A5316, Sigma−Aldrich). The results are presented as relative change in protein expression compared to corresponding controls in the same interval after PCN application. The data are shown as the mean ± SD acquired from five biological samples per group. For Western blotting experiments with primary human hepatocytes and cancer cell lines lysates, β-actin (A5316, Sigma−Aldrich), PXR (PA5-41170, Thermo Fisher Scientific), CYP3A4 (PA1-343, Thermo Fisher

Table 2 Primer sequences for RT-qPCR with SYBR green chemistry.

Gene	Forward primer	Reverse primer
CYP3A4	GCCTGGTGCTCCTCTATCTA	GGTGTTGACCATCATAAAG
NR1I2	GGACCAGCTGCAGGAGCAAT	CATGAGGGGCGTAGCAAAGG
RPLP0	TCGACAATGGCAGCATCTAC	GCCTTGACCTTTTCAGCAAG
GAPDH	GGTCGGAGTCAACGGATTTGGTCG	CAAAGTTGTCATGGATGACC

Scientific), and RXRα (5388, Cell Signaling Technology, Danvers, MA, USA) primary antibodies were used.

2.13. Statistical analysis

Statistical analyses were performed using GraphPad PRISM 7 software (GraphPad Software Inc., San Diego, CA, USA). Differences between groups were compared using Student's paired two-tailed *t* test. One-way analysis of variance (ANOVA) with Dunnett's test was applied to the data if more than two groups were analyzed. Nonparametric Wilcoxon matched-pairs tests and parametric paired *t* test were used to statistically compare paired expression data between control and PXR ligand-treated samples in human hepatocytes. Nonparametric Mann−Whitney test was used to compare unpaired data in mice.

3. Results

3.1. PXR ligands downregulate PXR expression in human hepatocytes

It has been shown that feed-back regulation is a common phenomenon among nuclear receptors[24]. Therefore, in our preliminary experiment, we explored whether treatment with a model PXR ligand, rifampicin, resulted in pronounced downregulation of *NR1I2* transcripts in one culture of Primary human hepatocytes (Liv13). We observed a time-dependent effect of rifampicin on *NR1I2* gene mRNA expression (Fig. 1A). Correspondingly, rifampicin induced expression of *CYP3A4*, the dominant PXR target gene, in the same hepatocyte culture (Fig. 1B). At the protein level, CYP3A4 was consistently upregulated by rifampicin at both 24 and 48 h intervals, however, PXR was apparently downregulated after 24 h treatment but not after 48 h. Expression of the PXR heterodimerzation partner, RXRα, was not affected by rifampicin treatment (Fig. 1C). In a follow-up study with PHH cultures, we confirmed that rifampicin (10 μmol/L) ($P = 0.001$) and another PXR activator, SR-12813 (3 μmol/L) ($P = 0.022$, paired *t* test), significantly suppress *NR1I2* mRNA expression 24 h after treatment (Fig. 1D), but the expression of *CYP3A4* mRNA was significantly upregulated in cultures of human hepatocytes (Fig. 1E). Phenobarbital (500 μmol/L) also significantly upregulated CYP3A4 mRNA expression even though the expression of *NR1I2* mRNA was not significantly affected in six hepatocyte cultures (Fig. 1D and E). The expression of both genes was highly variable among liver donors, reflecting known interindividual variability in hepatic *NR1I2* expression. The genetic variation in 3′UTR may also contribute to the variability.

3.2. PCN significantly downregulates mouse PXR in mice livers

A similar pattern of PXR expression was demonstrated in mice administered a single dose of PCN, the agonist of mouse PXR. Decreased expression of *Nr1i2* gene mRNA was observed 24 h after PCN exposure but was preceded by upregulation of *Nr1i2* transcript at 6 h (Fig. 2A). As expected, the prototypical murine PXR target gene *Cyp3a11* was significantly upregulated in response to PCN in murine livers (Fig. 2B). In agreement with these findings, the protein levels of PXR were significantly decreased at early intervals after PCN administration (Fig. 2C), but CYP3A11 protein was increased after 24, 72, and 168 h post-PCN application (Fig. 2D).

Herein, we can conclude that activation of PXR or its murine orthologue leads to time-dependent downregulation of *NR1I2* expression, which indicates feedback regulation of PXR.

3.3. Confirmation of NR1I2 promoter region and 3′-UTR in PXR regulation

To further examine PXR regulation, we used several luciferase reporter gene vectors with different promoter regions or the full-length 3′-UTR sequence of *NR1I2* gene mRNA (Fig. 3A). Our findings revealed that forced overexpression of PXR itself led to decreased activity of the pPXR1-Gluc vector bearing the promoter sequence from −1375 to +238 upstream of the human *NR1I2* gene. Similar findings were shown for the mutant T248D, which produces a constitutively active form of PXR[26] (Fig. 3B). The activity of the pPXR2-Gluc reporter vector bearing a shorter promoter region (−1147 to +238) of the *NR1I2* gene was statistically significantly downregulated with wild-type PXR construct. The sequence from −1375 to −1147 (228 nt) in pPXR1-Gluc, but not in pPXR2-Gluc, containing numerous putative response elements for HNF4α, PPARα, Sp1 and NF-κB transcription factors[9] (see schematic in Fig. 3A) thus does not seem to be important for the regulation although further detail experiments are needed. In subsequent experiments, we proved that the pmiRGLO-UTR construct harboring the 3′-UTR of the *NR1I2* mRNA was inhibited by PXR overexpression in HepG2 cells (Fig. 3C). Rifampicin treatment had no further significant effect on the observed inhibition. However, we observed significantly different activation of pmiRGLO-UTR after treatment with rifampicin, an agonist of PXR, or SPA70, a PXR antagonist (Fig. 3C). We thus suppose that ectopic PXR may be activated by endogenous ligands in HepG2 cells and that the activation can be abrogated by PXR antagonist SPA70.

These data demonstrate that both promoter and 3′-UTR regions of the *NR1I2* mRNA are important for PXR feed-back regulation (Fig. 3A).

3.4. The 3′-UTR of NR1I2 mRNA is involved in the suppression of PXR expression and the reduction of rifampicin-mediated upregulation of CYP3A4

It is widely recognized that miRNAs predominantly bind to responsive elements within the 3′-UTR of their target genes.

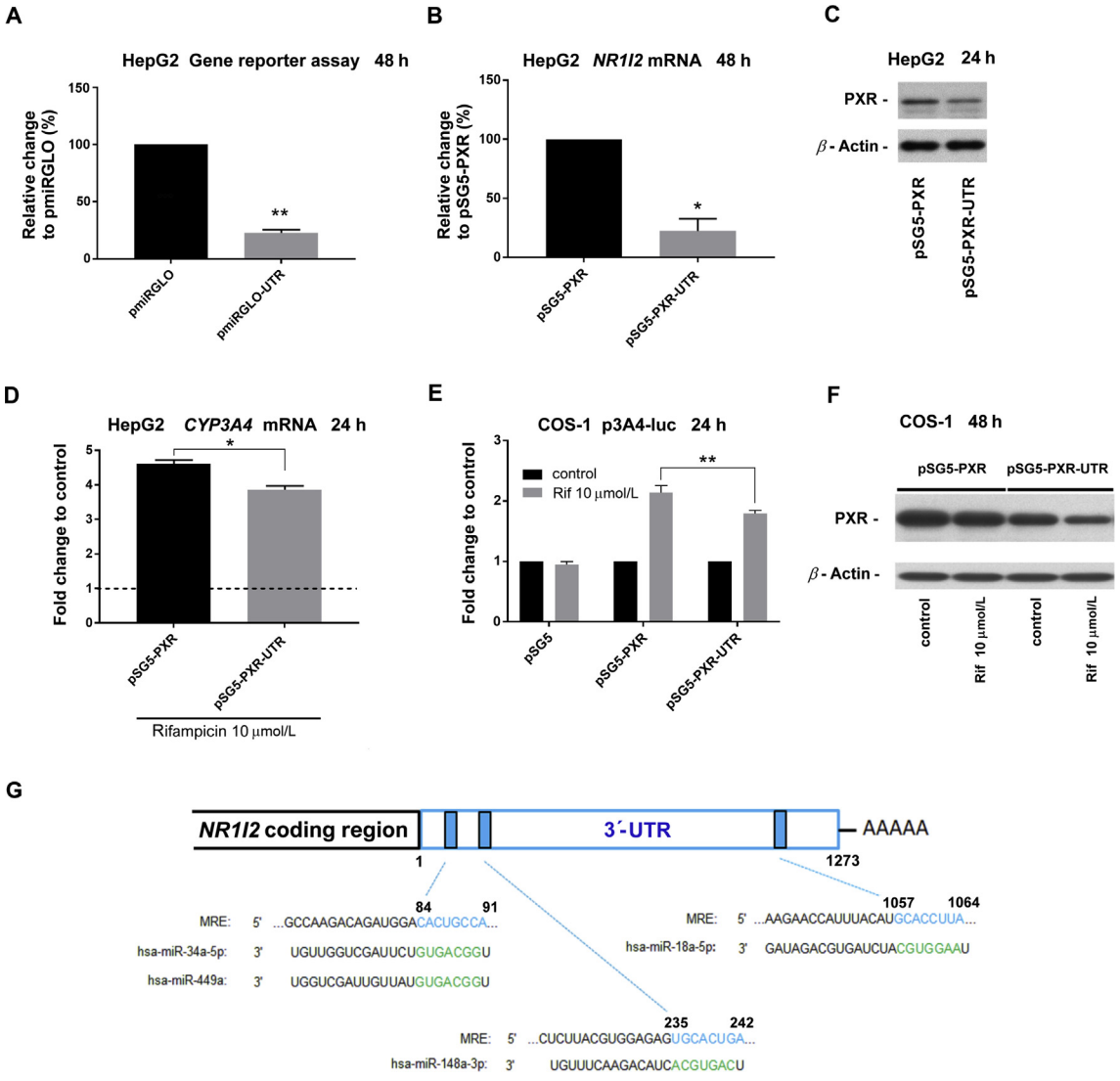

Figure 4 The 3′-UTR is involved in downregulation of PXR expression and decreased rifampicin-mediated upregulation of CYP3A4. (A) HepG2 cells were transiently transfected with either pmiRGLO-UTR or empty pmiRGLO vector (50 ng). After 48 h, the samples were analyzed by the Dual-Luciferase Reporter Assay kit. Firefly luciferase activities were normalized to *Renilla* luciferase activities. The data are shown as the mean ± SD ($n = 3$) and are expressed as relative change to an empty pmiRGLO vector defined as 100%. HepG2 cells were transiently transfected with either pSG5-PXR or pSG5-PXR-UTR vector (200 ng) and cotransfected with pSG5-RXRα (200 ng) for 24 h. After another 24 or 48 h in fresh medium, total RNA was isolated and analyzed for basal levels of *NR1I2* mRNA by RT-qPCR (B) or for PXR protein expression (C). The *HPRT* reference gene-normalized data are shown as the mean ± SD ($n = 3$) and are expressed as relative change to values gained from pSG5-PXR/pSG5-RXRα experiments (100%). Cell lysates were analyzed for expression of PXR protein by Western blotting. β-Actin was used as a loading control. Representative Western blotting results are shown. (D) HepG2 cells were transiently transfected with either pSG5-PXR or pSG5-PXR-UTR vector (200 ng) and cotransfected with pSG5-RXRα (200 ng). The next day, cells were treated with rifampicin (Rif, 10 µmol/L) for 24 h. Total RNA was isolated and analyzed for induction of *CYP3A4* mRNA by RT-qPCR. The *HPRT* reference gene-normalized data are shown as the mean ± SD ($n = 3$) and are expressed as fold change compared to vehicle-treated controls (defined as 1). (E) COS-1 cells were transiently transfected with a *CYP3A4* promoter luciferase reporter vector (p3A4-luc, 150 ng), pRL-TK (30 ng), and pSG5-RXRα (50 ng) together with either pSG5-PXR, pSG5-PXR-UTR or empty pSG5 vector (50 ng). The next day, cells were treated with rifampicin (Rif, 10 µmol/L) or vehicle (DMSO; 0.1%) for another 24 h. Then, samples were analyzed using a Dual-Luciferase Reporter Assay. Firefly luciferase activities were normalized to *Renilla* activities. The data are shown as the mean ± SD ($n = 3$) and are expressed as fold change compared to respective DMSO-treated controls. (F) COS-1 cells were transiently transfected with either a pSG5-PXR or pSG5-PXR-UTR construct (200 ng) and cotransfected with pSG5-RXRα (200 ng). The next day, cells were treated with vehicle (DMSO; 0.1%) or rifampicin (Rif, 10 µmol/L) for 48 h. Samples were analyzed for expression of PXR protein by Western blotting. β-Actin was used as a loading control. Representative Western blotting results are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ indicate statistically significant effects of the 3′-UTR region of *NR1I2* mRNA. (G). Schematic overview showing sequence complementarity between particular miRNAs and *in silico* predicted or experimentally confirmed miRNA-response elements (MREs) within the 3′-UTR of human *NR1I2* mRNA. Green letters depict seed regions of miRNAs. Blue letters refer to sequences within MREs complementary to miRNA seed regions. Numbers above sequences indicate a position of nucleotides downstream from the coding region of *NR1I2* mRNA.

Therefore, to address whether cellular miRNAs may affect the posttranscriptional regulation of PXR, HepG2 cells were transfected with either pmiRGLO (empty) or pmiRGLO-UTR vector. Detected luciferase activity was higher in cells transfected with the control empty vector (pmiRGLO) compared to cells transfected with the luciferase reporter construct bearing the 3′-UTR of the *NR1I2* gene mRNA (Fig. 4A). Consistently, transfection with the PXR expression vector harboring the 3′-UTR resulted in lower PXR expression at both the mRNA and protein levels in HepG2 cells (Fig. 4B and C). The same trend was also observed in undifferentiated Huh-7 cells, which are a near PXR-null cellular model (Supporting Information Figs. S1A–C). Taken together, it is plausible that some miRNAs in hepatic cell lines may cause general repressive effects on the 3′-UTR of *NR1I2* mRNA.

Next, we investigated whether this suppressive effect may affect the inducibility of the PXR target gene *CYP3A4* in HepG2 and COS-1 cells. Indeed, rifampicin-mediated upregulation of *CYP3A4* mRNA was mildly decreased in cells transfected with pSG5-PXR-UTR relative to cells transfected with the pSG5-PXR vector (Fig. 4D). This difference was also pronounced in the *CYP3A4* promoter luciferase reporter gene assay using COS-1 cells (Fig. 4E). Of particular interest, rifampicin enhanced the suppressive role of the 3′-UTR on PXR protein expression in COS-1 cells transfected with pSG5-PXR-UTR. However, rifampicin did not affect PXR protein expression in COS-1 cells transfected with pSG5-PXR (Fig. 4F). For this reason, we can assume that rifampicin-induced changes in the cellular miRNA expression profile may result in the suppression of *NR1I2* expression through its 3′-UTR region of *NR1I2* mRNA.

3.5. *MiR-18a-5p is a relevant regulator of NR1I2 expression and transcriptional activity*

To reveal which miRNAs might be implicated in rifampicin-induced *NR1I2* mRNA destabilization *via* its full-length 3′-UTR, we first screened the effect of a set of miRNA mimics on the pmiRGLO-UTR luciferase reporter vector. The tested miRNAs were selected based on previous literature reports and on our recent bioinformatics study[33]. An overview of sequence complementarity between candidate miRNAs and their putative miRNA-response elements (MREs) within 1273-bp-long 3′-UTR of *NR1I2* mRNA is shown in Fig. 4G.

Gene reporter assays performed in HepG2 cells indicated that all selected miRNA mimics, such as hsa-miR-18a-5p, hsa-miR-449a, hsa-miR-34a-5p, and hsa-miR-148a-3p, were able to attenuate pmiRGLO-UTR activity compared to the nonspecific control (cel-miR-39-3p) mimic (Fig. 5A). However, only miR-18a-5p and miR-148a-3p were potent negative regulators of rifampicin-induced and PXR-mediated activation of the CYP3A4-based luciferase reporter construct pER6-3A4-tat-luc in HepG2 cells (Fig. 5B). In contrast, hsa-miR-449a enhanced the effect of rifampicin on the PXR-responsive reporter construct. Hsa-miR-34a-5p had no effect on the activation of the reporter construct by rifampicin (Fig. 5B). The latter experiments were performed on the pER6-3A4-tat-luc plasmid, which carries three copies of the proximal *CYP3A4* promoter ER6 response element and displays minimal basal PXR-non-specific activation in HepG2 cells. Of note, miRNA mimics at a concentration of 25 nmol/L were used in these experiments in accordance with manufacturer's recommendation.

To further characterize the role of miR-18a-5p in this phenomenon, we performed a reporter assay with a set of luciferase reporter constructs containing the full-length 3′-UTR or native, reversed or miR-18a-5p complementary MREs (Fig. 5C, left panel). We found that the miR-18a-5p mimic has the ability to significantly decrease activation of constructs generated with both the full-length 3′-UTR and those with one copy of the native MRE for miR-18a-5p. The construct with the reversed MRE exerted no effects on miR-18a-5p in HepG2 (Fig. 5C, right panel) or COS-1 cells (Supporting Information Fig. S2A). In control experiments, we demonstrated functional transfection of the miR-18a-5p mimic and inhibitor into HepG2 cells (Fig. S2B) and significant endogenous activity of miR-18a-5p by employing the miR-18a-5p-Compl reporter vector (Fig. S2C). Additionally, treatment with rifampicin led to time-dependent changes in miR-18a-5p expression in HepG2 cells, with significant upregulation of miR-18a-5p at 6 h, suppression at 12 h and normalization to original levels between 24 and 48 h posttreatment (Fig. 5D). Moreover, HepG2 cells transiently transfected with the miR-18a-5p-Compl luciferase construct with the pSG5-PXR expression vector that were treated with rifampicin showed inhibited reporter construct activity, even though the effect was not statistically significant. Treatment with SPA70, a PXR antagonist, reversed the suppressive effect of PXR on pmiRGLO-18a-5p activation, but the effect was statistically significant only in comparison with rifampicin effect (Fig. 5E). These data confirm augmented miR-18a-5p functional expression in HepG2 cells in response to both overexpressed and activated PXR (Fig. 5E). Finally, miR-18a-5p mimic down-regulated PXR expression in HepG2 cells (data not shown), which is in agreement with published data in LS180 cells[20]. Taken together, these data suggest that miR-18a-5p is a direct regulator of *NR1I2* gene expression and that rifampicin-mediated changes in miR-18a-5p expression may help to explain the negative regulation of activated PXR.

3.6. *Activators of GR upregulate NR1I2 mRNA levels by both increasing NR1I2 mRNA stability via the 3′-UTR and by activating the NR1I2 promoter*

In the next series of experiments, we attempted to decipher the role of the 3′-UTR region of *NR1I2* mRNA in GR-mediated PXR expression regulation. To address this aim, pSG5-PXR-UTR was transfected into Huh-7 cells to explore the effect of subsequent treatment with dexamethasone. Huh-7 cells were selected due to their functional activity of GR signaling[34]. We observed significantly higher expression of *NR1I2* mRNA after treatment with dexamethasone (100 nmol/L) than in control experiments with the PXR expression vector lacking the 3′-UTR (Fig. 6A). Since dexamethasone has been demonstrated to stimulate PXR-mediated *CYP3A4* expression[10], we investigated whether the 3′-UTR of *NR1I2* mRNA may have any impacts on *CYP3A4* mRNA induction. Indeed, dexamethasone induced *CYP3A4* mRNA in the presence of pSG5-PXR expression vector, but *CYP3A4* mRNA upregulation was even greater in the vector with the 3′-UTR (Fig. 6B). We further showed that dexamethasone enhanced luciferase activity in response to the pmiRGLO-UTR vector, which was reversed by the GR antagonist RU486 in Huh-7 cells (Fig. 6C) and HepG2 cells (Supporting Information Figs. S3A and B). Finally, we found that the observed 3′-UTR stabilization in the pmiRGLO-UTR construct was not selective for dexamethasone but was also present in response to other glucocorticoids (Fig. 6D). These luciferase gene reporter experiments were performed in parallel with pmiRGLO-UTR and pmiRGLO (empty)

controls, and the final activation of the 3′-UTR was calculated as normalized activation (ratio).

In additional experiments, we confirmed previously reported findings that ligand-activated GR triggers promoter-dependent transcription of *NR1I2* gene by employing *NR1I2* promoter *Gaussia* reporter constructs in HepG2 cells (Fig. 6E and F). In summary, we propose that activated GR may have a dual role in PXR upregulation, as it stabilizes *NR1I2* mRNA *via* the 3′-UTR potentially by suppression of targeting miRNA(s) and activates *NR1I2* gene transcription from the *NR1I2* promoter (Fig. 6G).

3.7. *Dexamethasone decreases miR-18a-5p expression and activity*

To determine which miRNAs demonstrate altered expression in response to dexamethasone treatment, we performed screening analysis of 754 miRNAs in response to either dexamethasone- or control-treated samples of Huh-7 cells using a TaqMan Array Human MicroRNA Card. Profiling showed 11 upregulated ($\geq$2) and 22 downregulated ($\leq$0.5) miRNAs after 24 h treatment with dexamethasone (Fig. 7A and B). Closer inspection also revealed that hsa-miR-148a-3p was slightly upregulated, while hsa-miR-

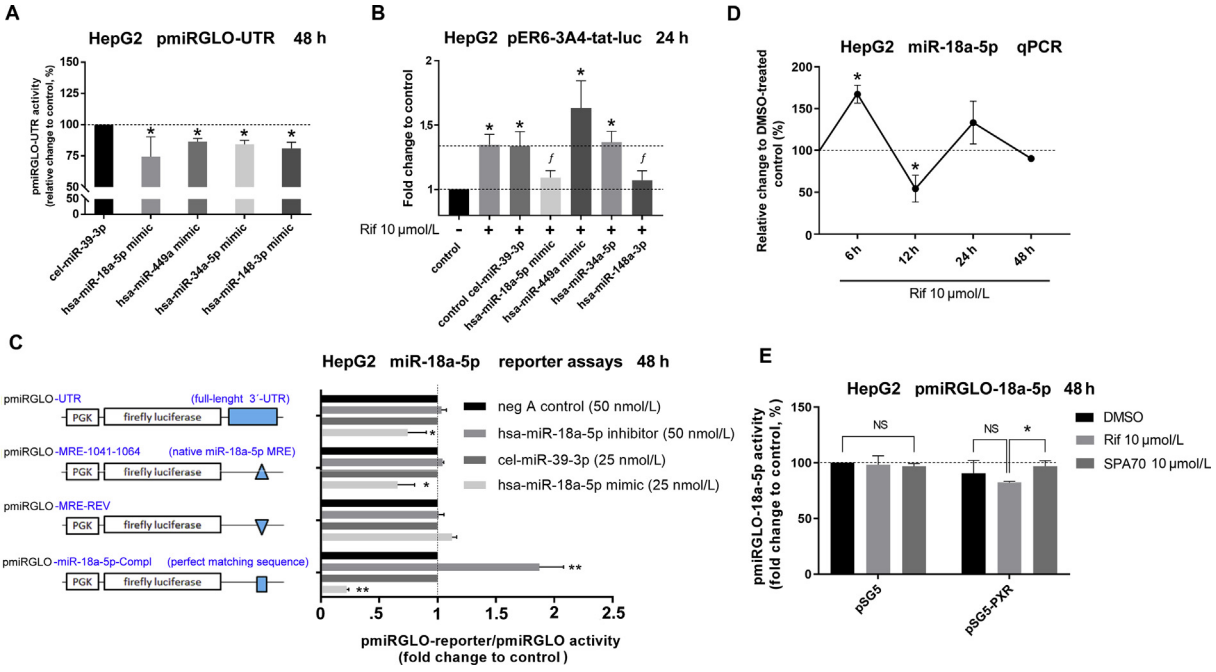

Figure 5 MiR-18a-5p is a relevant regulator of *NR1I2* expression and transcriptional activity. (A) Dual-Luciferase Reporter Assay in HepG2 cells transiently transfected with either pmiRGLO or pmiRGLO-UTR construct (50 ng) with one of the following miRNA mimic reagents such as cel-miR-39-3p (mimic control), hsa-miR-18a-5p mimic, hsa-miR-449a mimic, hsa-miR-34a-5p mimic or hsa-miR-148a-3p mimic (25 nmol/L) for 48 h. Firefly luciferase activities were normalized to *Renilla* activities and further normalized to the corresponding data with the empty pmiRGLO vector. The data are shown as the mean ± SD ($n = 3$) and are expressed as relative change compared to the miRNA mimic control cel-miR-39-3p (100%). (B) Dual-Luciferase Reporter Assay in HepG2 cells transiently transfected with pER6-3A4-tat-luc PXR-responsive luciferase reporter vector (150 ng) and pRL-TK (30 ng) together with either cel-miR-39-3p mimic control, hsa-miR-18a-5p mimic, hsa-miR-449a mimic, hsa-miR-34a-5p mimic or hsa-miR-148a-3p mimic (25 nmol/L) for 48 h. Cells were treated with rifampicin (Rif, 10 µmol/L) or vehicle (DMSO; 0.1%) for a further 24 h. Firefly luciferase activities were normalized to *Renilla* activities. The data are shown as the mean ± SD ($n = 3$) and are expressed as fold change compared to DMSO-treated control (defined as 1). (C) Dual-Luciferase Reporter Assay in HepG2 cells transiently transfected with either pmiRGLO, pmiRGLO-UTR, pmiRGLO-MRE-1041-1064, pmiRGLO-MRE-1041-1064-REV or pmiRGLO-18a-5p-Compl construct (50 ng) together with one of the miRNA inhibitor/mimic reagents, such as negative control A (inhibitor control), hsa-miR-18a-5p inhibitor (50 nmol/L), cel-miR-39-3p (mimic control) or hsa-miR-18a-5p mimic (25 nmol/L) for 48 h. Firefly luciferase activities were normalized to *Renilla* activities and further normalized to corresponding firefly luciferase activities obtained from empty pmiRGLO vector experiments. The data are shown as the mean ± SD ($n = 3$) and are expressed as fold change compared to inhibitor miRNA or mimic controls (defined as 1). (D) HepG2 cells were transiently transfected with the pSG5-PXR expression vector (400 ng) for 24 h. Then, cells were treated with vehicle (DMSO; 0.1%) or rifampicin (10 µmol/L) for 6, 12, 24, or 48 h, respectively. Total RNA was isolated and analyzed for miR-18a-5p expression by RT-qPCR. *U6* reference gene-normalized data are shown as the mean ± SD ($n = 3$) and are expressed as relative change compared to corresponding vehicle-treated controls (defined to be 100%). (E) HepG2 cells were transiently transfected with either pmiRGLO or pmiRGLO-18a-5p (50 ng) together with either the pSG5-PXR expression vector or empty pSG5 control vector (100 ng). The next day, cells were treated with rifampicin (Rif, 10 µmol/L), SPA70 (an PXR antagonist, 10 µmol/L) or vehicle (DMSO; 0.1%) for 48 h. Samples were subsequently analyzed by Dual-Luciferase Reporter Assay. Firefly luciferase activities were normalized to *Renilla* activities and further normalized to corresponding firefly luciferase activities obtained from the empty pmiRGLO vector. The data are shown as the mean ± SD ($n = 3$) and are expressed as relative change compared to DMSO-treated control transfected with empty pSG5 vector (defined as 100%). $^{*}P < 0.05$ or $^{**}P < 0.01$ indicates a statistically significant effect to vehicle-treated, negative controls or empty vector controls. $^{f}P < 0.05$ indicates a statistically significant effect of miRNA mimic on Rif-induced action.

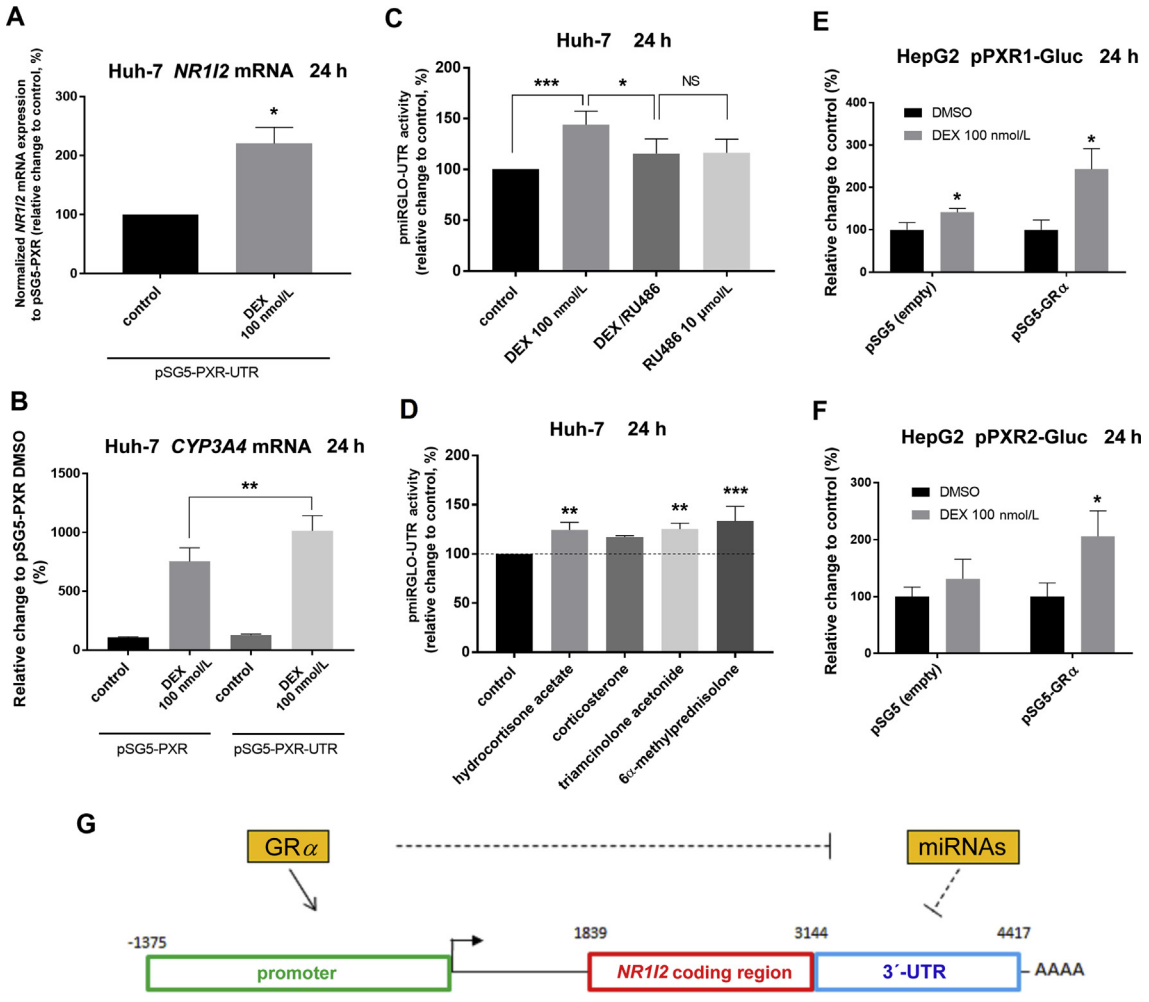

Figure 6 Activators of the glucocorticoid receptor upregulate *NR1I2* mRNA levels both by increasing PXR stability *via* the 3′-UTR and by *NR1I2* promoter activation. (A) Huh-7 cells were transiently transfected with pSG5-PXR or pSG5-PXR-UTR (200 ng) and cotransfected with the glucocorticoid receptor expression vector (pSG5-GRα, 600 ng). The next day, cells were treated with vehicle (DMSO; 0.1%) or dexamethasone (DEX, 100 nmol/L) for 24 h. Total RNA was isolated and analyzed for *NR1I2* mRNA expression by RT-qPCR. The data were first normalized to the *GAPDH* reference gene and were acquired from pSG5-PXR-UTR transfected samples, which were further normalized to corresponding experiments transfected with the PXR expression vector lacking the 3′-UTR. The data are shown as the mean ± SD ($n = 3$) and are expressed as relative change compared to DMSO-treated controls (defined as 100%). (B) Huh-7 cells were transiently transfected with either pSG5-PXR or pSG5-PXR-UTR (200 ng) and were cotransfected with the pSG5-GRα expression vector (600 ng). The next day, cells were treated with vehicle (DMSO; 0.1%) or DEX (100 nmol/L) for 24 h. Total RNA was isolated and analyzed for *CYP3A4* mRNA expression by RT-qPCR. *GAPDH* reference gene-normalized data are shown as the mean ± SD ($n = 3$) and are expressed as relative change compared to the mean of vehicle-treated pSG5-PXR/pSG5-GRα-transfected experiments (defined as 100%). $^{*}P < 0.05$, $^{**}P < 0.01$ indicates a statistically significant effect of the 3′-UTR region of *NR1I2* mRNA. Huh-7 cells were transiently transfected with pmiRGLO or pmiRGLO-UTR vector (50 ng) together with pSG5-GRα expression vector (150 ng). The next day, cells were treated with DEX (100 nmol/L), RU486 (a GR antagonist, 10 μmol/L), a combination of DEX (100 nmol/L)/RU486 (10 μmol/L) or vehicle (DMSO; 0.2%, *v/v*) (C) or with glucocorticoids hydrocortisone acetate, corticosterone, triamcinolone acetonide, and 6α-methylprednisolone (100 nmol/L) (D) for 24 h. After treatment, samples were analyzed by Dual-Luciferase Reporter Assay. Firefly luciferase activities were normalized to *Renilla* activities and further normalized to activation of the empty pmiRGLO vector. The data are shown as the mean ± SD ($n = 3$) and are expressed as relative change compared to vehicle-treated control (defined as 100%). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicates a statistically significant effect compared to vehicle-treated controls. (E) and (F) HepG2 cells were transiently transfected with either a pPXR1-Gluc or pPXR2-Gluc (150 ng) *NR1I2* promoter luciferase reporter construct concomitantly with pSG5-GRα expression vector or pSG5 empty control (100 ng). The next day, cells were treated with dexamethasone (DEX, 100 nmol/L) or vehicle (DMSO; 0.1%) for 24 h. Then, *Gaussia* luciferase and SEAP activities were assessed. The results are presented as relative change in SEAP-normalized *Gaussia* luciferase activities compared to DMSO-treated controls (defined as 100%). The data are shown as the mean ± SD ($n = 3$). $^{*}P < 0.05$ indicates a statistically significant effect of DEX. (G) The scheme summarizes hypothetical mechanisms for how activated GR enhances *NR1I2* mRNA expression. On one side, GR stabilizes the 3′-UTR of *NR1I2* mRNA and on the other hand, GR triggers *NR1I2* gene transcription.

34a-5p was unchanged (Fig. 7A). In contrast, hsa-miR-18a-5p and hsa-miR-449a were downregulated 24 h posttreatment with dexamethasone (Fig. 7B). Since hsa-miR-18a-5p was suppressed by dexamethasone and the functional miR-18a-5p responsive element was confirmed to be within the 3′-UTR of *NR1I2* mRNA (Fig. 5C, Fig. S2A, and reported in[20]), miR-18a-5p may be the candidate miRNA responsible for GR-mediated post-transcriptional stabilization of *NR1I2* mRNA. To examine this hypothesis, we transiently transfected HepG2 cells with the pmiRGLO-18a-5p-Compl and pSG5-GRα expression vectors followed by treatment with dexamethasone for either 24 or 48 h. As expected, dexamethasone increased normalized activity of the miR-18a-5p-sensitive luciferase reporter compared to vehicle, and this effect was reversed by treatment with RU486 (Fig. 8A and B). This was not the case for the pMIR-luc-34a-5p-Compl luciferase construct with a perfect matching sequence for miR-34a-5p (Supporting Information Fig. S4A). This finding is in agreement with the TaqMan Array analysis data, where dexamethasone did not alter hsa-miR-34a-5p expression (Fig. 7A), although miR-34a-5p appears to exhibit potent endogenous activity in HepG2 cells (Figs. S4B and C). Altogether, these data provide evidence that dexamethasone treatment in hepatic cell lines downregulates expression of hsa-miR-18a-5p, subsequently resulting in stabilization of the 3′-UTR of *NR1I2* mRNA and *NR1I2* mRNA upregulation (Fig. 9).

4. Discussion

Drug-metabolizing enzymes (DMEs) are involved in removal of xenobiotics, and maintain the homeostasis of certain endogenous compounds. To achieve balance between these metabolic processes, DMEs must be tightly regulated. Several nuclear receptors have been shown to be involved in mutual feed-back and feed-forward loops that are responsible for orchestrating these metabolic pathways.

In this study, we demonstrated that *NR1I2* expression may be under feed-back loop regulation through dual mechanisms involving both transcriptional and posttranscriptional regulation based on the following observations: (i) rifampicin represses PXR transcript and protein expression in a time-dependent manner in primary human hepatocytes (Fig. 1A and C); (ii) SR-12813, another PXR activator, downregulated *NR1I2* mRNA expression 24 h posttreatment (Fig. 1D); (iii) PCN, an agonist of mouse PXR, suppressed both mRNA and protein levels of PXR in mice 24 h after administration (Fig. 2A and C); and (iv) both the promoter and 3′-UTR regions of the *NR1I2* gene suppress PXR expression (Fig. 3B and C).

Our results are consistent with another study that used primary rat hepatocytes, where PCN-activated PXR attenuated its own expression at both the mRNA and protein levels after 48 h[35]. Negative PXR feed-back regulation was further supported in *NR1I2* promoter reporter assays using Huh-7 cells[35]. However, discordant data have also been reported in intestinal cell lines[20,36], in an immortalized human hepatocyte cell line[37] and in a few isolated human hepatocyte experiments[38,39]. Nevertheless, the extent of changes in *NR1I2* transcript was highly variable among human liver donors used in this study after treatment with PXR ligands. This is not surprising, since donor-dependent differences in the inducibility of PXR target genes have also been previously described[38,40]. Thus, different tissue models and small sample sizes in studies with human hepatocytes may be the reason for this discrepancy. Moreover, detailed time course analysis is missing in these reports.

In this study, we also confirmed that the overexpression of ectopic PXR suppresses promoter-mediated transcription or *NR1I2* gene. The mechanism explaining this phenomenon remains unclear since no putative binding site for PXR was found within its promoter in *in silico* analysis or in mechanistic studies either for human or mouse PXR[9]. It is interesting that PXR down-regulates its own transcription both *via* the promoter and 3′-UTR, regardless of whether PXR is activated by a ligand. We suppose that forced expression of exogenous PXR in the absence of a ligand may mimic activated PXR, which was previously reported[10]. One explanation may be that overexpression of PXR causes increased sensitivity to endogenous ligands. This hypothesis is further confirmed by no subsequent activation of PXR by rifampicin and by reversal of the effect by the PXR antagonist, SPA70, in the experiments (Fig. 3C).

In addition, we showed for the first time that PXR negatively affects the luciferase gene reporter construct carrying the full-length 3′-UTR of *NR1I2* mRNA. As a result of this finding, we aimed to uncover the precise role of the 3′-UTR in PXR

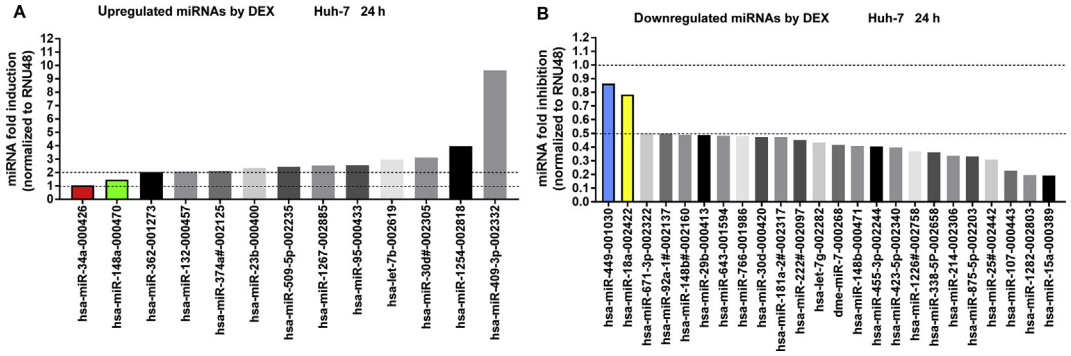

Figure 7 Dexamethasone-induced changes in miRNA expression profiles in Huh-7 cells. Huh-7 cells were transfected with pSG5-GRα (600 ng) overnight and then treated with DMSO (0.1%) or dexamethasone (DEX, 100 nmol/L) for 24 h. Total RNA was isolated and analyzed using a miRNA expression TaqMan array card. *RNU48* reference gene-normalized data are expressed as fold change compared to vehicle-treated experiments defined as 1. Only miRNAs with upregulated ($\geq$2) (A) or downregulated ($\leq$0.5) (B) expression in response to DEX treatment are shown. Additionally, the color bars indicate other miRNAs of interest potentially involved in PXR 3′-UTR regulation. Data of a representative experiments are shown.

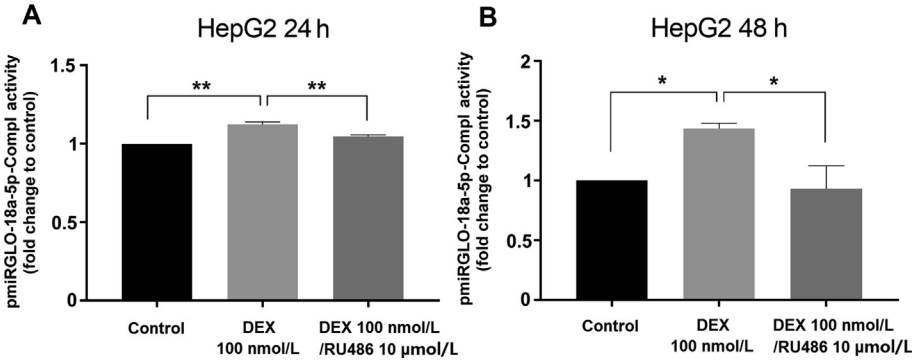

Figure 8 Dexamethasone treatment stabilizes miR-18a-5p-responsive luciferase construct. HepG2 cells were transiently transfected with pmiRGLO or pmiRGLO-18a-5p-Compl (50 ng) together with the glucocorticoid receptor expression vector (pSG5-GRα, 150 ng). The next day, cells were treated with dexamethasone (DEX, 100 nmol/L), a combination of DEX (100 nmol/L)/RU486 (10 µmol/L) or vehicle (DMSO; 0.2%) for 24 (A) or 48 h (B). After treatment, samples were analyzed with Dual-Luciferase Reporter Assay. Firefly luciferase activities were normalized to *Renilla* activities and were further normalized to the corresponding activity of the empty pmiRGLO vector. The data are shown as the mean ± SD ($n = 3$) and are expressed as fold change compared to DMSO-treated controls (defined as 1). $^{*}P < 0.05$, $^{**}P < 0.01$ indicate a statistically significant effect compared to vehicle-treated or DEX-treated samples.

regulation. It is known that the 3′-UTR of *NR1I2* mRNA is the primary site to which miRNAs bind and exert their negative posttranscriptional effects. Although more than one hundred unique miRNAs have been predicted to potentially target the 3′-UTR of *NR1I2* mRNA[33], there is still relatively scarce information on posttranscriptional regulation of PXR. As shown by Takagi et al.[13], levels of *NR1I2* mRNA expression does not correlate with PXR protein levels in human liver samples ($n = 25$), suggesting that PXR is subject to posttranscriptional regulation. However, in a follow-up correlation study, a linear relationship was observed between *NR1I2* mRNA and protein levels in liver samples ($n = 24$) obtained from a Chinese Han population, raising the question of potential ethnic differences in PXR expression regulation[41]. However, relatively small sample sizes have been enrolled in both these studies, impairing adequate statistical power. In a pioneering report, a functional responsive element for miR-148a was found in the 3′-UTR of *NR1I2* mRNA, and an inverse correlation of miR-148a expression with PXR protein/mRNA ratio has been reported[13]. However, this observation was not confirmed by others[41,42], and Lamba et al.[8] even showed a positive correlation.

In our experiments, we revealed that the 3′-UTR of *NR1I2* mRNA attenuates PXR expression (Fig. 4A–C) and suppresses rifampicin-mediated upregulation of *CYP3A4* mRNA in HepG2 cells (Fig. 4D). It is therefore plausible that endogenous miRNAs in these cells may result in a general repressive effect on the 3′-UTR. An analogous observation was reported for HNF4α[22]. Moreover, rifampicin enhanced the repressive role of the 3′-UTR in PXR protein expression (Fig. 4F), thus indicating that rifampicin-induced changes in cellular miRNAs profile may inhibit PXR expression *via* its 3′-UTR. In agreement with this hypothesis, a modulatory effect of rifampicin on miRNA expression profiles has been recently reported in primary human hepatocytes[43,44].

To shed light on the missing link between rifampicin and miRNA regulation of the PXR 3′-UTR, we decided to include miR-148a[13] and miR-18a-5p[20] in our subsequent analysis, as their binding sites were experimentally confirmed within the 3′-UTR of *NR1I2* mRNA. Additionally, miR-449a and miR-34a-5p, which share the same seed sequences (Fig. 4G), were also used as they were both predicted in our *in silico* analysis[33]. In addition, miR-34a-5p[8,22] and miR-449a[22] have been reported to be negatively associated with *NR1I2* mRNA expression. Both miR-34a-5p and miR-449a are also detectable in human hepatic cell line[22,45], which is in agreement with our experiments where endogenous miR-34a-5p downregulated a luciferase reporter construct with a cloned complementary sequence for miR-34a-5p in HepG2 (Fig. S4B). Among the tested miRNAs, we found that only miR-18a-5p and miR-148a mimics attenuated rifampicin-induced PXR-mediated transactivation of CYP3A4-derived luciferase

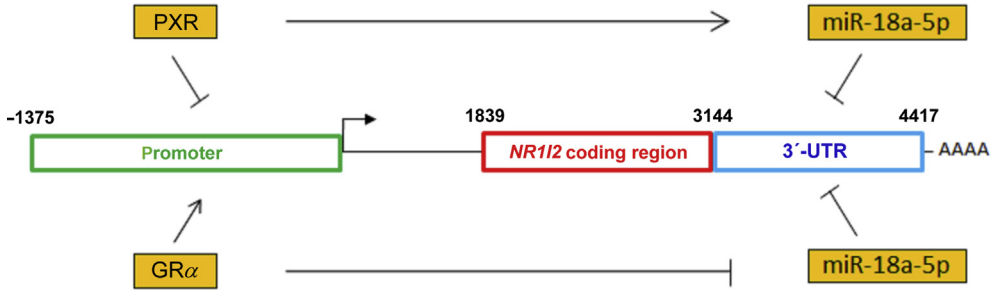

Figure 9 Summary of PXR expression regulation. PXR suppresses its own expression *via* a dual mechanism including promoter suppression and mRNA destabilization. The latter may be mediated at least partially *via* upregulation of miR-18a-5p expression. GR receptor triggers transcription of the *NR1I2* promoter and stabilizes *NR1I2* mRNA posttranscriptionally through downregulation of miR-18a-5p.

reporter construct (Fig. 5B), which is consistent with previous findings[13,20]. Notably, miR-449a even enhanced the activation of the CYP3A4-derived luciferase reporter construct in response to rifampicin, which indicates a possible indirect effect of miR-449a on other factors involved in the PXR-mediated transactivation.

In further experiments, we focused on miR-18a-5p as a potential culprit of PXR negative regulation. MiR-18a-5p is a member of the miR-17/92 cluster dysregulated in cancers and is highly expressed in hepatocellular carcinoma[46]. Very recently, miR-18a-5p was systematically described as a functional and direct regulator of PXR expression based on luciferase gene reporter studies, loss- and gain-of-function methods, and analysis of PXR-mediated inducibility of *CYP3A4* mRNA in LS180 human colorectal adenocarcinoma cells[20]. In the present study, the functional responsive element for miR-18a-5p within the 3′-UTR of *NR1I2* mRNA was confirmed with different luciferase reporter vectors, including the construct with the full-length 3′-UTR (Fig. 5C and Fig. S2A). In contrast to the report by Sharma et al.[20], we observed an oscillating pattern of miR-18a-5p regulation in HepG2 cells after treatment with rifampicin (Fig. 5D). During early intervals (6 h), rifampicin significantly induced expression of miR-18a-5p, which was followed by its significant reduction at 12 h and return to a slightly increased level after 24 h. The latter is in line with microarray experiments revealing only weak increases (1.64-fold change) in miR-18a-5 expression after 48 h treatment with rifampicin (10 μmol/L) in pooled human hepatocytes[20]. In intestinal LS180 cells, different time-dependent profiles of miR-18a-5p expression was reported with decreased expression during short intervals of treatment (3 and 6 h)[20]. In addition, authors did not observe significant down-regulation of *NR1I2* mRNA expression in any interval in LS180 cells after treatment with two PXR ligands. These findings highlight the tissue-specific manner of the regulation and the importance of time profiling of miRNA and mRNA expression with a stress on early periods of treatment. Additionally, HepG2 cells transfected with a pmiRGLO-18a-5p-Compl luciferase reporter construct with the pSG5-PXR expression vector showed downregulation of reporter vector activity after treatment with rifampicin and without treatment, which indicates an impact of overexpressed PXR on miR-18a-5p expression (Fig. 5E). In opposite, the PXR antagonist SPA70 displayed opposite effect that rifampicin in the experiments. Our results suggest that miR-18a-5p is a direct regulator of PXR and that rifampicin-mediated changes in miR-18a-5p expression likely contribute to negative regulation of activated PXR.

Additionally, we studied the role of the 3′-UTR region of *NR1I2* mRNA in GR-mediated regulation of PXR expression. In accordance with previous works[10,11], we confirmed that activated GR by dexamethasone triggers transcription of *NR1I2* gene from its promoter (Fig. 6E and F). Since it is unclear whether GR also induces posttranscriptional changes in *NR1I2* mRNA, we performed several experiments using reporter and expression vectors harboring the full-length 3′-UTR region of *NR1I2* mRNA. Interestingly, the expression of *NR1I2* mRNA was more enhanced in cells that were transfected with the PXR expression construct with the 3′-UTR region than without the 3′-UTR region after treatment with glucocorticoids (Fig. 6A). Moreover, dexamethasone-induced *CYP3A4* mRNA expression was significantly higher in the presence of the PXR vector with cloned 3′-UTR than with an expression vector lacking the 3′-UTR (Fig. 6B). We further observed that dexamethasone increased luciferase activity of pmiRGLO-UTR vector, which was reversed by the GR antagonist RU486 in Huh-7 (Fig. 6C) and HepG2 cells (Figs. S3A and B). Based on these results, we postulate that activation of GR leads to increased *NR1I2* mRNA *via* dual mechanisms involving *NR1I2* promoter activation and 3′-UTR *NR1I2* mRNA stabilization (Fig. 6G). Dexamethasone was used at nanomolar concentrations in our experiments, which is known to selectively activate GR but not PXR[10]. This experimental precaution limits the down-regulation of activated PXR on its 3′-UTR and helps to reveal the specific GR-mediated stabilization of 3′-UTR *NR1I2* mRNA.

Next, we proposed that activated GR may increase *NR1I2* mRNA expression through changes in miRNA expression levels. In our miRNA expression analysis, we revealed that miR-18a-5p is downregulated by dexamethasone. Dexamethasone-induced downregulation of miR-18a expression has been consistently observed in primary rat thymocytes and cultured leukemic cells[47]. As a miR-18a-5p responsive element was previously identified within the 3′-UTR of *NR1I2* mRNA, we believed that miR-18a-5p may also be a candidate miRNA responsible for GR-mediated posttranscriptional stabilization of *NR1I2* mRNA. We observed that dexamethasone increased normalized activation of pmiRGLO-18a-5p-Compl luciferase reporter construct, which was reversed by RU486. Taken together, these data provide insight indicating that dexamethasone treatment downregulates expression of hsa-miR-18a-5p, which may subsequently result in enhanced stability of *NR1I2* mRNA.

5. Conclusions

In conclusion, in this study, our data support the role of miRNAs in feed-back and feed-forward regulation and in glucocorticoid-mediated hormonal upregulation of PXR expression. Based on our results, it is tempting to suppose that PXR feed-back regulation could prevent overstimulation of PXR target genes or control augmented and time-limited metabolic elimination of xenobiotic from the body. This could be a protective mechanism that hinders the unfavorable effects of endogenous compounds in the presence of PXR ligands. In this work, we also propose that glucocorticoids increase PXR expression *via* a dual mechanism involving activation of the *NR1I2* gene promoter and by stabilization of the 3′-UTR of *NR1I2* mRNA for the first time. The positive effect of glucocorticoids on hepatic DME gene expression is an illustrative example of coordination and hormone-controlled liver response to xenobiotic exposure that occurs *via* a multifaceted process.

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Author contribution

Tomas Smutny, Stanislav Micuda, Sabine Gerbal-Chaloin and Petr Pavek designed the experiments. Jan Dusek, Lucie Hyrsova, Jana Nekvindova, and Alzbeta Horvatova performed cellular experiments. Stanislav Micuda performed animal studies, and Tomas Smutny, Stanislav Micuda, Sabine Gerbal-Chaloin and Petr Pavek performed statistical analyses.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2019.09.010.

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