

JPET # 252940

The HNF1 α -regulated lncRNA HNF1 α -AS1 is Involved in the Regulation of Cytochrome P450 Expression in Human Liver Tissues and Huh7 Cells

Yiting Wang*, Liang Yan*, Jingyang Liu, Shitong Chen, Guangming Liu, Yali Nie, Pei Wang, Weihong Yang, Liming Chen, Xiaobo Zhong, Shengna Han#, and Lirong Zhang#

Department of Pharmacology, School of Basic Medical Sciences, Zhengzhou University, Zhengzhou, Henan, China (Y.W., L.Y., J.L., S.C., G.L., Y.N., P.W., S.H., L.Z.); Department of Pharmacy, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China (L.Y.); Department of Forensic Medicine, School of Basic Medical Sciences, Zhengzhou University, Zhengzhou, Henan, China (W.Y.); Department of Pharmaceutical Sciences, School of Pharmacy, University of Connecticut, Storrs, Connecticut, USA (L.C., X.Z.)

* Yiting Wang and Liang Yan contributed equally to this work.

Dr. Lirong Zhang and Dr. Shengna Han are co-corresponding authors.

JPET # 252940

Running Title: Regulation of P450s by HNF1 α and HNF1 α -AS1

Address correspondence to: Dr. Lirong Zhang, Department of Pharmacology, School of Basic Medical Sciences, Zhengzhou University, Zhengzhou, Henan, China. Phone: +86 371 67781855; E-mail: zhanglirongzzu@126.com

Co-corresponding author: Dr. Shengna Han, Department of Pharmacology, School of Basic Medical Sciences, Zhengzhou University, Zhengzhou, Henan, China. E-mail: hanshengna@126.com

Number of text pages: 28

Number of figures: 7

Number of tables: 0

Number of references: 46

Number of words in Abstract: 241

Number of words in Introduction: 530

Number of words in Discussion: 1431

ABBREVIATIONS:

AhR: aryl hydrocarbon receptor; CAR: constitutive androstane receptor; CYPs: cytochrome P450s; DMEM: Dulbecco's modified Eagle's medium; DMSO: dimethyl sulfoxide; FBS: fetal bovine serum; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; HNF1 α : hepatocyte nuclear factor 1 alpha; HNF1 α -AS1: hepatocyte nuclear factor 1 alpha antisense 1; HNF4 α : hepatocyte nuclear factor 4 alpha;

JPET # 252940

HNF4 α -AS1: hepatocyte nuclear factor 4 alpha antisense 1; lncRNA: long non-coding RNA; miRNA: microRNA; PCR: polymerase chain reaction; PVDF: polyvinylidene fluoride; PXR: pregnane X receptor; qPCR, quantitative polymerase chain reaction

JPET # 252940

ABSTRACT

Expression of cytochrome P450s (CYPs) is regulated by epigenetic factors, such as DNA methylation, histone modifications, and non-coding RNAs through different mechanisms. Among these factors, long non-coding RNAs (lncRNAs) have been shown to play important roles in the regulation of gene expression; however, little is known about the effects of lncRNAs on the regulation of CYP expression. The aim of this study was to explore the role of lncRNAs in the regulation of CYP expression by using human liver tissues and hepatoma Huh7 cells. Through lncRNA microarray analysis and quantitative polymerase chain reaction in human liver tissues, we found that the lncRNA hepatocyte nuclear factor 1 alpha antisense 1 (HNF1 α -AS1), an antisense RNA of HNF1 α , is positively correlated with the mRNA expression of CYP2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 as well as pregnane X receptor (PXR) and constitutive androstane receptor (CAR). Gain- and loss-of-function studies in Huh7 cells transfected with siRNAs or overexpression plasmids showed that HNF1 α not only regulated the expression of HNF1 α -AS1 and CYPs, but also regulated the expression of CAR, PXR, and aryl hydrocarbon receptor (AhR). In turn, HNF1 α -AS1 regulated the expression of PXR and most CYPs without affecting the expression of HNF1 α , AhR, and CAR. Moreover, the rifampicin-induced expression of CYPs was also affected by HNF1 α and HNF1 α -AS1. In summary, the results of this study suggested that HNF1 α -AS1 is involved in the HNF1 α -mediated regulation of CYPs in the liver at both basal and drug-induced levels.

Introduction

Cytochrome P450 enzymes (CYPs) comprise monooxygenases that are responsible for the metabolism of most clinically used drugs (Nair et al., 2016). Significant interindividual variability in CYP-mediated drug metabolism leads to various responses to drugs in clinical practice (Zanger and Schwab, 2013). Factors contributing to the changes of CYP expression and function account for the inter-individual variability of CYP-mediated drug metabolism, including genetic, epigenetic, physiological, pathological, and environmental factors (Zhou et al., 2009; Zanger et al., 2014; Tracy et al., 2016; Yu et al., 2017). Uncovering the molecular mechanisms in the regulation of CYP expression is therefore likely to be beneficial for effective therapies and drug safety.

Genetic polymorphism constitutes an important mechanism affecting the expression and functions of CYPs, but can only explain a proportion (15–30%) of the inter-individual differences among global populations (Ingelman-Sundberg et al., 2007; Pinto and Dolan, 2011). Epigenetic mechanisms are also important for the regulation of CYP expression, including DNA methylation, histone modifications, and non-coding RNAs (Peng and Zhong, 2015; Tang and Chen, 2015). Both DNA methylation and histone modifications regulate the expression of CYPs at a transcriptional level, whereas non-coding RNAs can influence P450 expression at either the transcriptional or post-transcriptional level. Depending on size, a non-coding RNA can be grouped as either small non-coding RNA (<200 nt), including approximately 22 nt microRNA (miRNA), or long non-coding RNA (lncRNA, >200 nt). Regulation of CYPs by miRNAs has been well documented through either direct or indirect interactions between miRNAs and the 3'-untranslated regions of the *CYP* mRNAs (Tsuchiya et al., 2006; Pan et al., 2009) or their regulatory nuclear receptor mRNAs (Yu et al., 2016); however, the regulation of CYP expression by lncRNAs is under investigation.

LncRNAs can act as either activators or repressors in the regulation of gene expression by directly binding to transcriptional factors or recruiting chromatin-remodeling complexes. In some cases, lncRNAs recruit histone modification enzymes such as WD repeat domain 5/myeloid-lymphoid leukemia protein complexes to promoter regions and activate the transcription of target genes by driving histone H3 lysine 4 trimethylation (Wang et al., 2011). In other situations, lncRNAs inactivate the transcription of target genes by recruiting polycomb repressive complex-2 and increasing histone H3 lysine 27 trimethylation in promoter regions (Kaneko et al., 2014). Moreover, lncRNAs can also affect the post-transcriptional and translational regulation of target genes by influencing the splicing process of mRNA or binding to translation factors and ribosomes (Ma et al., 2013).

Expression of CYPs can also be regulated by lncRNAs. Recently, we have reported that the lncRNAs hepatocyte nuclear factor 1 alpha antisense 1 (HNF1 α -AS1) and HNF4 α -AS1, together with nuclear receptors, comprise a regulatory network to control the basal and drug-induced expression of CYPs in HepaRG cells (Chen et al., 2018). In the current study, we provide a systematic analysis to determine the role of HNF1 α -AS1 as well as its neighbor HNF1 α in the regulation of CYP expression in human liver samples and hepatocarcinoma Huh7 cells. We found that HNF1 α -AS1 under the control of HNF1 α is involved in the regulation of basal and drug-induced expression of numerous CYPs as well as the transcriptional regulator pregnane X receptor (PXR), constitutive androstane receptor (CAR), and aryl hydrocarbon receptor (AhR).

Materials and Methods

Chemicals and Reagents. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Corning Inc. (Armonk, NY). Opti-MEM were purchased from Thermo Fisher Scientific (51985034, Carlsbad, CA). TriPure isolation reagent was purchased from Roche (Basel, Switzerland). Penicillin and streptomycin mixture, dimethyl sulfoxide (DMSO), and other chemical reagents were provided by Solarbio Science & Technology Co. (Beijing, China). Primers, SYBRTM Select Master Mix, siRNAs for HNF1 α (A01003), siRNAs for HNF1 α -AS1 (HSS178564), and control siRNAs (A06001) were provided by Thermo Fisher Scientific (Carlsbad, CA). Polyvinylidene fluoride (PVDF) membranes were purchased from EMD Millipore (Billerica, MA). HNF1 α expression plasmid was purchased from Genecopoeia (Guangzhou, Guangdong, China), HNF1 α -AS1 plasmid was provided by GeneChem Co., Ltd. (Shanghai, China). HNF1 α primary antibody (ab174653, Abcam, Cambridge, UK), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary antibody (60004-1-Ig, Proteintech, Wuhan, China), and secondary antibodies (SA00001-1, SA00001-2, Proteintech, Wuhan, China) were used in western blot analysis.

Human Liver Tissues. The uses of the liver tissues were approved by the Medical Ethical Committee of First Affiliated Hospital of Zhengzhou University and written consents were obtained from the participating patients. A total of 43 human liver tissues were collected and prepared as described in our previous studies (He et al., 2016; Nie et al., 2017). The information of the 43 liver samples used in this study has been provided in Supplemental Tables 3 and 4, including age, sex, race, and pathological condition.

Cell Culture and Transfection. Human hepatoma cell line Huh7 is a commercially available cell line that constitutes a frequently used in vitro system to study gene regulation (Sivertsson et al., 2010). Huh7 cells were provided by the Type Culture Collection of the

JPET # 252940

Chinese Academy of Sciences (Catalog number: TcHu182, Shanghai, China) and cultured in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin mixture. For gene silencing or overexpression experiments, Huh7 cells were transiently transfected with specific siRNAs or expression plasmids using LipofectamineTMRNAiMAX Transfection Reagent according to the manufacturer's instructions (Thermo Fisher Scientific, US). Briefly, Huh7 cells were allowed to grow for up to 24 h on 6-well plates and reached 80–90% confluence prior to transfection. Then, 40 pmol of siRNAs targeting HNF1 α , HNF1 α -AS1, or control siRNAs were mixed with RNAiMAX reagent in MEM and added into the culture medium. In overexpression studies, 2.5 μ g of plasmids were used for each well. At 24 h after transfection, the culture medium was replaced with DMEM supplemented with 2% FBS and incubated for another 24 h. For drug induction studies, transfected cells were incubated with rifampicin (10 μ M) or DMSO (0.1%, v/v) for 24 h before harvested.

RNA Isolation and Real-time Quantitative Polymerase Chain Reaction (qPCR). Total RNA from liver tissues or cultured cells was isolated using the TriPure isolation reagent according to the manufacturer's instruction (Basel, Switzerland). The quality and concentrations of RNAs were analyzed by a Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, US). For mRNA expression analysis, total RNAs were reversely transcribed using a PrimeScript RT reagent kit and qPCR reactions were performed by a SYBR method as previously described (Nie et al., 2017). Primers are shown in Supplemental Table S1.

LncRNA Microarray Analysis. Total RNAs from the selected liver tissues were isolated using TRIzol reagent (10296028, Thermo Fisher Scientific, Carlsbad, CA) and the expression profiles of lncRNAs were determined by lncRNA microarray chips (4 \times 180k, Agilent Technologies, Santa Clara, CA). Data were extracted with Feature Extraction software 10.7 (Agilent Technologies, Santa Clara, CA) and raw data were normalized using the Quantile

JPET # 252940

algorithm, GeneSpring Software 11.0 (Agilent Technologies). LncRNA microarray and data analysis were performed by Shanghai Biotechnology Corporation (Shanghai, China).

Western Blot Analysis. Total proteins of the liver tissues or treated cells were prepared using a RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) and protein concentrations were determined using a previously described method (Nie et al., 2017; Yan et al., 2017). Protein samples were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. After blocked for 2 h in 5% nonfat milk, membranes were incubated with primary antibodies for HNF1 α or GAPDH overnight at 4°C. Primary antibodies were diluted as follows: anti-HNF1 α (1:1000; rabbit polyclonal) and anti-GAPDH (1:10000; mouse monoclonal). The membranes were then incubated in horseradish peroxidase-labeled secondary antibodies in blocking buffer for 2 h and visualized with an enhanced chemiluminescence method. GAPDH protein was used as a loading control.

Statistical Analysis. All described in vitro experiments with Huh7 cells were performed as three independent experiments. Data are shown as the means \pm SD (standard deviation). Statistical significances between groups were analyzed by two-tailed unpaired Student's *t* test using SPSS version 17.0 (IBM, Armonk, NY). Pearson's correlation analysis was performed to assess the correlations of gene expression between HNF1 α -AS1 and CYPs as well as nuclear receptors in the 43 liver tissue samples using Prism 6 from GraphPad (La Jolla, CA).

Results

LncRNA Expression Profiles in Liver Tissues. The expression levels of mRNA and protein of CYP3A4 in liver tissues were measured by qPCR and western blot. Two groups were set according to the expression levels of CYP3A4: Group A contained three samples with a high level of CYP3A4 expression for both mRNA and protein and Group B contained three samples with a low level of CYP3A4 (Fig. 1A and 1B). The differences of mean mRNA and protein between group A and B were 3.0- and 3.1-fold, respectively. Then, expression profiles were analyzed in each group using a microarray assay specific for lncRNAs. A total of 112 lncRNA transcripts were identified to be differently expressed between the two groups (fold difference >2.0 , $p < 0.05$, Fig. 1C). Among these, 89 lncRNA transcripts were higher in Group A than in Group B, whereas 23 transcripts were lower. Among the top 10 increased lncRNAs in Group A, lncRNA HNF1 α -AS1 was ranked at sixth with a 3.3-fold difference (Fig. 1D).

Evolutionary Conservation of HNF1 α and HNF1 α -AS1 DNA Sequences. The *HNF1 α -AS1* gene is located at chromosome 12 between 120,941,728 and 120,980,771 in the annotated Human GRCh38/hg38 Genome with a genomic sequence of 39.04 kb spanning 2 exons and 1 intron on the antisense strand (Fig. 2A). In the 3'-direction, the next gene is that encoding HNF1 α at chromosome 12 between 120,978,543 and 121,002,512 in the Human GRCh38/hg38 Genome with a genomic sequence of 23.97 kb spanning 10 exons and 9 introns on the sense strand. The genomic locus of *HNF1A* and *HNF1 α -AS1* genes forms a typical pair of sense coding gene and neighbor antisense non-coding gene. Analysis of the conservation levels of the DNA sequences of HNF1 α and HNF1 α -AS1 was performed using HomoloGene from NCBI (www.ncbi.nlm.nih.gov/homologene/459), UCSC genomic browser (www.genome.ucsc.edu), and NONCODE (www.noncode.org). The results indicated that the human DNA sequences of HNF1 α are highly conserved with those of other mammals

JPET # 252940

(approximately 90%) and birds (approximately 75%) as well as fish (zebrafish, approximately 60%) (Fig. 2B and 2C), indicating its likely importance in physiological processes across the species. However, the human HNF1 α -AS1 sequence showed a much less level of conservation with that of other species, being only conserved within mammals (>60%) and showing no conservation with birds and fish (Fig. 2B and 2C).

Tissue-specific Expression Patterns of *HNF1A* and *HNF1 α -AS1* RNAs. The protein encoded by the *HNF1A* gene is a well-studied transcription factor controlling the expression of numerous liver-specific genes. A specific tissue distribution pattern of *HNF1A* mRNA was retrieved from the RNA-Seq Expression Data GTEx in 53 tissues from 570 donors (Consortium, 2013), which showed relatively high expression levels in the stomach, liver, pancreas, small intestine, colon, and kidney, the major organs in the gastrointestinal tract (Supplemental Fig. S1A). A very similar tissue distribution pattern in the gastrointestinal tract organs was also found for HNF1 α -AS1 (Supplemental Fig. S1B), indicating that HNF1 α -AS1 is expressed in the same organs as HNF1 α . These results suggested that HNF1 α may control general physiological processes for gastrointestinal functions, whereas HNF1 α -AS1 is possibly involved in physiological processes for gastrointestinal functions in a more species-specific manner in mammals. These results further supported the value of examining the roles of HNF1 α and HNF1 α -AS1 in the regulation of drug-metabolizing CYP enzymes.

Correlations between HNF1 α -AS1 and CYPs as well as Transcriptional Regulators in Liver Tissues. To further study the relationships between HNF1 α -AS1 and CYPs as well as transcriptional regulators in human liver, the RNA levels of HNF1 α -AS1, HNF1 α , CYPs (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4), and transcriptional regulators (PXR, CAR, and AhR) were measured in 43 liver tissues using qPCR and the correlations between them were analyzed using Pearson's correlation coefficient (Fig. 3A-I, Supplemental Fig. S2,

JPET # 252940

and Supplemental Table S2). The results indicated that the RNA levels of the target genes presented considerable individual differences among the 43 samples (Supplemental Fig. S2) and the correlations between different mRNAs were varied (Supplemental Table S2). Specifically, the expression of *HNF1 α -AS1* RNA showed a statistically significant correlation with the expression of *HNF1A* mRNA (Fig. 3A, $r = 0.447$, $p = 0.002$) and major examined CYPs, including CYP2C8 (Fig. 3B, $r = 0.498$, $p = 0.0007$), 2C9 (Fig. 3C, $r = 0.535$, $p = 0.0002$), 2C19 (Fig. 3D, $r = 0.360$, $p = 0.018$), 2D6 (Fig. 3E, $r = 0.538$, $p = 0.0002$), 2E1 (Fig. 3F, $r = 0.391$, $p = 0.009$), and 3A4 (Fig. 3G, $r = 0.503$, $p = 0.0006$) as well as PXR (Fig. 3H, $r = 0.602$, $p < 0.0001$) and CAR (Fig. 3I, $r = 0.676$, $p < 0.0001$).

HNF1 α Regulates the Expression of HNF1 α -AS1, CYPs, and Nuclear Receptors in Huh7 cells. To uncover the impact of HNF1 α on the transcriptional expression of HNF1 α -AS1, CYPs, as well as transcriptional regulators, silencing and overexpression of HNF1 α were performed in Huh7 cells. A decrease of HNF1 α mRNA and protein was confirmed after siRNA knockdown of HNF1 α (Fig. 4A and 4B). A decrease of HNF1 α -AS1 (10% of the control) was also observed after knocking down the expression of HNF1 α (Fig. 4A). In addition, the mRNA levels of CYP2B6, 2C8, 2C9, 2D6, 2E1, and 3A4 as well as CAR and PXR were also decreased in the HNF1 α knockdown cells, whereas the expression of CYP1A2 and AhR was increased (Fig. 4C and 4D). Moreover, knockdown of the expression of HNF1 α also reduced the induction fold of CYP2B6, 2D6, 2E1, and 3A4 by rifampicin, but increased the induction of CYP1A2 (Fig. 4E).

Overexpression of HNF1 α mRNA and protein levels was confirmed by transfection of an expression plasmid containing the *HNF1A* gene (Fig. 5A). An increase of HNF1 α -AS1 (Fig. 5B) as well as CYP2B6, 2C9, 2D6, and 3A4 (Fig. 5D) was found in the cells with overexpression of HNF1 α in comparison to the control plasmid, whereas a decrease of

JPET # 252940

CYP1A2 and *AHR* mRNA expression was observed (Fig. 5C and 5D). The induction of *CYP2B6* and *2D6* by rifampicin were also increased by HNF1 α overexpression (Fig. 5E). These findings suggested that the alterations of HNF1 α expression resulted in changes of basal and rifampicin-induced expression of major CYPs via changes of HNF1 α -AS1 and the transcriptional regulators PXR, CAR, and AhR.

HNF1 α -AS1 Regulates the Expression of CYPs and PXR, but not HNF1 α , CAR, and AhR. To explore the role of HNF1 α -AS1 in the regulation of CYPs and transcriptional regulators, RNA interference and overexpression were performed in Huh7 cells by using transient transfection of siRNAs or expression plasmids of HNF1 α -AS1. SiRNA treatment showed the knockdown of HNF1 α -AS1 to approximately 50% of siRNA control (Fig. 6A). After knockdown of HNF1 α -AS1 expression, no alteration was observed for the mRNA levels of HNF1 α , AhR, or CAR (Fig. 6A and 6B), whereas the expression of *PXR* mRNA as well as the mRNAs of *CYP2B6*, *2C8*, *2C9*, *2D6*, *2E1*, and *3A4* was reduced, whereas that of *CYP1A2* was increased (Fig. 6B and 6C). The induction of most CYPs by rifampicin was also decreased after knockdown of HNF1 α -AS1, including *CYP1A2*, *2C8*, *2C19*, *2D6*, *2E1*, and *3A4* (Fig. 6D).

Overexpression of HNF1 α -AS1 by transfection with an HNF1 α -AS1 plasmid in Huh7 cells showed a 30-fold increase of HNF1 α -AS1 in comparison to that from a control plasmid (Fig. 7A). An increase of *PXR* mRNA without an effect on the expression of *AHR* and *CAR* mRNA was observed (Fig. 7B). However, the basal expression levels of most CYPs mRNA remained unchanged except *CYP1A2* and *2E1* (Fig. 7C). Furthermore, no effect of HNF1 α -AS1 overexpression was observed on rifampicin-induced expression of CYPs except an increase in *CYP1A2* induction (Fig. 7D). These results suggested that endogenous expression of HNF1 α -AS1 is needed in the regulation of PXR as well as most CYPs and the regulation of the

JPET # 252940

transcription of CYPs by HNF1 α may be mediated by alteration of both HNF1 α -AS1 and transcriptional regulators.

Discussion

Expression of CYPs in liver cells is largely regulated at transcriptional levels by nuclear receptors. HNF1 α and HNF4 α comprise two key transcription factors in the regulation of basal expression of CYPs (Liu and Gonzalez, 1995; Jover et al., 2001; Cheung et al., 2003; Kamiyama et al., 2007), whereas PXR and CAR represent two key transcription factors in the control of drug-induced expression of CYPs (Goodwin et al., 1999; Waxman, 1999; Tompkins and Wallace, 2007). PXR and CAR often require crosstalk with HNF1 α and HNF4 α as coactivators (Tirona et al., 2003; Li and Chiang, 2006). HNF1 α is a liver-enriched transcription factor, the overexpression of which in HepG2 cells enhances the expression of CYP3A4, 1A1, and 2C9 (Chiang et al., 2014). In the current study, strong correlations between the mRNA level of HNF1 α and transcriptional regulators PXR, CAR, and AhR as well as several CYPs in human liver tissues were observed (Supplemental Table S2). Loss- and gain-of-function studies showed that alteration of the HNF1 α expression directly resulted in changes of the mRNA levels of PXR, CAR, and AhR (Fig. 4C and 5C). The regulatory mechanisms may be associated with direct binding of HNF1 α on the target promoters, as it has been reported that an HNF1 α -binding site is located in the *PXR* promoter (Uno et al., 2003; Aouabdi et al., 2006). More importantly, the expression of HNF1 α also showed obvious impact on the basal and rifampicin-induced expression of several CYPs (Fig. 4D, 4E, 5D, and 5E). These results were in accordance with our recently published study in HepaRG cells (Chen et al., 2018). Together with previous studies, the current findings support HNF1 α as a key regulator of both transcriptional regulators and CYPs.

However, the regulatory mechanisms behind the impact of HNF1 α on the expression of transcriptional regulators and CYPs are far from being well understood. In this study, the role of lncRNA HNF1 α -AS1 in the HNF1 α -mediated regulation of CYP expression in liver cells

JPET # 252940

was determined. LncRNAs have attracted much more attentions owing to their irreplaceable functions in the regulation of many physiological processes (Khorkova et al., 2015). LncRNAs can interact with a wide range of biological molecules to regulate gene expression, such as proteins, DNAs, and RNAs (Villegas and Zaphiropoulos, 2015). In particular, a large group of proteins that interact with lncRNAs are transcriptional factors. For example, the lncRNA linc-YY1, derived from the promoter of transcriptional factor YY1, interacts with YY1 to remove the YY1/Polycomb repressive complex from target promoters, which leads to activation of downstream genes and promotes muscle regeneration (Zhou et al., 2015). Interactions of transcription factors with their genomic neighboring lncRNAs, particularly for sense-antisense pairs, have been considered as a general biological phenomenon (Kung et al., 2013; Herriges et al., 2014). Transcriptional factor-derived lncRNAs often participate in the regulatory activities of their paired transcriptional factors (Zhou et al., 2015). Therefore, it is logical to speculate that the HNF1 α -mediated transcription regulation of CYPs and transcriptional regulators may require involvement of its neighboring antisense lncRNAs.

The involvement of lncRNAHNF1 α -AS1 in HNF1 α regulatory function was first determined in an initial study wherein we screened differently expressed lncRNAs associated with differential expression of CYP3A4. Differentially expressed lncRNAs determined via microarray analysis identified a set of candidate lncRNAs (Fig. 1C), which included lncRNAHNF1 α -AS1 in a list of the top associated lncRNAs (Fig. 1D). The *HNF1 α -AS1* gene is located next to the *HNF1A* gene on the antisense strand in chromosome 12 (Fig. 2A). Compared to the *HNF1A* DNA sequence, the *HNF1 α -AS1* sequence is much less conserved through evolution outside of mammals (Fig. 2B and 2C), implying a certain extent of involvement of HNF1 α -AS1 in HNF1 α regulatory function in the mammalian liver.

The involvement of HNF1 α -AS1 in HNF1 α regulatory function was further demonstrated

JPET # 252940

in a correlation study with 43 human liver tissue samples (Fig. 3A-I). Expression of HNF1 α -AS1 was found to be statistically significantly correlated with the mRNA expression levels of most selected CYPs as well as that of HNF1 α , CAR, and PXR in the human liver tissues (Fig. 3A-I, $p < 0.05$, 0.01, or 0.001 in Pearson correlation analysis).

The involvement of HNF1 α -AS1 in HNF1 α regulatory function on CYP expression is supported by loss- or gain-of-function studies in human Huh7 cells. Alterations of HNF1 α -AS1 expression by either siRNA knockdown or plasmid overexpression directly resulted in significant changes of the mRNA levels of numerous tested CYPs as well as transcriptional regulators (Fig. 6 and 7) without a concomitant change of HNF1 α , suggesting that a network composed of HNF1 α and HNF1 α -AS1 is the upstream regulator of nuclear receptors, which then further mediate the basal and drug-induced CYP expression. Knockdown of the endogenous expression of HNF1 α -AS1 showed similar effects on the basal expression of CYPs compared with that obtained from silencing of the expression of HNF1 α (Fig. 4D and 6C). However, among the three transcriptional regulators, only *PXR* mRNA was affected by knockdown of HNF1 α -AS1 (Fig. 6B). We found that knockdown of HNF1 α -AS1 in Huh7 cells did not affect the AhR mRNA level but significantly increased the CYP1A2 mRNA level, and inconsistent with liver tissue in Supplementary Figure 2. Functional experiments may not be sufficient to detect CAR and AhR effects, because the basal expression level of CAR and AhR is lower in huh7 cells (data not show), there may be differences in expression of transcription factors between different cell types. We have previously reported that lncRNA, transcription factors and receptors form a complex regulatory network that regulates CYP enzyme expression (Chen et al., 2018). Therefore, we speculate that the regulation of CYP1A2 is a complex process that requires additional in-depth study. In addition, the impact of HNF1 α -AS1 knockdown on rifampicin-induced expression of CYPs was also not identical as that

JPET # 252940

obtained with HNF1 α knockdown. Specifically, different impact on the induction of CYP1A2, 2C8, and 2C19 by HNF1 α -AS1 knockdown was observed compared with that from knockdown of HNF1 α (Fig. 4E and Fig. 6D). Moreover, exogenous expression of HNF1 α -AS1 also showed different effects on the basal and rifampicin-induced expression of CYPs compared with that consequent to HNF1 α overexpression (Fig. 5D, 5E, 7C, and 7D), although the same effect on the transcription of PXR was observed (Fig. 7B). We have not yet found a reasonable explanation for these data and will continue to determine the underlying mechanisms for the induction of CYP1A2 in the HNF1 α -AS1 overexpression experiment. However, it could be concluded that the HNF1 α and HNF1 α -AS1 pair is involved in PXR-mediated basal expression of CYP genes, but may function in different ways with regard to their induced expression.

A detailed determination of the mechanisms by which HNF1 α -AS1 is involved in the HNF1 α -mediated regulatory function in liver cells will require further analyses. The physiological functions of HNF1 α -AS1 were first identified in the regulation of cell proliferation and migration in esophageal adenocarcinoma cells (Yang et al., 2014). The role of HNF1 α -AS1 in the promotion of cancer progression and metastasis in gastrointestinal tract organs has been reported, including in pancreatic cancer (Muller et al., 2015), gastric cancer (Dang et al., 2015), and hepatocellular carcinoma (Liu et al., 2016). HNF1 α -AS1 has been considered as an oncogene (Liu et al., 2016) and may serve as a biomarker for cancer prognosis (Zhang et al., 2017). Mechanistically, HNF1 α -AS1 may function as a competing endogenous RNA to repress miRNA-mediated post-transcriptional regulation in cancer progression (Fang et al., 2017). Transcriptional regulation of HNF1 α -AS1 by HNF1 α has also been reported in hepatocellular carcinoma (Ding et al., 2018). HNF1 α -AS1 was further shown to directly bind to the c-terminus of Scr homology region 2 domain-containing phosphatase 1 and increase the

JPET # 252940

phosphatase activity of this protein, which can reverse the malignancy of hepatocellular carcinoma (Ding et al., 2018).

Given that we have found that HNF1 α and lncRNAHNF1 α -AS1 constitute a regulatory network involved in receptor-mediated regulation of CYP enzyme expression, and based on current reports, it therefore appears that HNF1 α -AS1 acts at the core of this regulatory mechanism. Moreover, such studies have provided potential future directions to further illustrate the molecular mechanisms of HNF1 α -AS1 in the involvement of the HNF1 α -mediated regulatory function in liver cells. A clear understanding of these mechanisms is necessary to support the clinical application of epigenetic regulation of drug metabolism from the perspective of regulatory networks, by facilitating the discovery of novel targets and providing novel strategies for the development of new drugs. For example, future research may reveal that lncRNAs regulate the expression of CYPs by acting as a bridge, although the detailed mechanisms have not been established and should be further explored.

In conclusion, this study demonstrates that lncRNAHNF1 α -AS1, an antisense RNA of HNF1 α , is involved in the HNF1 α -mediated regulation of the expression of CYPs and nuclear receptors in human liver cells.

JPET # 252940

Authorship Contributions:

Participated in research design: Yan, Zhong, Han, and Zhang.

Conducted experiments: Wang, Yan, Liu, Chen, Liu, Nie, Wang, and Yang.

Performed data analysis: Wang, Yan, Chen, Zhong, and Zhang.

Wrote or contributed to the writing of the manuscript: Yan, Zhong, and Zhang

JPET # 252940

References

- Aouabdi S, Gibson G and Plant N (2006) Transcriptional regulation of the PXR gene: identification and characterization of a functional peroxisome proliferator-activated receptor alpha binding site within the proximal promoter of PXR. *Drug Metab Dispos* **34**:138-144.
- Chen L, Bao Y, Piekos SC, Zhu K, Zhang L and Zhong XB (2018) A Transcriptional Regulatory Network Containing Nuclear Receptors and Long Noncoding RNAs Controls Basal and Drug-Induced Expression of Cytochrome P450s in HepaRG Cells. *Mol Pharmacol* **94**:749-759.
- Cheung C, Akiyama TE, Kudo G and Gonzalez FJ (2003) Hepatic expression of cytochrome P450s in hepatocyte nuclear factor 1-alpha (HNF1alpha)-deficient mice. *Biochem Pharmacol* **66**:2011-2020.
- Chiang TS, Yang KC, Chiou LL, Huang GT and Lee HS (2014) Enhancement of CYP3A4 activity in Hep G2 cells by lentiviral transfection of hepatocyte nuclear factor-1 alpha. *PLoS One* **9**:e94885.
- Consortium GT (2013) The Genotype-Tissue Expression (GTEx) project. *Nat Genet* **45**:580-585.
- Dang Y, Lan F, Ouyang X, Wang K, Lin Y, Yu Y, Wang L, Wang Y and Huang Q (2015) Expression and clinical significance of long non-coding RNA HNF1A-AS1 in human gastric cancer. *World J Surg Oncol* **13**:302.
- Ding CH, Yin C, Chen SJ, Wen LZ, Ding K, Lei SJ, Liu JP, Wang J, Chen KX, Jiang HL, Zhang X, Luo C and Xie WF (2018) The HNF1alpha-regulated lncRNA HNF1A-AS1 reverses the

JPET # 252940

malignancy of hepatocellular carcinoma by enhancing the phosphatase activity of SHP-1.

Mol Cancer **17**:63.

Fang C, Qiu S, Sun F, Li W, Wang Z, Yue B, Wu X and Yan D (2017) Long non-coding RNA HNF1A-AS1 mediated repression of miR-34a/SIRT1/p53 feedback loop promotes the metastatic progression of colon cancer by functioning as a competing endogenous RNA.

Cancer Lett **410**:50-62.

Goodwin B, Hodgson E and Liddle C (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module.

Mol Pharmacol **56**:1329-1339.

He H, Nie YL, Li JF, Meng XG, Yang WH, Chen YL, Wang SJ, Ma X, Kan QC and Zhang LR (2016) Developmental regulation of CYP3A4 and CYP3A7 in Chinese Han population.

Drug Metab Pharmacokinet **31**:433-444.

Herriges MJ, Swarr DT, Morley MP, Rathi KS, Peng T, Stewart KM and Morrissey EE (2014) Long noncoding RNAs are spatially correlated with transcription factors and regulate lung development. *Genes Dev* **28**:1363-1379.

Ingelman-Sundberg M, Sim SC, Gomez A and Rodriguez-Antona C (2007) Influence of cytochrome P450 polymorphisms on drug therapies: pharmacogenetic, pharmacoepigenetic and clinical aspects. *Pharmacol Ther* **116**:496-526.

Jover R, Bort R, Gomez-Lechon MJ and Castell JV (2001) Cytochrome P450 regulation by hepatocyte nuclear factor 4 in human hepatocytes: a study using adenovirus-mediated antisense targeting. *Hepatology* **33**:668-675.

JPET # 252940

Kamiyama Y, Matsubara T, Yoshinari K, Nagata K, Kamimura H and Yamazoe Y (2007) Role of human hepatocyte nuclear factor 4alpha in the expression of drug-metabolizing enzymes and transporters in human hepatocytes assessed by use of small interfering RNA. *Drug Metab Pharmacokinet* **22**:287-298.

Kaneko S, Bonasio R, Saldana-Meyer R, Yoshida T, Son J, Nishino K, Umezawa A and Reinberg D (2014) Interactions between JARID2 and noncoding RNAs regulate PRC2 recruitment to chromatin. *Mol Cell* **53**:290-300.

Khorkova O, Hsiao J and Wahlestedt C (2015) Basic biology and therapeutic implications of lncRNA. *Adv Drug Deliv Rev* **87**:15-24.

Kung JT, Colognori D and Lee JT (2013) Long noncoding RNAs: past, present, and future. *Genetics* **193**:651-669.

Li T and Chiang JY (2006) Rifampicin induction of CYP3A4 requires pregnane X receptor cross talk with hepatocyte nuclear factor 4alpha and coactivators, and suppression of small heterodimer partner gene expression. *Drug Metab Dispos* **34**:756-764.

Liu SY and Gonzalez FJ (1995) Role of the liver-enriched transcription factor HNF-1 alpha in expression of the CYP2E1 gene. *DNA Cell Biol* **14**:285-293.

Liu Z, Wei X, Zhang A, Li C, Bai J and Dong J (2016) Long non-coding RNA HNF1A-AS1 functioned as an oncogene and autophagy promoter in hepatocellular carcinoma through sponging hsa-miR-30b-5p. *Biochem Biophys Res Commun* **473**:1268-1275.

Ma L, Bajic VB and Zhang Z (2013) On the classification of long non-coding RNAs. *RNA Biol* **10**:925-933.

JPET # 252940

- Muller S, Raulefs S, Bruns P, Afonso-Grunz F, Plotner A, Thermann R, Jager C, Schlitter AM, Kong B, Regel I, Roth WK, Rotter B, Hoffmeier K, Kahl G, Koch I, Theis FJ, Kleeff J, Winter P and Michalski CW (2015) Next-generation sequencing reveals novel differentially regulated mRNAs, lncRNAs, miRNAs, sdRNAs and a piRNA in pancreatic cancer. *Mol Cancer* **14**:94.
- Nair PC, McKinnon RA and Miners JO (2016) Cytochrome P450 structure-function: insights from molecular dynamics simulations. *Drug Metab Rev* **48**:434-452.
- Nie YL, He H, Li JF, Meng XG, Yan L, Wang P, Wang SJ, Bi HZ, Zhang LR and Kan QC (2017) Hepatic expression of transcription factors affecting developmental regulation of UGT1A1 in the Han Chinese population. *Eur J Clin Pharmacol* **73**:29-37.
- Pan YZ, Gao W and Yu AM (2009) MicroRNAs regulate CYP3A4 expression via direct and indirect targeting. *Drug Metab Dispos* **37**:2112-2117.
- Peng L and Zhong X (2015) Epigenetic regulation of drug metabolism and transport. *Acta Pharm Sin B* **5**:106-112.
- Pinto N and Dolan ME (2011) Clinically relevant genetic variations in drug metabolizing enzymes. *Curr Drug Metab* **12**:487-497.
- Tang X and Chen S (2015) Epigenetic Regulation of Cytochrome P450 Enzymes and Clinical Implication. *Curr Drug Metab* **16**:86-96.
- Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, Parviz F, Duncan SA, Inoue Y, Gonzalez FJ, Schuetz EG and Kim RB (2003) The orphan nuclear receptor HNF4alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. *Nat Med* **9**:220-

224.

Tompkins LM and Wallace AD (2007) Mechanisms of cytochrome P450 induction. *J Biochem Mol Toxicol* **21**:176-181.

Tracy TS, Chaudhry AS, Prasad B, Thummel KE, Schuetz EG, Zhong XB, Tien YC, Jeong H, Pan X, Shireman LM, Tay-Sontheimer J and Lin YS (2016) Interindividual Variability in Cytochrome P450-Mediated Drug Metabolism. *Drug Metab Dispos* **44**:343-351.

Tsuchiya Y, Nakajima M, Takagi S, Taniya T and Yokoi T (2006) MicroRNA regulates the expression of human cytochrome P450 1B1. *Cancer Res* **66**:9090-9098.

Uno Y, Sakamoto Y, Yoshida K, Hasegawa T, Hasegawa Y, Koshino T and Inoue I (2003) Characterization of six base pair deletion in the putative HNF1-binding site of human PXR promoter. *J Hum Genet* **48**:594-597.

Villegas VE and Zaphiropoulos PG (2015) Neighboring gene regulation by antisense long non-coding RNAs. *Int J Mol Sci* **16**:3251-3266.

Wang KC, Yang YW, Liu B, Sanyal A, Corces-Zimmerman R, Chen Y, Lajoie BR, Protacio A, Flynn RA, Gupta RA, Wysocka J, Lei M, Dekker J, Helms JA and Chang HY (2011) A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression. *Nature* **472**:120-124.

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

Wu Y, Liu H, Shi X, Yao Y, Yang W and Song Y (2015) The long non-coding RNA HNF1A-AS1 regulates proliferation and metastasis in lung adenocarcinoma. *Oncotarget* **6**:9160-

9172.

Yan L, Wang Y, Liu J, Nie Y, Zhong XB, Kan Q and Zhang L (2017) Alterations of Histone Modifications Contribute to Pregnane X Receptor-Mediated Induction of CYP3A4 by Rifampicin. *Mol Pharmacol* **92**:113-123.

Yang X, Song JH, Cheng Y, Wu W, Bhagat T, Yu Y, Abraham JM, Ibrahim S, Ravich W, Roland BC, Khashab M, Singh VK, Shin EJ, Yang X, Verma AK, Meltzer SJ and Mori Y (2014) Long non-coding RNA HNF1A-AS1 regulates proliferation and migration in oesophageal adenocarcinoma cells. *Gut* **63**:881-890.

Yu AM, Ingelman-Sundberg M, Cherrington NJ, Aleksunes LM, Zanger UM, Xie W, Jeong H, Morgan ET, Turnbaugh PJ, Klaassen CD, Bhatt AP, Redinbo MR, Hao P, Waxman DJ, Wang L and Zhong XB (2017) Regulation of drug metabolism and toxicity by multiple factors of genetics, epigenetics, lncRNAs, gut microbiota, and diseases: a meeting report of the 21(st) International Symposium on Microsomes and Drug Oxidations (MDO). *Acta Pharm Sin B* **7**:241-248.

Yu AM, Tian Y, Tu MJ, Ho PY and Jilek JL (2016) MicroRNA Pharmacoepigenetics: Posttranscriptional Regulation Mechanisms behind Variable Drug Disposition and Strategy to Develop More Effective Therapy. *Drug Metab Dispos* **44**:308-319.

Zanger UM, Klein K, Thomas M, Rieger JK, Tremmel R, Kandel BA, Klein M and Magdy T (2014) Genetics, epigenetics, and regulation of drug-metabolizing cytochrome p450 enzymes. *Clin Pharmacol Ther* **95**:258-261.

Zanger UM and Schwab M (2013) Cytochrome P450 enzymes in drug metabolism: regulation

JPET # 252940

of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther* **138**:103-141.

Zhang X, Xiong Y, Tang F, Bian Y, Chen Y and Zhang F (2017) Long noncoding RNA HNF1A-AS1 indicates a poor prognosis of colorectal cancer and promotes carcinogenesis via activation of the Wnt/beta-catenin signaling pathway. *Biomed Pharmacother* **96**:877-883.

Zhou L, Sun K, Zhao Y, Zhang S, Wang X, Li Y, Lu L, Chen X, Chen F, Bao X, Zhu X, Wang L, Tang LY, Esteban MA, Wang CC, Jauch R, Sun H and Wang H (2015) Linc-YY1 promotes myogenic differentiation and muscle regeneration through an interaction with the transcription factor YY1. *Nat Commun* **6**:10026.

Zhou SF, Liu JP and Chowbay B (2009) Polymorphism of human cytochrome P450 enzymes and its clinical impact. *Drug Metab Rev* **41**:89-295.

JPET # 252940

Footnotes:

This work was supported by the National Natural Science Foundation of China [Grant 81773815 and U1604163] (L.Z.) and the National Institute of Health National Institute of General Medical Sciences of USA [Grant R01GM-118367] (X.Z).

Figure legends

Fig. 1. LncRNA expression profiles in liver tissues. (A) The mRNA levels of CYP3A4 in six liver tissues subjected to lncRNA microarray. (B) The protein levels of CYP3A4 in six liver tissues subjected to lncRNA microarray. Data are shown as the mean \pm SD, $*p < 0.05$, two-tailed unpaired Student's *t* test. (C) Heat map of the expression profiles of lncRNAs in six liver tissues with fold changes in \log_2 scale. Colors represent the higher (red) or lower (green) expression of lncRNAs. (D) The top 10 upregulated lncRNAs in Group A, which contain three samples with higher levels of CYP3A4.

Fig. 2. DNA sequence conservation of *HNF1 α* and *HNF1 α -AS1* across various species. (A) Genomic location of the *HNF1A* and *HNF1 α -AS1* genes in the Human GRCh38/h38 Genome. (B-C) Conservation levels (% identity) of the entire gene sequences of *HNF1 α* (B) and *HNF1 α -AS1* (C) among species throughout evolution in comparison to human. The sequences are derived from the NCBI and NONCODE databases. NA: not available.

Fig. 3. Correlations of the RNA level of *HNF1 α -AS1* with that of *HNF1 α* (A), *CYP2C8* (B), *2C9* (C), *2C19* (D), *2D6* (E), *2E1* (F), *3A4* (G), *PXR* (H), and *CAR* (I) in 43 human liver tissue samples. Pearson's correlation coefficients (*r*) were calculated by two-tailed Pearson's correlation analysis.

Fig. 4. Effects of *HNF1 α* knockdown on the mRNA expression of the studied genes. (A) mRNA expression of *HNF1 α* and RNA level of *HNF1 α -AS1* in control si-NC- and si-*HNF1 α* -transfected Huh7 cells. (B) Protein level of *HNF1 α* in control si-NC- and si-*HNF1 α* -transfected Huh7 cells. (C) Impact of *HNF1 α* knockdown on the expression of *AHR*, *CAR*, and *PXR*

JPET # 252940

mRNAs. (D) Impact of HNF1 α knockdown on the basal expression of *CYP* mRNAs. (E) Impact of HNF1 α knockdown on the rifampicin-induced expression of *CYP* mRNAs. Data points from three independent experiments are shown as dots along with the mean \pm SD, * p < 0.05, ** p < 0.01 in comparison of si-HNF1 α with the si-NC group by two-tailed unpaired student's t test.

Fig. 5. Effects of HNF1 α overexpression on the mRNA levels of the studied genes. (A) mRNA and protein levels of HNF1 α in control and HNF1 α -overexpressing Huh7 cells. (B) Impact of HNF1 α overexpression on the expression of *HNF1 α -AS1* RNA in control and HNF1 α -overexpressing Huh7 cells. (C) Impact of HNF1 α overexpression on the expression of *AHR*, *CAR*, and *PXR* mRNAs. (D) Impact of HNF1 α overexpression on the basal expression of *CYP* mRNAs. (E) Impact of HNF1 α overexpression on the rifampicin-induced expression of *CYP* mRNAs. Data points from three independent experiments are shown as dots along with the mean \pm SD, * p < 0.05, ** p < 0.01 in comparison of overexpressing HNF1 α with the control group by two-tailed unpaired student's t test.

Fig. 6. Effects of HNF1 α -AS1 knockdown on the mRNA expression of the studied genes. (A) Expression levels of HNF1 α -AS1 and HNF1 α in the control si-NC- and si-HNF1 α -AS1-transfected Huh7 cells. (B) Impact of HNF1 α -AS1 knockdown on the expression of *AHR*, *CAR*, and *PXR* mRNAs. (C) Impact of HNF1 α -AS1 knockdown on the basal expression of *CYP* mRNAs. (D) Impact of HNF1 α -AS1 knockdown on the rifampicin-induced expression of *CYP* mRNAs. Data points from three independent experiments are shown as dots along with the mean \pm SD, * p < 0.05, ** p < 0.01 in comparison of si-HNF1 α -AS1 with the control si-NC by

JPET # 252940

two-tailed unpaired student's *t* test.

Fig. 7. Effects of HNF1 α -AS1 overexpression on mRNA levels of the studies genes. (A) Expression levels of HNF1 α -AS1 in control and HNF1 α -AS1 overexpressing Huh7 cells. (B) Impact of HNF1 α -AS1 overexpression on the expression of *HNF1A*, *AHR*, *CAR*, and *PXR* mRNAs. (C) Impact of HNF1 α -AS1 overexpression on the basal expression of *CYP* mRNAs. (D) Impacts of HNF1 α -AS1 overexpression on the rifampicin-induced expression of *CYP* mRNAs. Data points from three independent experiments are shown as dots along with the mean \pm SD, **p* < 0.05, ***p* < 0.01 in comparison of HNF1 α -AS1 overexpression with the control group by two-tailed unpaired student's *t* test.

Fig . 1

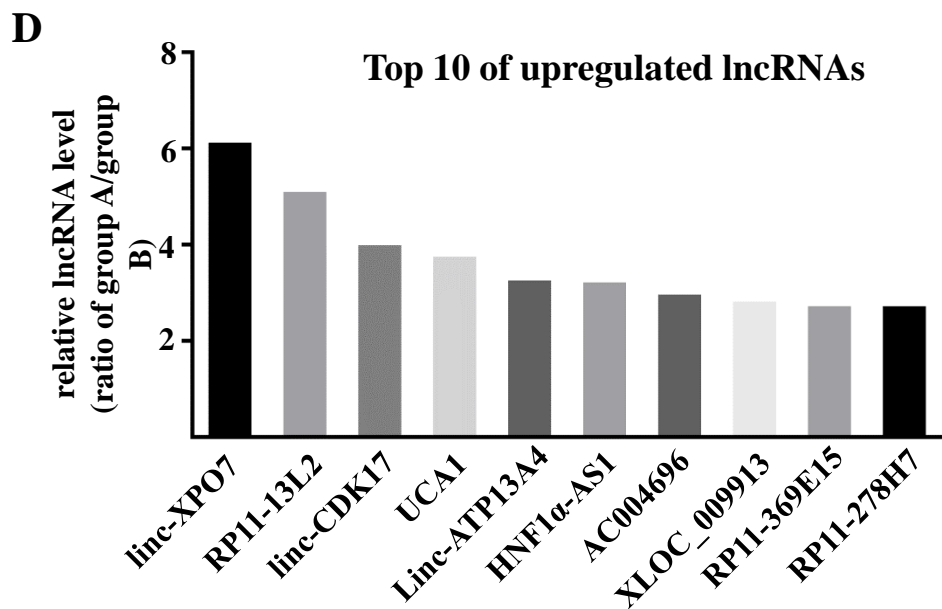
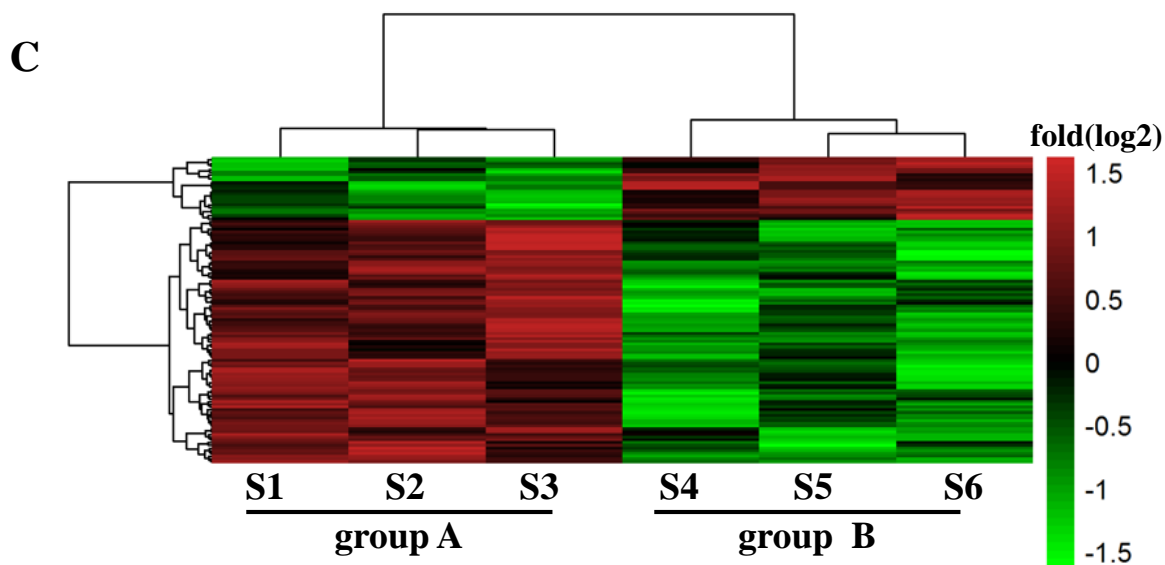
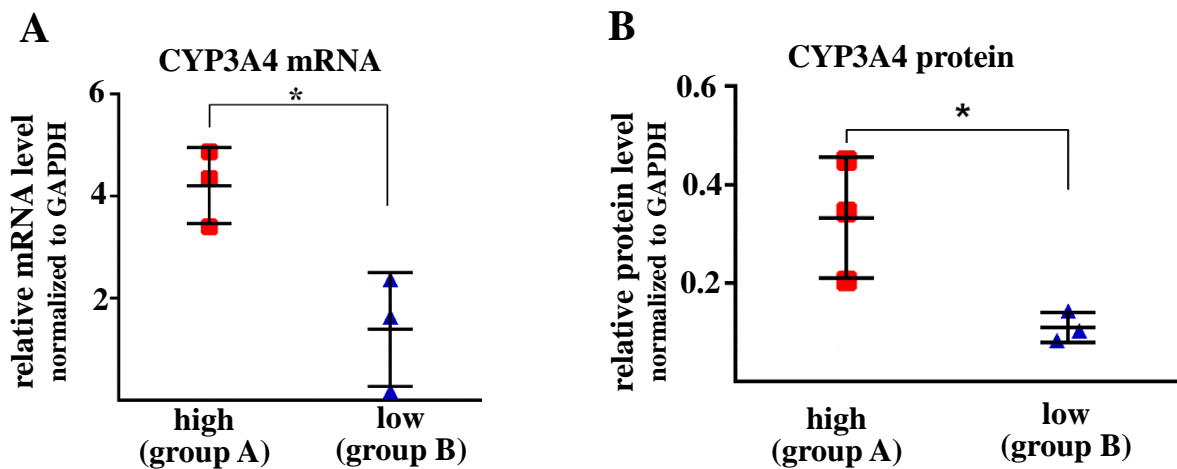
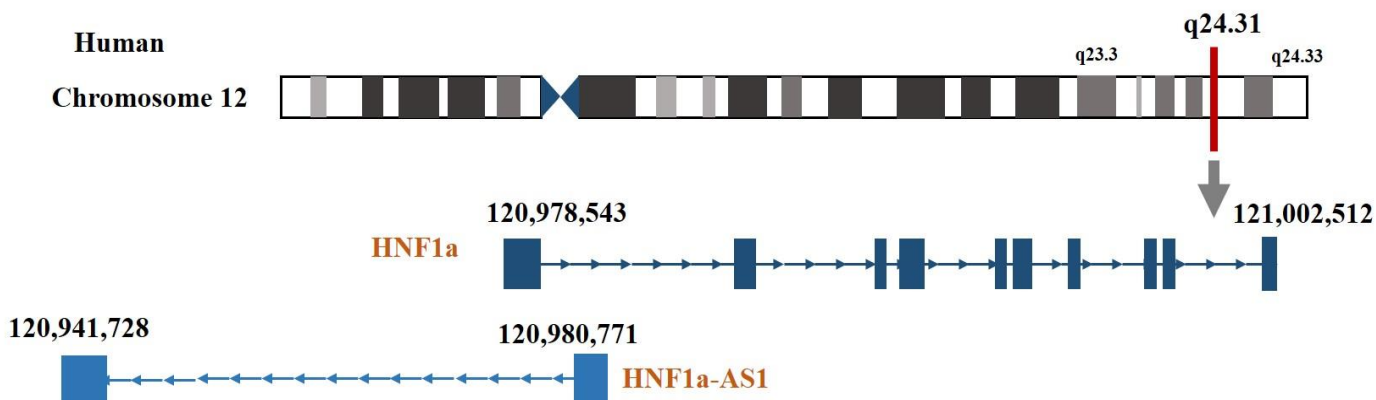
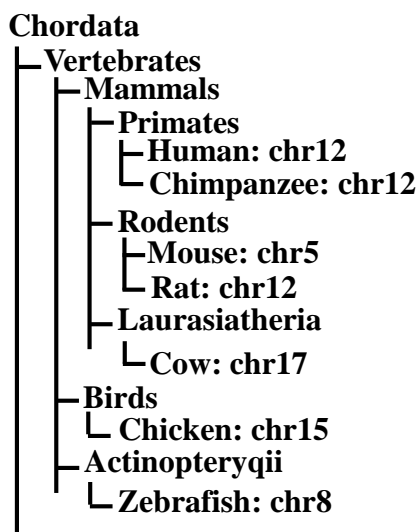


Fig . 2



B Conservation of *HNF1a*



C Conservation of *HNF1a-AS1*

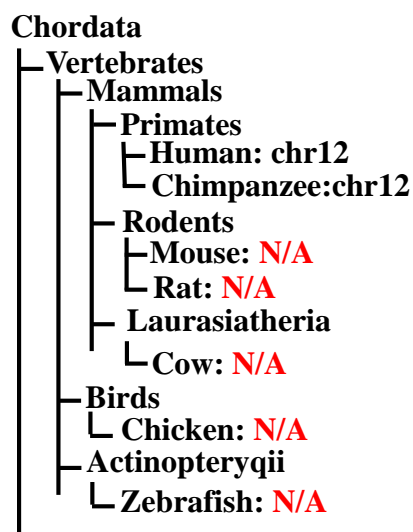


Fig . 3

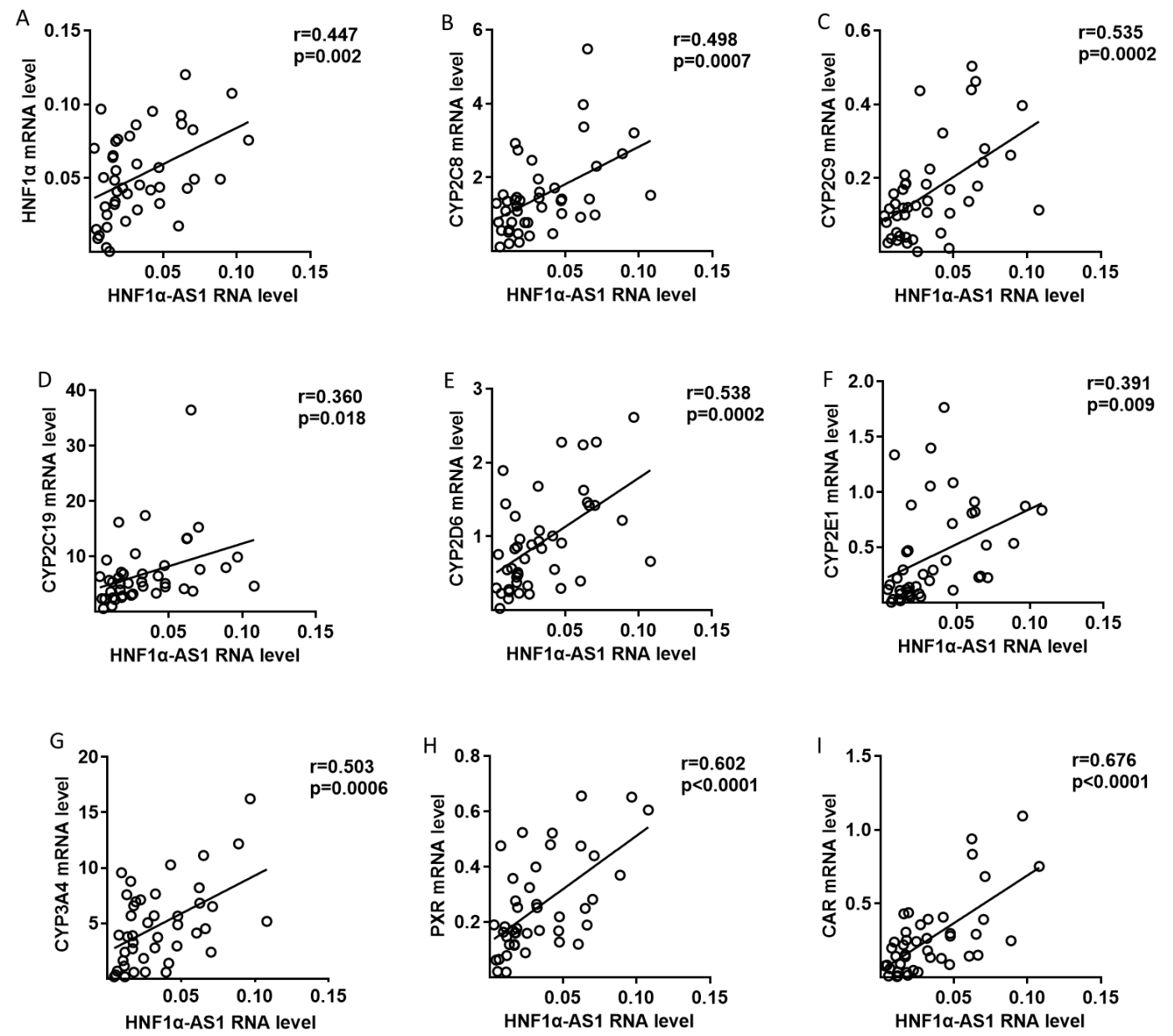


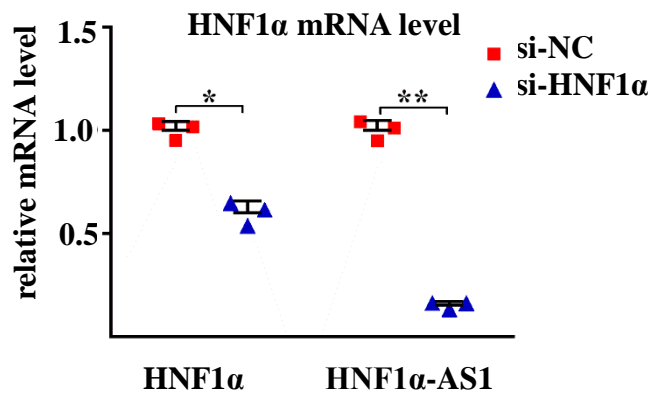
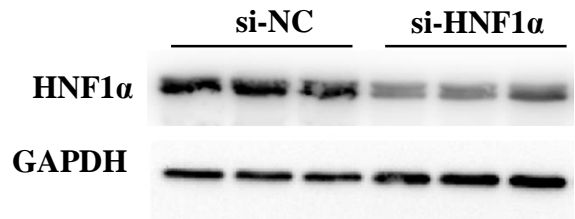
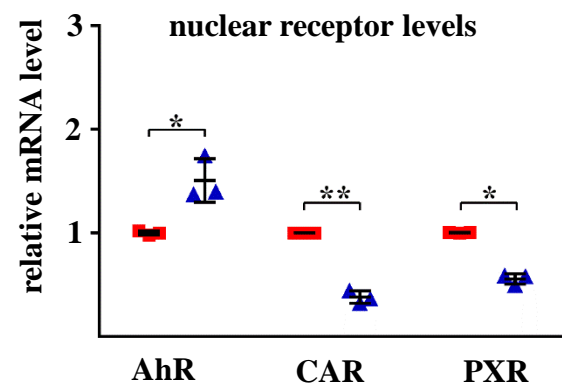
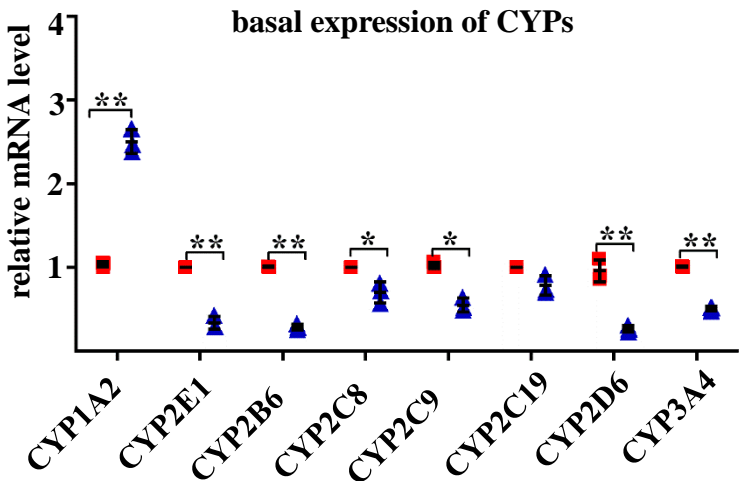
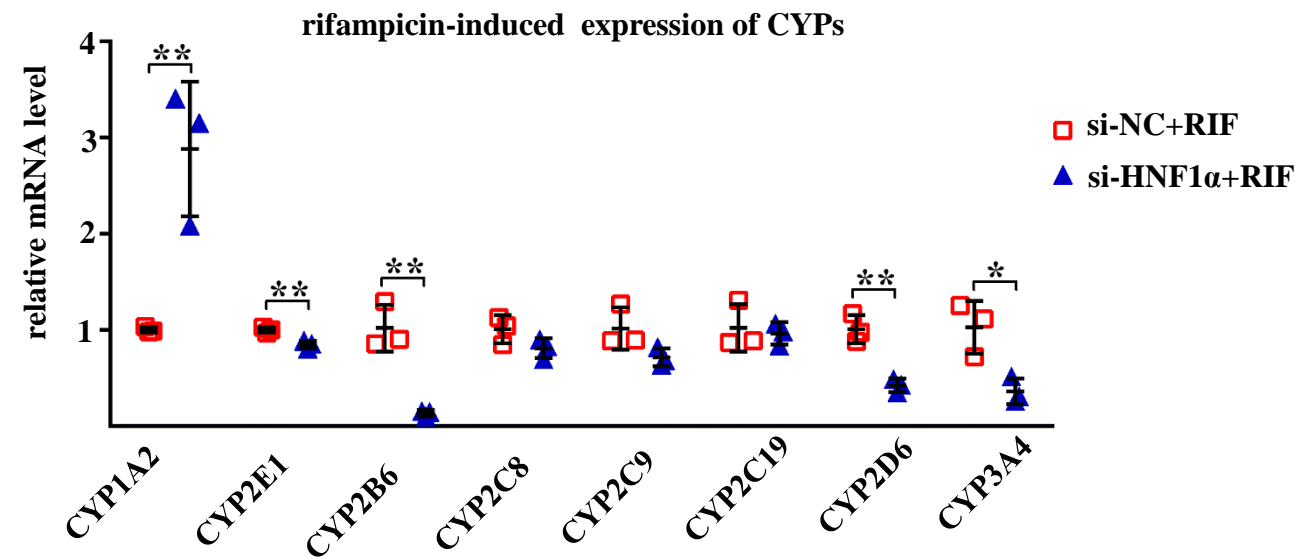
Fig . 4**A****B****C****D****E**

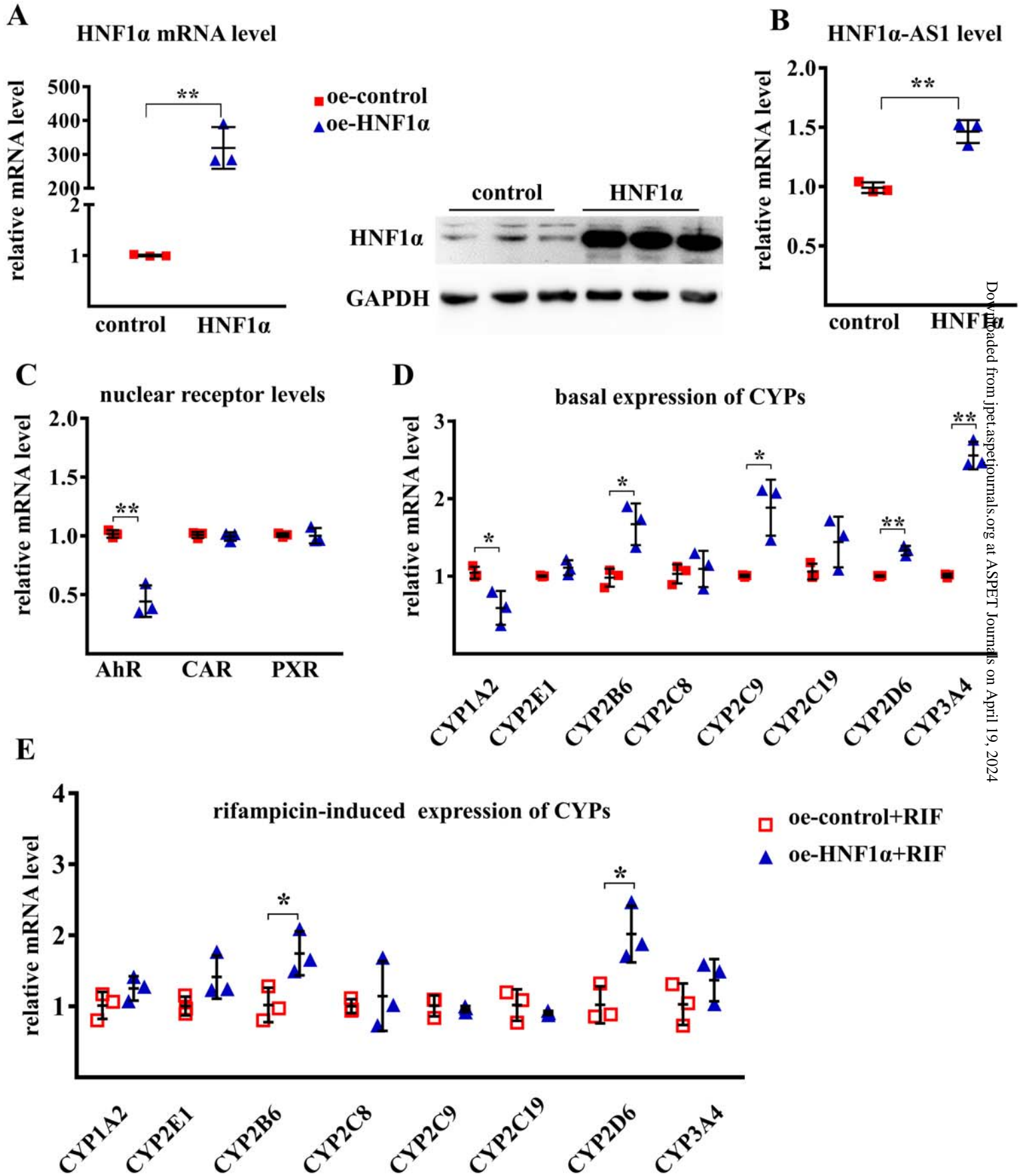
Fig . 5

Fig . 6

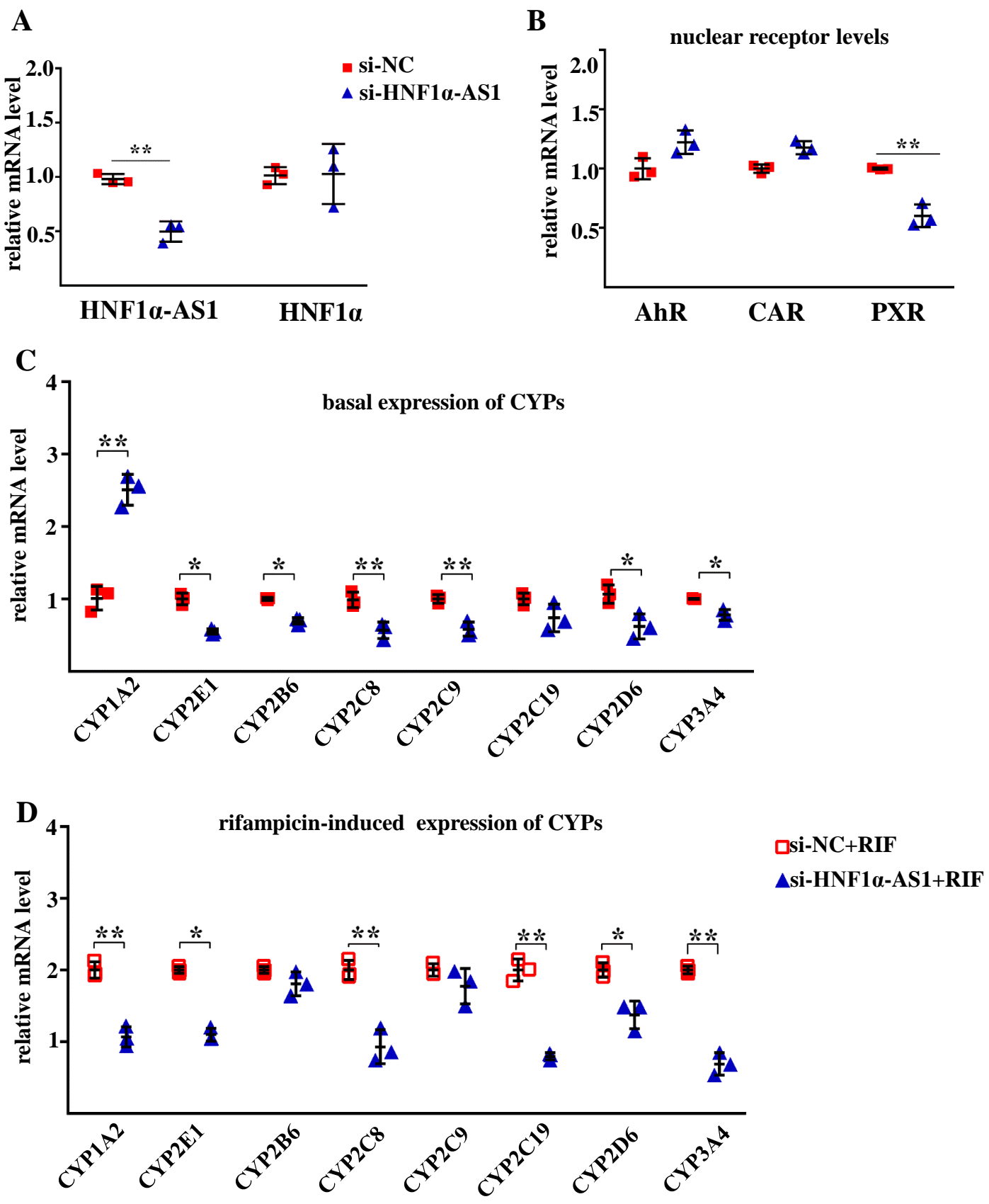
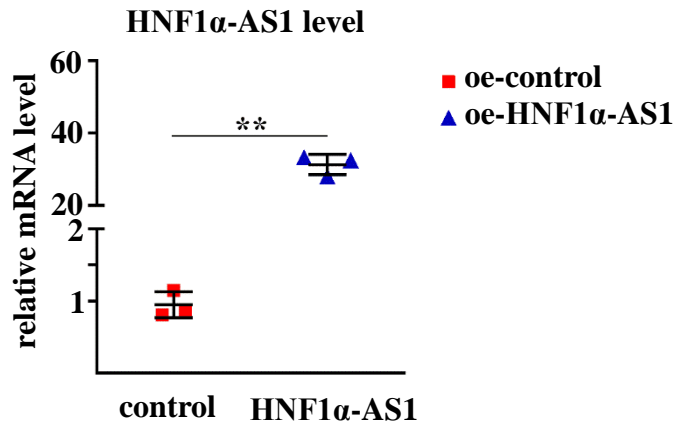
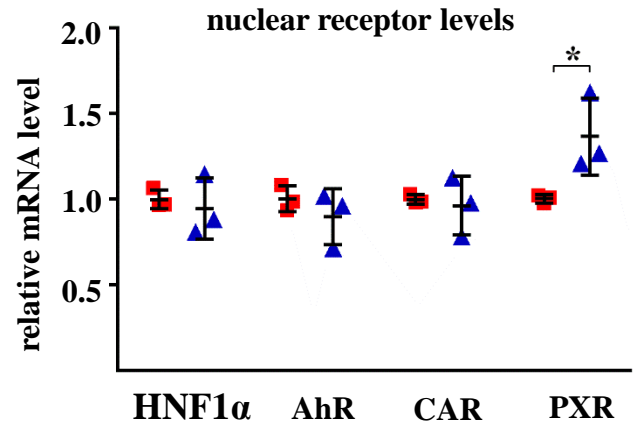
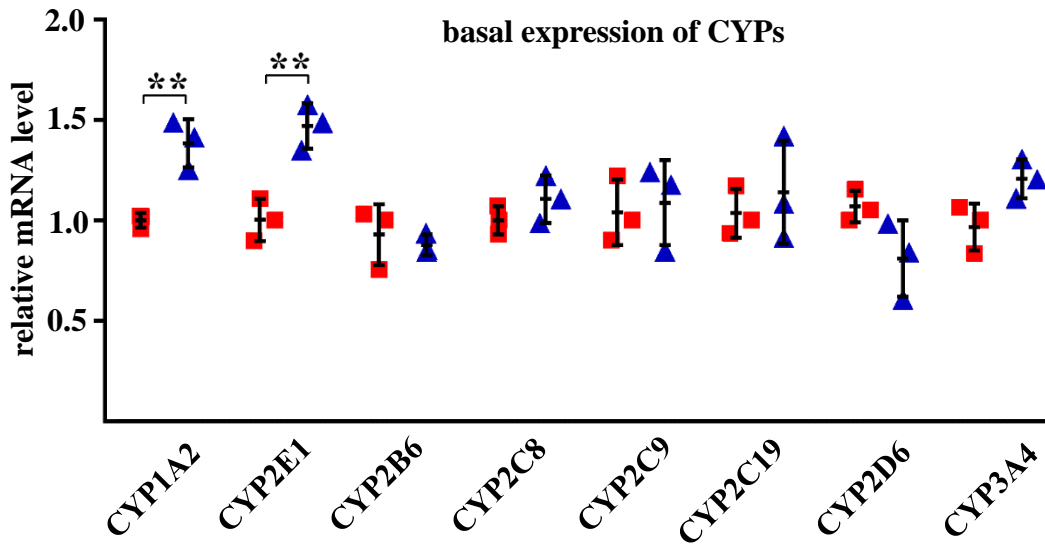


Fig . 7**A****B****C****D**