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The Long Noncoding RNA Hepatocyte Nuclear Factor 4α Antisense RNA 1 Negatively Regulates Cytochrome P450 Enzymes in Huh7 Cells via Histone Modifications

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ABSTRACT

The maintenance of homeostasis of cytochromes P450 enzymes (P450s) under both physiologic and xenobiotic exposure conditions is ensured by the action of positive and negative regulators. In the current study, the hepatocyte nuclear factor 4α (HNF4A) antisense RNA 1 (HNF4A-AS1), an antisense long noncoding RNA of *HNF4A*, was found to be a negative regulator of the basal and rifampicin (RIF)-induced expression of nuclear receptors and downstream P450s. In Huh7 cells, knockdown of HNF4A-AS1 resulted in elevated expression of HNF4A, pregnane X receptor (PXR), and P450s (including CYP3A4) under both basal and RIF-induced conditions. Conversely, overexpression of HNF4A-AS1 led to decreased basal expression of constitutive androstane receptor, aryl hydrocarbon receptor, PXR, and all studied P450s. Of note, significantly diminished induction levels of PXR and CYP1A2, 2C8, 2C19, and 3A4 by RIF were also observed in HNF4A-AS1 plasmid-transfected Huh7 cells. Moreover, the negative feedback of HNF4A on HNF4A-AS1–mediated gene expression was validated using a loss-of-function experiment in this study. Strikingly, our data showed that increased enrichment levels of histone 3 lysine 4 trimethylation and HNF4A in the *CYP3A4* promoter contribute to the elevated CYP3A4 expression after HNF4A-AS1 knockdown. Overall, the current study reveals that histone modifications contribute to the negative regulation of nuclear receptors and P450s by HNF4A-AS1 in basal and drug-induced levels.

SIGNIFICANCE STATEMENT

Utilizing loss-of-function and gain-of-function experiments, the current study systematically investigated the negative regulation of HNF4A-AS1 on the expression of nuclear receptors (including HNF4A, constitutive androstane receptor, aryl hydrocarbon receptor, and pregnane X receptor) and P450s (including CYP1A2, 2E1, 2B6, 2D6, 2C8, 2C9, 2C19, and 3A4) in both basal and rifampicin-induced levels in Huh7 cells. Notably, this study is the first to reveal the contribution of histone modification to the HNF4A-AS1–mediated expression of CYP3A4 in Huh7 cells.

Introduction

Cytochrome P450 enzymes (P450s), the predominant phase I enzymes in human liver, are responsible for the metabolism of approximately 75% of the clinically used drugs; additionally, P450s are of paramount importance for the detoxification of xenobiotics (Pikuleva, 2006; Fisher et al., 2009). Considerable interindividual variabilities in the expression and induction of P450s have been observed, which markedly affect the effectiveness and safety of therapeutic agents (Tang et al., 2005; Zanger and Schwab, 2013). Thus, elucidating the precise mechanism underlying the basal and drug-induced expression of P450s is essential for precision medicine.

It is well studied that nuclear receptors such as hepatocyte nuclear factor 4α (HNF4A) and 1α (HNF1A), aryl hydrocarbon receptor (AHR), pregnane X receptor (PXR), and constitutive androstane receptor (CAR) are crucial transcriptional regulators of P450s (Cheung et al., 2003; Kamiyama et al., 2007; Aleksunes and Klaassen, 2012; Hakkola et al., 2018). Notably, HNF4A is considered to be at the top hierarchy of the transcription factor network (Kuo et al., 1992; Kamiya et al., 2003). HNF4A was reported to be involved in HNF1A-mediated transcriptional activation (Eeckhoute et al., 2004). Additionally, the indispensable role of HNF4A in the PXR- and CAR-mediated induction of P450s has also been reported (Kamiya et al., 2003; Tirona et al., 2003; Li and

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ABBREVIATIONS: AHR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; ChIP, chromatin immunoprecipitation; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H3K4me3, histone 3 lysine 4 trimethylation; HNF1A, hepatocyte nuclear factor 1α; HNF4A, hepatocyte nuclear factor 4α; HNF4A-AS1, HNF4A antisense RNA 1; HRE, HNF4A response element; OE-HNF4A-AS1, overexpression of HNF4A-AS1; OE-NC, overexpression plasmid of negative control; P450, cytochrome P450; PXR, pregnane X receptor; qRT-PCR, quantitative real-time polymerase chain reaction; RIF, rifampicin; si-HNF4A, siRNA against HNF4A; si-HNF4A-AS1, siRNA against HNF4A-AS1; si-NC, negative control siRNA; siRNA, small interfering RNA.

Chiang, 2006; Takezawa et al., 2012); however, these studies are all focused on positive regulatory mechanisms in P450 expression. Far less information is available on the negative regulation of P450s, thereby limiting our knowledge on the precise mechanism underlying the maintenance of the homeostasis of P450 expression in normal and xenobiotic (including drug) exposure conditions.

Long noncoding RNAs (lncRNAs) are noncoding RNAs with a length of more than 200 nucleotides (Atkinson et al., 2012; Yang et al., 2014; Ning et al., 2019). Recent studies have reported that lncRNAs participate in the regulation of neighboring genes (Halley et al., 2014; Villegas et al., 2014; Villegas and Zaphiropoulos, 2015). Our previous study has unveiled that HNF4A antisense RNA 1 (HNF4A-AS1), a neighboring antisense lncRNA of *HNF4A*, negatively regulated the mRNA expression of nuclear receptors, including HNF4A and PXR, and the downstream P450s at the basal level (Chen et al., 2018). However, the underlying mechanism and the role of HNF4A-AS1 in the drug-induced expression of P450s have not been elucidated thus far.

lncRNAs can act as cofactors for transcriptional factors and jointly regulate gene expression (Zhao et al., 2014; Mi et al., 2016). Additionally, lncRNAs can also regulate gene expression by recruiting complexes to the promoters of target genes and causing alterations of histone modifications of genes (Wang et al., 2011). Of note, studies conducted by us and other groups have shown that transcription factors can affect gene transcription by regulating histone modifications in gene promoter regions (Xie et al., 2009; Yan et al., 2017). Therefore, we speculate that HNF4A-AS1 can regulate the expression of P450s via altering the histone modification status of P450 genes, thus affecting the binding of HNF4A.

In the current study, we performed a systematic analysis to investigate the role of HNF4A-AS1 in the expression of transcription factor–mediated P450s in Huh7 cells. Importantly, the underlying mechanism was elucidated in this study. Our findings reveal that HNF4A-AS1 negatively regulates the basal and rifampicin (RIF)-induced expression of P450s. Moreover, the elevated expression of CYP3A4 after HNF4A-AS1 knockdown is related to the increased enrichment levels of histone 3 lysine 4 trimethylation (H3K4me3) and of HNF4A in the promoter of *CYP3A4*.

Materials and Methods

Chemicals and Reagents. Huh7 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). RIF was obtained from Sigma-Aldrich (St. Louis, MO). Small interfering RNAs (siRNAs), including siRNA against HNF4A (si-HNF4A), HNF4A-AS1 (si-HNF4A-AS1), and a negative control siRNA (si-NC), were provided by GenePharma Co., Ltd. (Shanghai, China). HNF4A-AS1 expression plasmids were obtained from GeneChem Co., Ltd. (Shanghai, China). LipofectAMINE 3000, opti-MEM, and SYBR Select Master Mix were purchased from Thermo Fisher Scientific (Carlsbad, CA). Nanofecter transfection reagent was obtained from Procell Co., Ltd. (Wuhan, China). The TriPure isolation reagent and protease inhibitor cocktail were purchased from Roche (Basel, Switzerland). Primers were synthesized by SunYa Co. (Zhejiang, China). Antibodies against HNF4A (ab174653); PXR (bs-2334R); and CYP3A4 (ab124921), 1A2 (ab151728), 2C9 (ab150364), and 2C19 (ab185213) were obtained from Abcam (Cambridge, MA). Horseradish peroxidase–linked secondary antibodies (SA00001-1 and SA00001-2) and the antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (60004-1-Ig) were purchased from Proteintech (Wuhan, China). The antibody recognizing H3K4me3 (17-678) and polyvinylidene fluoride membranes were obtained from MD Millipore (Billerica, MA). Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Corning Inc. (Armonk, NY). Radio immunoprecipitation assay reagent, penicillin and streptomycin mixture, DMSO, and other chemical reagents were provided by Solarbio Science & Technology Co. (Beijing, China).

Cell Culture and Transfection. Huh7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin mixture at 37°C with 5% CO_2. Before transfection, Huh7 cells were allowed to grow for up to 24 hours on six-well plates reaching 80%–90% confluence. For gene knockdown experiments, 40 pmol per well of siRNAs targeting HNF4A, HNF4A-AS1, or control siRNAs were transfected into Huh7 cells with the LipofectAMINE 3000 reagent according to the manufacturer's instructions. For gene overexpression experiments, Nanofecter transfection reagent was used to transfect 2.5 μg per well of plasmids into Huh7 cells following the manufacturer's protocol. Cells were harvested 48 hours after transfection.

Drug Treatment. At 24 hours after transfection, cells were incubated with 10 μM RIF or 0.1% DMSO (vehicle) for 24 hours before harvesting.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction. Total RNAs were isolated from Huh7 cells using a TriPure isolation reagent according to the manufacturer's instruction. The quality and concentrations of RNAs were measured by a Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific). Total RNAs (1 μg) were reverse-transcribed using a PrimeScript RT reagent kit. qRT-PCR was performed using a SYBR Select Master Mix with specific primers (Table 1) in an Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). Relative RNA expression levels were determined by normalizing focused gene expression against GAPDH expression using the $2^{-\Delta\Delta Ct}$ method.

Protein Isolation and Western Blot Analysis. Whole protein homogenates of Huh7 cells were prepared using a radio immunoprecipitation assay reagent supplemented with a protease inhibitor cocktail. Protein concentrations were determined by a bicinchoninic acid method according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Hangzhou, China). Western blot analysis was performed using 80 μg total protein samples as described previously (Yan et al., 2017; Wang et al., 2019a) with minor modifications. The dilutions of primary antibodies against HNF4A, PXR, and CYP3A4 were 1:1000. The dilutions of primary antibodies against CYP1A2, 2C9, and 2C19 were 1:2000. The dilutions of the primary antibody against GAPDH were 1:5000. Horseradish peroxidase–linked secondary antibodies were diluted 1:10,000. Protein bands were visualized using an enhanced chemiluminescence method with a FluorChem E system (Proteinsample, San Jose, CA). The protein expression levels of focused genes were normalized against GAPDH.

TABLE 1

Primer sequences for qRT-PCR analysis

Gene	Sequence (5′-3′)	Orientation
GAPDH	GCACCGTCAAGGCTGAGAAC	Sense
	TGGTGAAGACGCCAGTGGA	Antisense
HNF4A-AS1	TGGAGCTGGGATCTGACACT	Sense
	ATGACCGGTGTGCAGTCAAG	Antisense
HNF4A	CGTGCTGCTCCTAGGCAA	Sense
	GTCAAGGATGCGTATGGACAC	Antisense
HNF1A	TGGGTCCTACGTTCACCAAC	Sense
	TCTGCACAGGTGGCATGAGC	Antisense
CAR	CAGAAGCGGTGCATACCTCA	Sense
	AGATGGCACTGACTCTGCAA	Antisense
AhR	ACATCACCTACGCCAGTCGC	Sense
	TCTATGCCGCTTGGAAGGAT	Antisense
PXR	CAACCTACATGTTCAAAGGCATC	Sense
	ACACTCCCAGGTTCCAGTCTC	Antisense
CYP1A2	CCAACGTCATTGGTGCCATG	Sense
	GTGATGTCCCGGACACTGTTC	Antisense
CYP2E1	TCCTAGGGCACAGTCGTAGT	Sense
	AGGGAAGGTACTGCCTCTGAT	Antisense
CYP2B6	AGACGCCTTCAATCCTGACC	Sense
	CCTTCACCAAGACAAATCCGC	Antisense
CYP2D6	CCTACGCTTCCAAAAGGCTTT	Sense
	AGAGAACAGGTCAGCCACCACT	Antisense
CYP2C8	GGACTTTATGGATTGCTTCCTG	Sense
	CCATATCTCAGAGTGGTGCTTG	Antisense
CYP2C9	GACATGAACAACCCTCAGGACTTT	Sense
	TGCTTGTCGTCTCTGTCCCA	Antisense
CYP2C19	GAACACCAAGAATCGATGGACA	Sense
	TCAGCAGGAGAAGGAGAGCATA	Antisense
CYP3A4	CCCTTTGGAAGTGGACCCAG	Sense
	ACGGTGCCATCCCTTGACTC	Antisense

TABLE 2

Primer sequences for ChIP-qPCR

Primer	Position	Sequence (5′-3′)	Orientation
Primer 1	−362 ~ +53	GCACCGTCAAGGCTGAGAAC	Sense
		GCCTGAGAACACCAGAGACC	Antisense
Primer 2	−6093 ~ −6574	CAGCAGACAGAGAACCCAGA	Sense
		GCCTGAGAACACCAGAGACC	Antisense

Chromatin Immunoprecipitation Analysis. ChIP was performed as described previously (Yan et al., 2017; Wang et al., 2019a) with minor modifications. Briefly, siRNA-transfected cells were incubated with 1% formaldehyde for 10 minutes; fixation was halted by glycine (125 mM) for 5 minutes at room temperature. Cells were then washed twice with cold phosphate-buffered saline and lysed using a sodium dodecyl sulfate lysis buffer (0.1% SDS, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1% Triton X) with a proteinase inhibitor cocktail on ice for 15 minutes. Cell lysates were sonicated with a Bioruptor Pico sonication system (Diagenode, Seraing, Belgium) to obtain DNA fragments ranging between ~200 and 1000 bp. DNA fragments were immunoprecipitated using antibodies against H3K4me3 and HNF4A. Mouse IgG was used as a negative control. The purification of DNA was performed with a PCR purification kit (QIAGEN GmbH, Hilden, Germany) followed by qRT-PCR analysis with specific primers (Table 2) using a SYBR green method. Enrichment was calculated as the percentage of chromatin input and normalized against IgG.

Statistical Analysis. All studies with Huh7 cells described here were performed as three independent experiments. Data are shown as means ± S.D. Statistical significances between two groups were analyzed by a two-tailed unpaired Student's *t* test using SPSS 21.0 software (IBM Corp., Armonk, NY).

Results

Negative Regulation of HNF4A-AS1 on the Basal Expression of Nuclear Receptors and P450s in Huh7 Cells. To uncover the role of HNF4A-AS1 in the regulation of nuclear receptors and P450s, loss-of-function and gain-of-function studies were performed in Huh7 cells. The expression of HNF4A-AS1, five nuclear receptors (HNF4A, HNF1A, CAR, AHR, and PXR), and eight P450s (CYP1A2, 2E1, 2B6, 2D6, 2C8, 2C9, 2C19, and 3A4) was measured.

The knockdown efficiency of HNF4A-AS1 after siRNA transfection into Huh7 cells was confirmed by qRT-PCR. As seen in Fig. 1A, the expression of HNF4A-AS1 decreased approximately 57% after si-HNF4A-AS1 transfection. Knockdown of HNF4A-AS1 led to increased expression of HNF4A at both mRNA and protein levels (Fig. 1, A and B). For the focused nuclear receptors, 1.7-fold increased expression of PXR was observed by HNF4A-AS1 knockdown, whereas the basal expression of HNF1A, CAR, and AHR was unchanged at the mRNA level (Fig. 1C). Meanwhile, the basal mRNA expression of CYP1A2, 2C8, 2C9, 2C19, and 3A4 was also significantly higher in the HNF4A-AS1 knockdown group than in the control group (Fig. 1D). There was no significant difference in the mRNA expression of CYP2E1, 2B6, and 2D6 after HNF4A-AS1 knockdown (Fig. 1D). Importantly, consistent with the increased expression of PXR and CYP3A4 at the mRNA level, the protein expression of PXR and CYP3A4 was also significantly

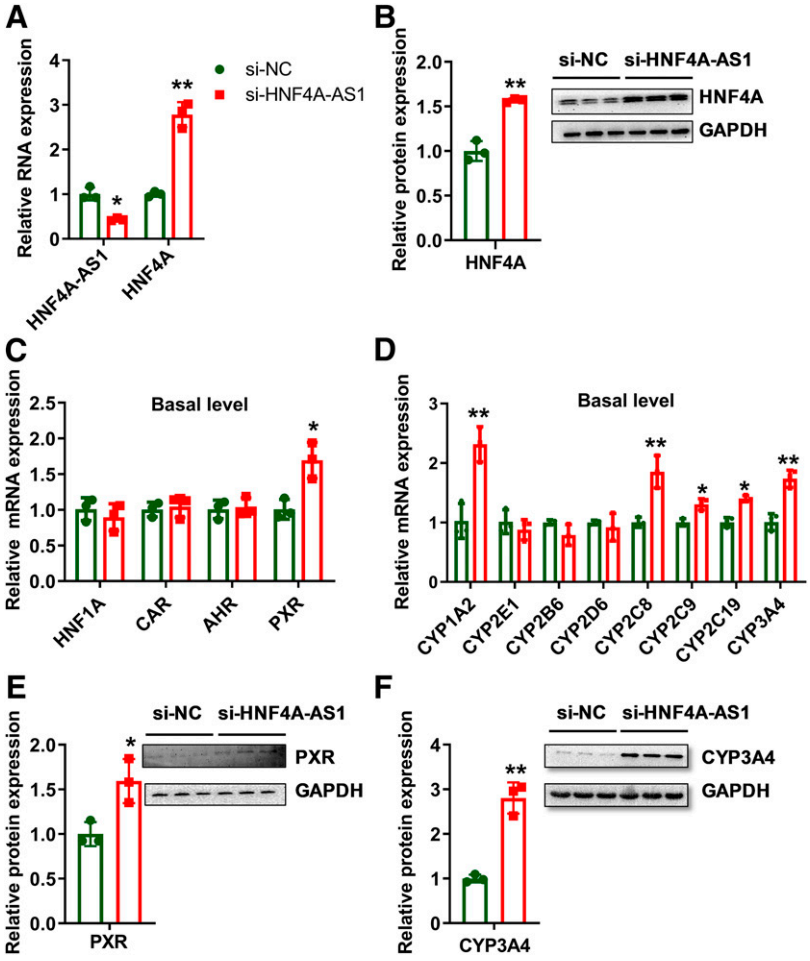

Fig. 1. Impact of HNF4A-AS1 knockdown on the basal expression of genes in Huh7 cells. si-HNF4A-AS1 or negative control siRNA was transfected into Huh7 cells for 24 hours. The RNA and protein expression levels of genes after HNF4A-AS1 knockdown were measured by qRT-PCR and Western Blot, respectively. (A) Expression of HNF4A and HNF4A-AS1 at the RNA level. (B) The protein expression of HNF4A. (C) The relative mRNA expression of selected nuclear receptors. (D) The relative mRNA expression of P450s. (E) The protein expression of PXR. (F) The protein expression of CYP3A4. Data are shown as means ± S.D. of three independent experiments. Statistical analysis was performed using an unpaired Student's *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. the si-NC group.

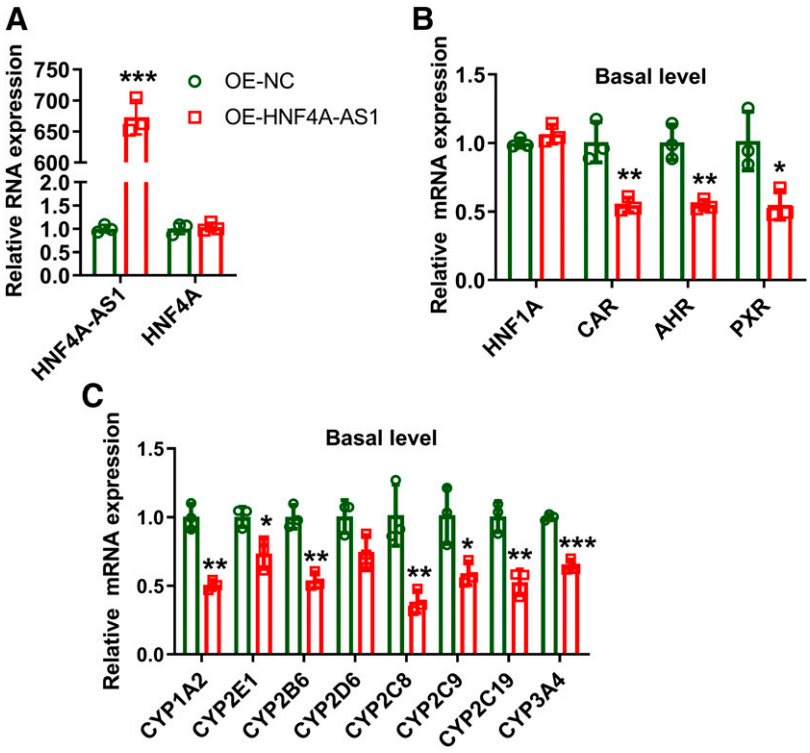

Fig. 2. Impact of HNF4A-AS1 overexpression on the basal expression of genes in Huh7 cells. HNF4A-AS1 expression plasmid or negative control plasmid was transfected into Huh7 cells for 24 hours. The RNA expression of genes after HNF4A-AS1 overexpression was measured by qRT-PCR. (A) Expression of HNF4A and HNF4A-AS1 at the RNA level. (B) The relative mRNA expression of selected nuclear receptors. (C) The relative mRNA expression of P450s. Data are shown as means ± S.D. of three independent experiments. Statistical analysis was performed using an unpaired Student's *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. the OE-NC group. OE-HNF4A-AS1, overexpression of HNF4A-AS1. OE-NC, overexpression plasmid of negative control.

elevated after HNF4A-AS1 knockdown (Fig. 1, E and F). Moreover, higher protein expression levels of CYP1A2 and 2C9 were also observed in the HNF4A-AS1 group ($P < 0.05$), whereas the protein expression of CYP2C19 remained unchanged ($P > 0.05$) (Supplemental Fig. 1).

The expression of HNF4A-AS1 in the overexpression of HNF4A-AS1 (OE-HNF4A-AS1) group was approximately 673 times that in the control group (Fig. 2A), which confirmed the overexpression efficiency of HNF4A-AS1. Compared with the control group, the mRNA expression of CAR, AHR, and PXR was approximately 50% lower in the HNF4A-AS1 overexpression group (Fig. 2B). However, the mRNA expression of HNF4A and HNF1A was not affected by HNF4A-AS1 overexpression ($P > 0.05$) (Fig. 2, A and B). Additionally, as shown in Fig. 2C, HNF4A-AS1 overexpression also resulted in significantly decreased expression of studied P450s except for CYP2D6 at the mRNA level. These findings indicate that HNF4A-AS1 acts as a negative regulator in the basal expression of P450s, probably via the negative regulation of nuclear receptors.

HNF4A-AS1 Attenuates the RIF-Induced Expression of P450s in Huh7 Cells. To further explore the role of HNF4A-AS1 in the drug-induced expression of P450s, siRNA- or plasmid-transfected Huh7 cells were treated with RIF. As seen in Fig. 3A, knockdown of HNF4A-AS1 abolished the induction of HNF4A-AS1 expression by RIF, whereas it elevated that of HNF4A. Significantly higher induction levels of PXR were also observed in the HNF4A-AS1 knockdown group ($P < 0.05$), whereas the RIF-induced expression of other nuclear receptors (including HNF1A, CAR, and PXR) was unaffected by the HNF4A-AS1 knockdown ($P > 0.05$) (Fig. 3B). Additionally, after HNF4A-AS1 knockdown, the induction of CYP1A2, 2C8, 2C9, 2C19, and 3A4 expression by RIF was significantly increased ($P < 0.05$), whereas that of CYP2E1, 2B6, and 2D6 was not affected ($P > 0.05$) (Fig. 3C).

Conversely, overexpression of HNF4A-AS1 led to higher levels of HNF4A-AS1 and lower levels of PXR induced by RIF (Fig. 3, D and E). However, the RIF-induced expression of HNF4A, HNF1A, CAR, and AHR remained unchanged after HNF4A-AS1 overexpression ($P > 0.05$) (Fig. 3, D and E). For P450s, the induction levels of CYP1A2, 2C8, 2C19, and 3A4 decreased after HNF4A-AS1 overexpression (Fig. 3F). Altogether, these results suggest that HNF4A-AS1 attenuates the drug-induced expression of P450s.

Negative Feedback of HNF4A on HNF4A-AS1–Mediated Gene Expression. To verify the role of HNF4A in the transcriptional regulation of HNF4A-AS1 and the related P450s, knockdown of HNF4A was performed in Huh7 cells. After effective knockdown of HNF4A, the expression of HNF4A-AS1 decreased (Fig. 4, A and B). Lower expression levels of all studied nuclear receptors, including HNF1A, CAR, AHR, and PXR, were also observed at the mRNA level after HNF4A knockdown (Fig. 4C). Additionally, the knockdown of HNF4A also led to reduced mRNA expression of P450s (Fig. 4D). Consistent with the decreased expression at the mRNA level, the protein expression of PXR and CYP3A4 was lower in the HNF4A knockdown group than in the control group (Fig. 4, E and F). Moreover, as seen in Fig. 5, the RIF-induced expression of most focused genes, including HNF1A and P450s, was significantly diminished after HNF4A knockdown at the RNA level. There was also a trend of lower induced expression levels of CAR, AHR, and PXR by RIF in HNF4A knockdown versus control cells ($P > 0.05$) (Fig. 5B). These findings demonstrate that HNF4A participates in the transcriptional regulation of HNF4A-AS1, nuclear receptors, and P450s under both basal and RIF-induced expression-level conditions.

HNF4A-AS1 Knockdown Leads to Elevated Enrichment Levels of Histone Modifications and HNF4A in the Promoter of *CYP3A4*. To explore the underlying mechanism by which HNF4A-AS1 regulated the expression of CYP3A4, ChIP-qPCR experiments were conducted in si-NC– or si-HNF4A-AS1–transfected Huh7 cells. As shown in Fig. 6A, two pairs of primers around the HNF4A response elements (HREs) in the promoter of *CYP3A4* were synthesized. Knockdown of HNF4A-AS1 resulted in elevated enrichment levels of H3K4me3, a gene activation marker, in the HREs of the *CYP3A4* promotor, which was

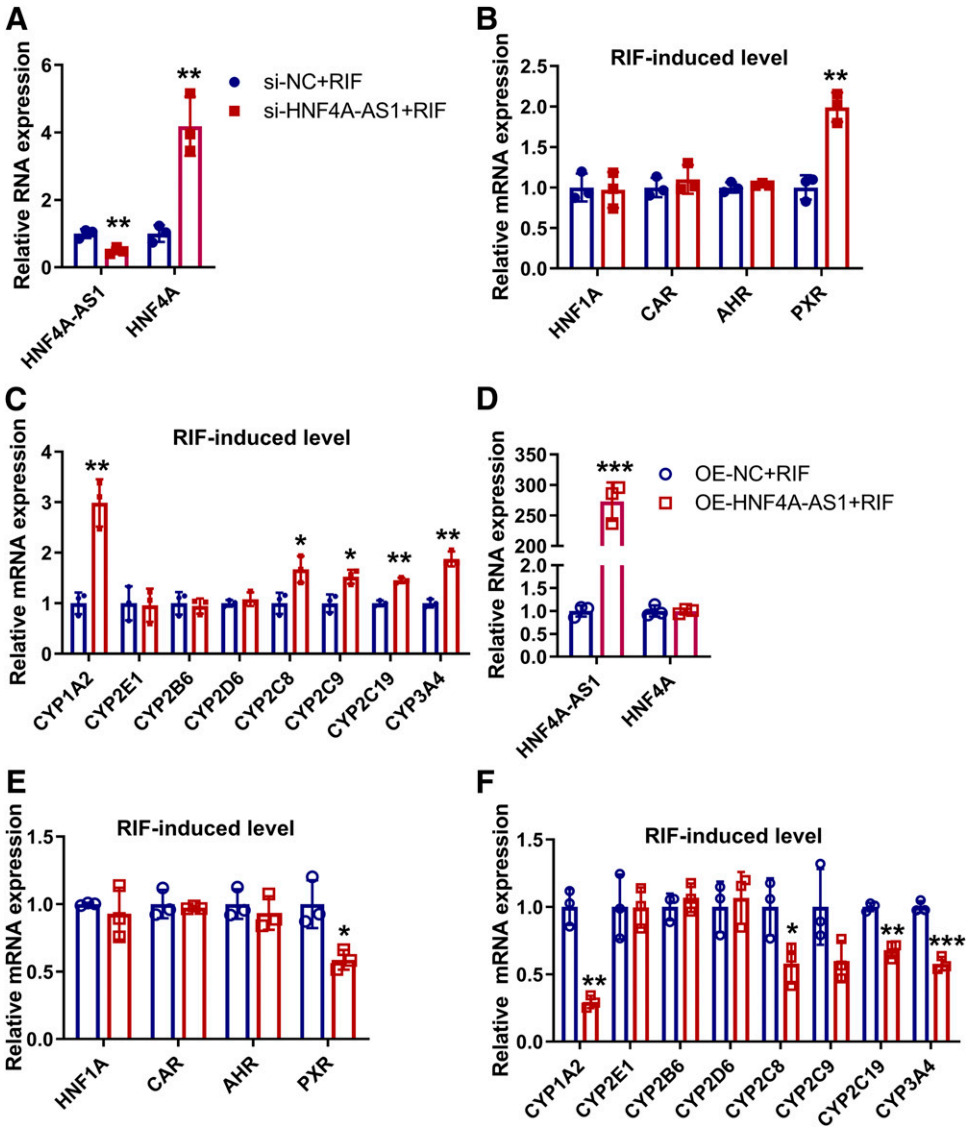

Fig. 3. HNF4A-AS1 repressed the RIF-induced expression of genes in Huh7 cells. si-HNF4A-AS1–, si-NC–, OE-HNF4A-AS1–, or OE-NC–transfected Huh7 cells were treated with RIF for 24 hours. The RIF-induced expression of genes after HNF4A-AS1 knockdown or overexpression at the RNA level was detected by qRT-PCR method. (A–C) Knockdown of HNF4A-AS1 elevated the RIF-induced expression of genes. (D–F) Overexpression of HNF4A-AS1 diminished the RIF-induced expression of genes. Data are shown as means ± S.D. of three independent experiments. Statistical analysis was performed using an unpaired Student's *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. the control group. OE-NC, overexpression plasmid of negative control.

consistent with the higher expression of CYP3A4 in the HNF4A-AS1 knockdown group (Fig. 6B). Moreover, the enrichment level of HNF4A also increased after HNF4A-AS1 knockdown (Fig. 6C). Altogether, these findings indicate that HNF4A-AS1 might regulate the expression of CYP3A4 by a scaffold mechanism.

Discussion

It is well known that the expression of P450s is transcriptionally regulated by nuclear receptors (Honkakoski and Negishi, 2000; Jover et al., 2009; Nie et al., 2017). As a master regulator, HNF4A plays a crucial role in the complex regulatory network of basal and drug-induced P450 expression (Tirona et al., 2003; Kamiyama et al., 2007). Recently, an increasing number of studies have elucidated the epigenetic regulation of P450s (Klaassen et al., 2011; Tang and Chen, 2015; Li et al., 2019). Notably, as promising epigenetic regulators, lncRNAs were reported to be important for multiple biologic or physiologic processes (Fatica and Bozzoni, 2014; Schmitt and Chang, 2016; Ghafouri-Fard et al., 2020; Zhou et al., 2020). In the current study, utilizing loss-of-function and gain-of function experiments, we systemically verified that HNF4A-AS1 was involved in the negative regulation of HNF4A-mediated gene expression (including that of P450s) at the basal level and under drug-induced conditions in Huh7 cells. Importantly, the alteration of histone modification status was proved as the underlying mechanism for the HNF4A-AS1–mediated negative regulation of P450s in this study.

Mounting evidence suggests that lncRNAs can be involved in the regulation of their neighboring genes (Villegas and Zaphiropoulos, 2015; Gibbons et al., 2018; Qi et al., 2019). In particular, lncRNAs are known to interact on their antisense strands with transcription factors (Villegas and Zaphiropoulos, 2015; Gil and Ulitsky, 2020). Our previous studies proved that the *HNF1A* antisense lncRNA HNF1A antisense RNA 1 acted as a positive regulator of HNF1A-mediated gene expression in Huh7 and HepaRG cells (Chen et al., 2018; Wang et al., 2019b). In the present study, using loss-of-function and gain-of-function studies, HNF4A-AS1 was validated to have opposite regulatory effects to HNF1A antisense RNA 1 on the basal expression of most studied genes in Huh7 cells (Figs. 1 and 2), which confirmed our previous findings in HNF4A-AS1 knockdown HepaRG cells (Chen et al., 2018).

Although higher mRNA expression of most P450s (including CYP1A2, 2C9, 2C19, and 3A4) after HNF4A-AS1 knockdown was observed in both Huh7 and HepaRG cells (Fig. 1) (Chen et al., 2018), differential impacts on genes were noticed. For instance, after efficient knockdown of HNF4A-AS1, the basal mRNA expression of HNF4A

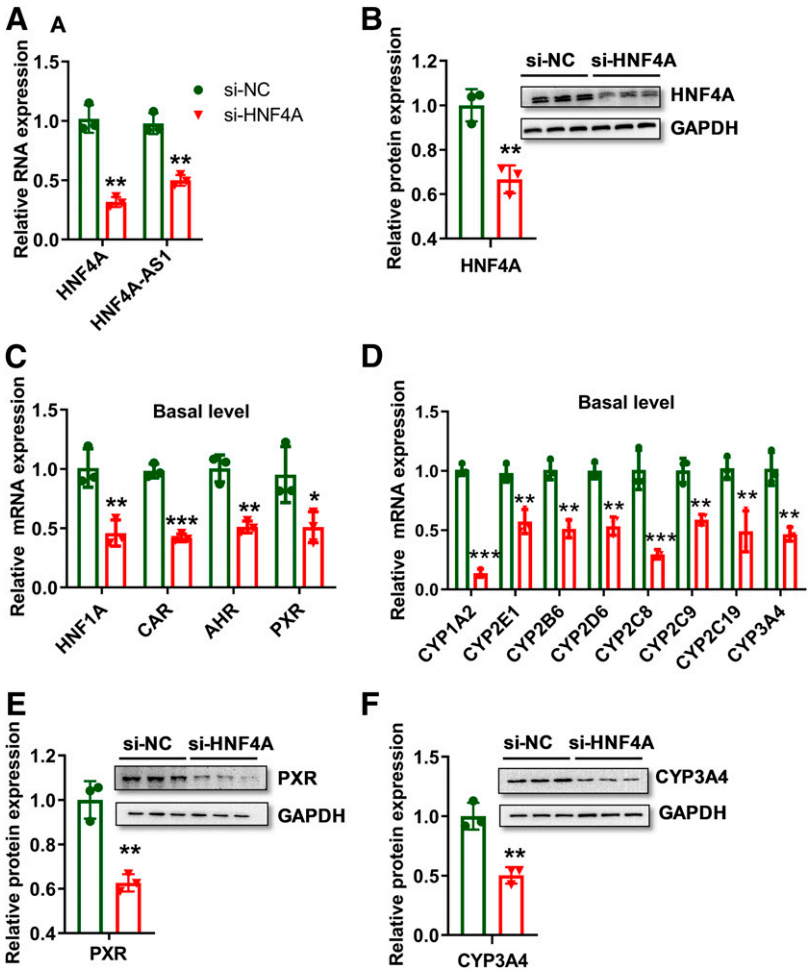

Fig. 4. Impact of HNF4A knockdown on the basal expression of genes in Huh7 cells. si-HNF4A or negative control siRNA was transfected into Huh7 cells for 24 hours. The RNA and protein expression of selected genes after HNF4A knockdown were measured by qRT-PCR and Western Blot method, respectively. (A) Expression of HNF4A and HNF4A-AS1 at the RNA level. (B) The protein expression of HNF4A. (C) The relative mRNA expression of selected nuclear receptors. (D) The relative mRNA expression of P450s. (E) The protein expression of PXR. (F) The protein expression of CYP3A4. Data are shown as means ± S.D. of three independent experiments. Statistical analysis was performed using an unpaired Student's *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. the si-NC group.

and PXR was significantly elevated in Huh7 cells (Fig. 1, A and C) but remained unchanged in HepaRG cells (Chen et al., 2018). Increased protein expression of HNF4A and PXR by HNF4A-AS1 knockdown was also observed in Huh7 cells (Fig. 1, B and E). However, overexpression of HNF4A-AS1 in Huh7 cells did not affect the mRNA expression of HNF4A (Fig. 2A). This may be attributed to the low intrinsic expression of HNF4A in Huh7 cells. Another possible explanation for the abovementioned findings is that HNF4A-AS1 may serve as a central platform for the binding of effector molecules repressing the expression of HNF4A in Huh7 cells. That is, the knockdown of HNF4A-AS1 may lead to dismantling of the lncRNA effector scaffold, thus increasing the expression of HNF4A, whereas overexpression of HNF4A-AS1 may have no impact on the scaffolding complexes, and consequently on the expression of HNF4A. However, these are aspects that warrant further investigation. Additionally, transfection of siRNA against HNF4A-AS1 into HepaRG cells resulted in the altered mRNA expression of CAR and AHR (Chen et al., 2018), whereas in Huh7 cells, the expression of CAR and PXR was not affected after HNF4A-AS1 knockdown, although it decreased after overexpression of HNF4A-AS1 (Fig. 1C; Fig. 2B). The differential impacts of HNF4A-AS1 on the basal expression of genes in Huh7 and HepaRG cells may be ascribed to differences in the intrinsic expression of HNF4A-AS1, HNF4A-related nuclear receptors, and P450s. Overall, both studies confirmed the negative regulation of HNF4A-AS1 in the basal and drug-induced expression of HNF4A-mediated genes.

It is well documented that PXR and CAR are xenobiotic receptors that play crucial roles in the induction or inhibition of P450s (Timsit and Negishi, 2007; Hernandez et al., 2009; Chai et al., 2013). Additionally, several studies have reported that HNF4A is indispensable for the PXR- and CAR-mediated induction of P450s (Tirona et al., 2003; Li and Chiang, 2006; Takezawa et al., 2012). Based on our previous findings, we speculated that HNF4A-AS1 could be involved in the drug-induced expression of P450s. Importantly, consistent with the elevated basal expression of HNF4A, PXR, and P450s (such as CYP1A2 and 3A4) in HNF4A-AS1 knockdown Huh7 cells, the induction of these genes by RIF was improved after HNF4A-AS1 knockdown (Fig. 3, A–C). Conversely, overexpression of HNF4A-AS1 resulted in diminished induction levels of PXR and CYP1A2, 2C8, 2C19, and 3A4 in Huh7 cells (Fig. 3, D–F). These results indicate that HNF4A-AS1 is involved in the negative regulation of drug-induced P450 expression. Under both normal physical and xenobiotic exposure conditions, HNF4A-AS1 may act as a "break" to sustain constant intracellular levels of P450s. Importantly, to better understand the human implications of these findings, studies using primary human hepatocytes, human induced pluripotent stem cell–derived hepatocytes, or HepaRG cells should be conducted in the future.

In a recent study using dual-luciferase assay, Guo and Lu (2019) found that the endogenous expression of HNF4A-AS1 in HepG2 and HEK293 cells was transcriptionally regulated by P1-HNF4A, the predominant subtype of HNF4A in adult liver. Thus, in this study, we also assessed the impacts of HNF4A on the expression of HNF4A-AS1, other nuclear receptors, and P450s under basal and RIF-induced conditions. As shown in Fig. 4, knockdown of HNF4A led to decreased expression of HNF4A-AS1, nuclear receptors (HNF1A, CAR, and

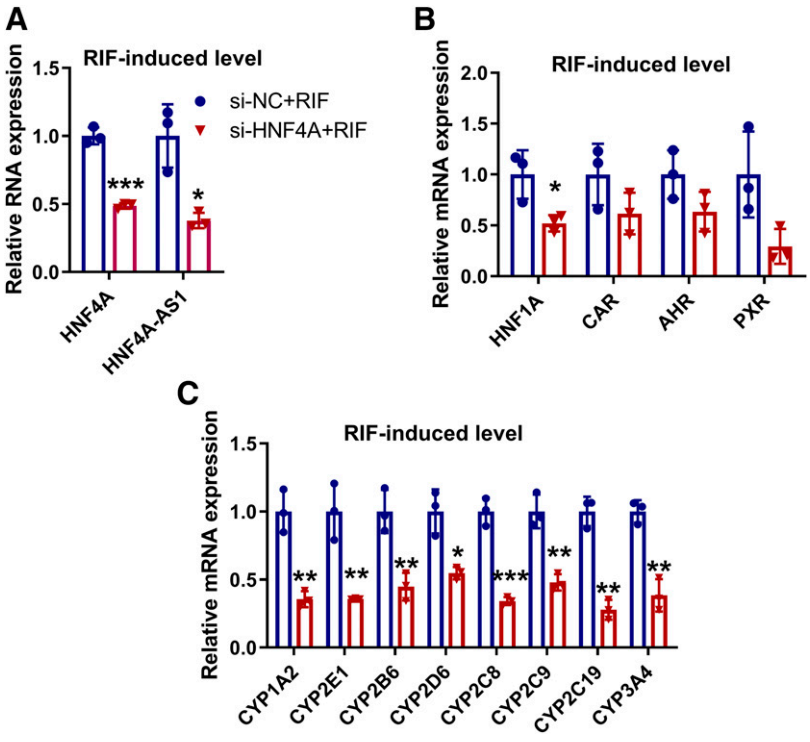

Fig. 5. Effect of HNF4A knockdown on the RIF-induced expression of genes in Huh7 cells. si-HNF4A– or si-NC–transfected Huh7 cells were treated with RIF for 24 hours. The RIF-induced expression of genes after HNF4A knockdown in the RNA level was determined by qRT-PCR method. (A) Expression of HNF4A and HNF4A-AS1 at the RNA level. (B) The relative mRNA expression of selected nuclear receptors. (C) The relative mRNA expression of P450s. Data are shown as means ± S.D. of three independent experiments. Statistical analysis was performed using an unpaired Student's *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. the control group.

PXR), and P450s (including CYP2E1, 2B6, 2C8, 2C19, and 3A4) in Huh7 cells, which was consistent with our previous findings in HepaRG cells. Similarly, the RIF-induced expression of these P450s was also diminished after HNF4A knockdown in both Huh7 (Fig. 5) and HepaRG cells (Chen et al., 2018). These findings verified the previously published results and further indicated that there was a negative feedback loop in the HNF4A-AS1/HNF4A-P450s axis. However, the precise mechanisms need to be elucidated in further researches.

The involvement of histone modifications in the regulation of P450s has been proven by more and more studies (Yan et al., 2017; Wang et al., 2019a; Pande et al., 2020). For instance, histone 3 lysine 27 acetylation was reported to be involved in HNF4A-mediated CYP2C9 expression. Englert et al. (2015) found that HNF4A could recruit the coregulator mediator complex 25 to the *CYP2C9* promoter, thus promoting the transcription of CYP2C9. However, alterations in histone modification status are usually nonspontaneous, and their activation requires the action of upstream regulatory factors. Therefore, we speculate that HNF4A-AS1, acting as a cofactor, would interact with HNF4A by recruiting histone modification enzymes to the HREs in the *CYP3A4* promoter, thereby regulating the expression of P450s. Because it has the highest expression level among all P450s in liver, CYP3A4 was selected as a representative gene to uncover the underlying mechanisms of how HNF4A-AS1 regulated the expression of P450s in this study. After the efficient knockdown of HNF4A-AS1, the HREs (−7836 to −6093 bp and −362 to +53 bp) in the *CYP3A4* promoter were found to have higher enrichment levels of H3K4me3, a gene-active factor. In addition, interference with HNF4A-AS1 resulted in elevated enrichment levels of HNF4A in the promoter of *CYP3A4*. Altogether, these findings validate our hypothesis and indicate that HNF4A-AS1 may regulate the expression of CYP3A4 by recruiting histone methylation enzymes to the promoter of *CYP3A4*, which alters the histone modification status, thereby affecting the binding of HNF4A to *CYP3A4* promoter. However, whether HNF4A-AS1 interacts with HNF4A and histone modification enzymes was not elucidated in this study. That said, these findings provide potential future directions for the investigation of the molecular mechanisms of HNF4A-AS1 in HNF4A-mediated regulatory functions in the human liver.

In summary, this study observed a negative regulation of HNF4A-AS1 on the basal and drug-induced expression of HNF4A, P450s, and other nuclear receptors. Our study also revealed that elevated enrichment levels of H3K4me3 and HNF4A in the *CYP3A4* promotor was responsible for the induced CYP3A4 expression after HNF4A-AS1 knockdown. Therefore, the HNF4A-AS1/HNF4A-P450s axis is an

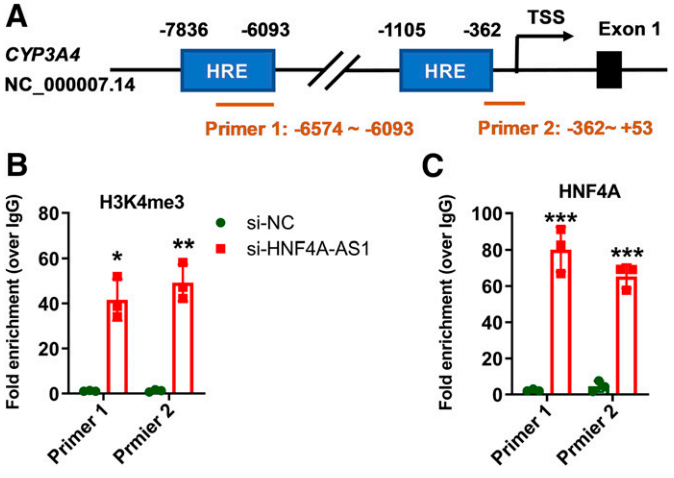

Fig. 6. Suppression of HNF4A-AS1 elevated the enrichment levels of histone modifications and HNF4A in the promoter of *CYP3A4* in Huh7 cells. (A) Schematic locations of HREs and ChIP-qPCR primers in the *CYP3A4* promoter. (B) Enrichment levels of H3K4me3 around the HREs in *CYP3A4* promoter. (C) Enrichment levels of HNF4A around the HREs in *CYP3A4* promoter. ChIP-qPCR analysis was conducted to measure the enrichment levels of H3K4me3 and HNF4A in the promoter of *CYP3A4*, and data were presented as fold enrichment over IgG. Data are shown as means ± S.D. of three independent experiments. Statistical analysis was performed using an unpaired Student's *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. the si-NC group. TSS, transcriptional start site.

epigenetically dependent novel negative feedback regulatory loop that should be considered for maintenance of the homeostasis of P450s in the liver.

Authorship Contributions

Participated in research design: P. Wang, Yan, Zhong, Han, Zhang.

Conducted experiments: P. Wang, Chen, Y. Wang, X. Wang, Yan, Yang.

Performed data analysis: P. Wang, Chen, Zhang.

Wrote or contributed to the writing of the manuscript: P. Wang, Chen, Zhong, Zhang.

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