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Knockdown of Long Noncoding RNAs Hepatocyte Nuclear Factor 1α Antisense RNA 1 and Hepatocyte Nuclear Factor 4α Antisense RNA 1 Alters Susceptibility of Acetaminophen-Induced Cytotoxicity in HepaRG Cells[S]

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ABSTRACT

Acetaminophen (APAP) is a commonly used over-the-counter drug for its analgesic and antipyretic effects. However, APAP overdose leads to severe APAP-induced liver injury (AILI) and even death as a result of the accumulation of *N*-acetyl-*p*-benzoquinone imine, the toxic metabolite of APAP generated by cytochrome P450s (P450s). Long noncoding RNAs HNF1α antisense RNA 1 (HNF1α-AS1) and HNF4α antisense RNA 1 (HNF4α-AS1) are regulatory RNAs involved in the regulation of P450 expression in both mRNA and protein levels. This study aims to determine the impact of HNF1α-AS1 and HNF4α-AS1 on AILI. Small hairpin RNAs were used to knock down HNF1α-AS1 and HNF4α-AS1 in HepaRG cells. Knockdown of these lncRNAs altered APAP-induced cytotoxicity, indicated by MTT and LDH assays. Specifically, HNF1α-AS1 knockdown decreased APAP toxicity with increased cell viability and decreased LDH release, whereas HNF4α-AS1 knockdown exacerbated APAP toxicity, with opposite effects in the MTT and LDH assays. Alterations on gene expression by knockdown of HNF1α-AS1 and HNF4α-AS1 were examined in several APAP metabolic pathways, including CYP1A2, CYP2E1, CYP3A4, UGT1A1, UGT1A9, SULT1A1, GSTP1, and GSTT1. Knockdown of HNF1α-AS1 decreased mRNA expression of CYP1A2, 2E1, and 3A4 by 0.71-fold, 0.35-fold, and 0.31-fold, respectively, whereas knockdown of HNF4α-AS1 induced mRNAs of CYP1A2, 2E1, and 3A4 by 1.3-fold, 1.95-fold, and 1.9-fold, respectively. These changes were also observed in protein levels. Knockdown of HNF1α-AS1 and HNF4α-AS1 had limited effects on the mRNA expression of UGT1A1, UGT1A9, SULT1A1, GSTP1, and GSTT1. Altogether, our study suggests that HNF1α-AS1 and HNF4α-AS1 affected AILI mainly through alterations of P450–mediated APAP biotransformation in HepaRG cells, indicating an important role of the lncRNAs in AILI.

SIGNIFICANCE STATEMENT

The current research identified two lncRNAs, hepatocyte nuclear factor 1α antisense RNA 1 and hepatocyte nuclear factor 4α antisense RNA 1, which were able to affect susceptibility of acetaminophen (APAP)-induced liver injury in HepaRG cells, possibly through regulating the expression of APAP-metabolizing cytochrome P450 enzymes. This discovery added new factors, lncRNAs, which can be used to predict cytochrome P450–mediated drug metabolism and drug-induced toxicity.

Introduction

Acetaminophen (APAP), or *N*-acetyl-*p*-aminophenol, is one of the most commonly used over-the-counter drugs for its antipyretic or analgesic effects in the treatment of fever or management of pain (Bunchorntavakul and Reddy, 2013). APAP is a safe drug if used properly. The maximal dose of 4000 mg/d suggested by the US Food and Drug Administration is considered to be safe and generally does not cause liver toxicity (Yoon et al., 2016). However, APAP overdose can result in severe APAP-induced liver injury (AILI). Previous reports have indicated that AILI is one of the most common causes of liver damage and acute liver failure in the United States (Ostapowicz et al., 2002; Herndon and Dankenbring,

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ABBREVIATIONS: AILI, APAP-induced liver injury; APAP, acetaminophen; BSA, bovine serum albumin; CAR, constitutive androstane receptor; CI, confidence interval; DHR123, dihydrorhodamine 123; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione *S*-transferase; GSTP1, glutathione *S*-transferase π1; GSTT1, glutathione *S*-transferase θ1; HNF1α, hepatocyte nuclear factor 1α; HNF1α-AS1, HNF1α antisense RNA 1; HNF4α, hepatocyte nuclear factor 4α; HNF4α-AS1, HNF4α antisense RNA 1; LDH, lactate dehydrogenase; lncRNA, long noncoding RNA; MEM, minimal essential medium; miRNA, micro RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAPQI, *N*-acetyl-*p*-benzoquinone imine; P450, cytochrome P450; PCR, polymerase chain reaction; PXR, pregnane X receptor; ROS, reactive oxygen species; RT-PCR, real-time PCR; shHNF1α-AS1, shRNA targeting HNF1α-AS1; shHNF4α-AS1, shRNA targeting HNF4α-AS1; shNC, negative control shRNA; shRNA, small hairpin RNA; SNP, single nucleotide polymorphism; SULT1A1, sulfotransferase 1A1; UGT1A1, UDP glucuronosyltransferase family 1 subfamily A member 1; UGT1A9, UDP glucuronosyltransferase family 1 subfamily A member 9.

2014). Furthermore, APAP-related deaths, mostly caused by liver failure, are much more than those liver failure fatalities caused by all other prescription drugs combined (Lee, 2017). Considerable efforts have been made to understand the mechanisms responsible for AILI in different in vivo and in vitro models. *N*-acetyl-*p*-benzoquinone imine (NAPQI), the active metabolite of APAP by cytochrome P450s(P450), has been proven to cause cellular stress and damage through several pathways, including induction of oxidative stress (Xie et al., 2014). In this case, the metabolism of APAP by P450s to NAPQI has been regarded as a critical step in the development of AILI (Laine et al., 2009).

P450s are a group of heme-containing enzymes, which catalyze the metabolism of a broad range of endogenous compounds, environmental chemicals, and drugs (Gonzalez, 1988). The expression and function of P450s are critical factors in the maintenance of human health and the therapeutic efficacy of drugs. However, great interindividual variability has been observed in P450 expression and function as well as P450–mediated drug metabolism (Tracy et al., 2016). Several P450 subfamily members, including CYP1A2, 2E1, and 3A4, have been proven to mediate biotransformation of APAP to NAPQI, which are important mediators for predicting AILI (Tonge et al., 1998).

Multiple regulatory factors and mechanisms are involved in the regulation of P450 expression. Genetic and epigenetic regulations are among the most widely studied factors contributing to differential metabolism of drugs among individuals (Gomez and Ingelman-Sundberg, 2009; Zanger and Schwab, 2013; Tang and Chen, 2015). Furthermore, recent studies also showed that several factors known to affect APAP-metabolizing P450 enzymes are able to alter APAP metabolism and AILI outcome (Court et al., 2017). However, these studies mainly focus on the roles of genetic polymorphisms in the P450 genes, which can only account for a small portion of the interindividual differences in expression of P450s and their ability to metabolize drugs (Pinto and Dolan, 2011). More factors and mechanisms are needed to be discovered to fully understand this process.

lncRNAs are RNA transcripts from noncoding genes with a length of more than 200 nucleotides (Cabili et al., 2011). Recent studies have shown that the overwhelmingly abundant lncRNAs, compared with coding RNAs, in humans and other species have important functions in multiple physiologic processes, including development, cell differentiation, and immune response (Fatica and Bozzoni, 2014; Perry and Ulitsky, 2016; Agirre et al., 2019; Fernandes et al., 2019). Increasing evidence shows that lncRNAs are also important for the metabolism processes (Kornfeld and Bruning, 2014; Li et al., 2019). However, how lncRNAs regulate P450–mediated drug metabolism and the toxicological consequences on P450–generated metabolites are still not fully understood.

Neighborhood antisense lncRNAs are a common phenomenon in multiple living organisms, including humans (Villegas and Zaphiropoulos, 2015). Several examples have suggested that the existence of neighborhood antisense lncRNAs is critical for the function of their neighborhood coding genes (Zhou et al., 2015; Khyzha et al., 2019). lncRNAs hepatocyte nuclear factor 1α (HNF1α) antisense RNA 1 (HNF1α-AS1) and HNF4α antisense RNA 1 (HNF4α-AS1) are neighborhood antisense lncRNAs of the transcription factors HNF1α and HNF4α, respectively. These two lncRNAs have been reported to regulate mRNA levels of several P450s (including CYP1A2, 2E1, and 3A4) in opposing manners in in vitro models (Chen et al., 2018; Wang et al., 2019). Based on this observation, we hypothesize that the lncRNAs HNF1α-AS1 and HNF4α-AS1 have opposite effects that modulate AILI via alterations in APAP metabolism by P450s. To test the hypothesis, transfection of small hairpin RNA (shRNA)-containing vectors were used to knock down HNF1α-AS1 and HNF4α-AS1 in HepaRG cells, a reliable in vitro model to study hepatotoxicity caused by APAP (McGill et al., 2011). Cytotoxicity generated by APAP was determined by several different toxicity assays, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lactate dehydrogenase (LDH) release, and dihydrorhodamine 123 (DHR123) staining assays. Alterations in mRNA and protein levels of APAP-metabolizing phase I and II enzymes were determined by quantitative real-time PCR (RT-PCR) and Western blots, respectively.

Materials and Methods

Chemicals and Reagents. HepaRG cells, HepaRG growth additives (catalog number: ADD710), and HepaRG differentiation additives (catalog number: ADD720) were obtained from Biopredic International (Rennes, France). Williams' E medium, collagen I–coated T-25 flasks, collagen I–coated 12-well plates, collagen I–coated chamber slides, Glutamax supplement, Opti-Minimal Essential Medium (MEM) medium, Lipofectamine stem transfection reagent, MTT, Pierce LDH Cytotoxicity Assay Kit, and DHR123 were obtained from Thermo Fisher Scientific (Carlsbad, CA). An shRNA negative control (shNC) and shRNAs targeting HNF1α-AS1 (shHNF1α-AS1) or HNF4α-AS1 (shHNF4α-AS1) were obtained from GeneCopoeia (Rockville, MD). APAP was obtained from Sigma-Aldrich (St. Louis, MO). An antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Abcam (Cambridge, MA). Antibodies against CYP1A2 and 2E1 were purchased from Proteintech (Rosemont, IL). An anti-rabbit IgG antibody was obtained from Cell Signaling Technology (Danvers, MA). The TRIzol reagent was obtained from Invitrogen (Carlsbad, CA).

Cell Culture. HepaRG cells were cultured according to the provider's protocol. Briefly, the HepaRG cells were cultured in a three-step manner. Thawed cells were first cultured in a HepaRG growth medium (Williams' E medium supplied with Glutamax and growth additives) for 2 weeks until cells became fully confluent. Cells were then kept in a mixture of HepaRG growth medium and HepaRG differentiation medium (Williams' E medium supplied with Glutamax and differentiation additives) for another week. Lastly, cells were cultured in a HepaRG differentiation medium for one more week when cells were fully differentiated. Cells were incubated in a humidified cell incubator at 37°C with 5% CO_2.

shRNA Transfection. To generate lncRNA loss-of-function HepaRG models, plasmid vector–containing shRNAs with different targets were designed and purchased from GeneCopoeia. The transfection processes were performed according to a previous study, with minor modifications (Brauze et al., 2017). Briefly, HepaRG cells are seeded in collagen I–coated six-well plates with a concentration of ~50,000 cells per well. Plasmid transfection was performed when the cells reached ~90% confluence. Liposomes were prepared by mixing 1 μg of shRNA-containing vector in 50 μl of Opti-MEM medium with 5 μl of LipofectAMINE stem transfection reagent in 50 μl of Opti-MEM. After incubation for 20 minutes at room temperature, the DNA-lipid complexes were added to HepaRG cells. A puromycin selection (3 μg/ml) was performed after the transfection to select transfected cells. Cells were then cultured to a fully differentiated status.

Drug Treatment. Differentiated HepaRG cells (transfected with shNC, shHNF1α-AS1, or shHNF4α-AS1) were seeded into collagen I–coated 96-well plates with a density of 20,000 cells per well. Cells were incubated overnight for attachment before treatment. Cells were then treated with 0, 10, 30, or 100 mM of APAP in PBS for 24 hours.

MTT Assay. Cell viability was measured by the MTT assay according to the manufacturer's protocol. Briefly, after APAP treatment, 20 μl of MTT solution (4 mg/ml) was added to each well, and the plates were incubated for 3.5 hours in a 37°C incubator. After incubation, the remaining solution was removed carefully from the plates, and 100 μl of DMSO was added to each well to dissolve formed crystal. The plates were then agitated on an orbital shaker for 15 minutes for completed dissolution. Absorbance of solution was then measured at 570 nm with a spectrophotometric plate reader. Cell viability was calculated as a percentage of the control group.

LDH Assay. Cell damage was measured by the LDH release assay according to the manufacturer's protocol. As with the MTT assay above, one set of cells serving as a positive control was added with 10 μl of lysis buffer after APAP treatment, whereas another set of cells was added with 10 μl of sterile water. Cells were then incubated for 45 minutes in a cell incubator. After incubation, 50 μl of supernatant from each sample was transferred into a new 96-well flat-bottom plate. A reaction mixture (50 μl) was then added to each sample, followed by gentle mixing. Plates were then incubated at room temperature for 30 minutes with protection from light. After a final incubation, 50 μl of the stop solution was added to each sample with gentle mixing. Absorbance at 490 and 680 nm was measured with a spectrophotometer, and LDH activity was calculated as the difference in absorbance between 680 and 490 nm. The level of cell damage was represented by the ratio of LDH activities between a sample and its positive control.

DHR123 Staining. Oxidative stress in HepaRG cells after APAP treatment was measured by DHR123 staining. Differentiated HepaRG cells (transfected with shNC, shHNF1α-AS1, or shHNF4α-AS1) were seeded into collagen I–coated chamber slides with a density of 100,000 cells per well and incubated overnight for attachment. Cells were then treated with 10 mM of APAP in a 500-μl culturing medium for 24 hours. After the treatment, the medium was removed, and cells were rinsed once with PBS. Diluted DHR123 solution (5 μM) was then added to each well, and plates were incubated for 45 minutes with protection from light. After incubation, the DHR123 solution was replaced with PBS. Images of fluorescent stained cells at ×400 magnification were taken using an EVOS Fluorescence Microscope (Thermo Fisher Scientific). Quantification of fluorescence signals was performed with ImageJ software (NIH).

RNA Isolation and Quantitative RT-PCR. Total RNAs were isolated from HepaRG cells using a TRIzol reagent according to the manufacturer's protocol. RNA concentration was measured by a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) at 260 nm, and RNA integrity was evaluated using an Agilent 2200 Tape Station (Agilent Technologies, Santa Clara, CA). One microgram of total RNAs was subjected to cDNA synthesis using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). RT-PCR was performed using a CFX96 Real-Time System (Bio-Rad Laboratories) with the primer sequences shown in Supplemental Table 1. RNA or mRNA levels of GAPDH, HNF1α-AS1, CYP1A2, CYP2E1, CYP3A4, SULT1A1, UGT1A1, UGT1A9, GSTP1, and GSTT1 were measured using an iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories). RNA level of HNF4α-AS1 was measured by a TaqMan Gene expression assay (Life Technologies, Carlsbad, CA). Relative mRNA levels were determined by normalizing examined gene expression against mRNA level of GAPDH using the $2^{-\Delta\Delta Ct}$ method.

Protein Sample Preparation and Western Blotting. Cell lysates were prepared from HeapRG cells cultured in a collagen I–coated T-25 flask with a radioimmunoprecipitation assay buffer (supplied with protease inhibitor cocktail). Protein concentrations were determined using a Qubit 2.0 Fluorometer (Invitrogen). Eighty micrograms of protein was loaded and run on a polyacrylamide gel using a Mini-PROTEAN Tetra System (Bio-Rad Laboratories). Proteins were then transferred onto polyvinylidene difluoride membranes and blocked in 5% bovine serum albumin (BSA) for 1 hour. After blocking, membranes were incubated with primary antibodies diluted in 2.5% BSA (anti-GAPDH 1:4000, anti-CYP1A2 1:1000, and anti-CYP2E1 1:1000) overnight. Then, membranes were incubated in an anti-rabbit IgG antibody (1:5000) diluted in 2.5% BSA. Protein bands were visualized using a ChemiDoc MP Imaging System (Bio-Rad).

Statistical Analysis. The data are shown as means ± S.D. A two-tailed unpaired Student's t test was used to determine the significance of differences in lncRNA expression after shRNA transfection. A two-way ANOVA followed by Tukey's test was used to determine the significance of differences in the MTT and LDH assays. A one-way ANOVA followed by Dunnett's test was used to determine the significance of differences in the DHR123 staining and RT-PCR results. Statistical analyses were performed using Prism7, version 7.01, from GraphPad Software Inc. (La Jolla, CA). Differences were regarded as statistically significant if $P < 0.05$.

Results

Impact on APAP-Induced Cytotoxicity by Knockdown of HNF1α-AS1 and HNF4α-AS1. To study the roles of the lncRNAs HNF1α-AS1 and HNF4α-AS1 in affecting the cytotoxicity of APAP, HepaRG cells were stably transfected with shRNAs targeting these two lncRNAs as well as a negative control. Several assays measuring APAP cytotoxicity, including MTT, LDH release, and HDR123 staining assays, were performed. Knockdown of HNF1α-AS1 and HNF4α-AS1 by shRNA transfection in HepaRG cells yielded a decrease in their RNA levels to 0.41-fold [95% confidence interval (CI) = 0.24–0.58, $^{***}P < 0.001$] and 0.46-fold (CI = 0.23–0.71, $^{**}P < 0.01$) compared with their control groups (cells transfected with shNC), respectively, which is indicative of successful knockdown (Fig. 1). Cells with stable lncRNA knockdown were then treated with different concentrations of APAP for the assessment of cytotoxicity.

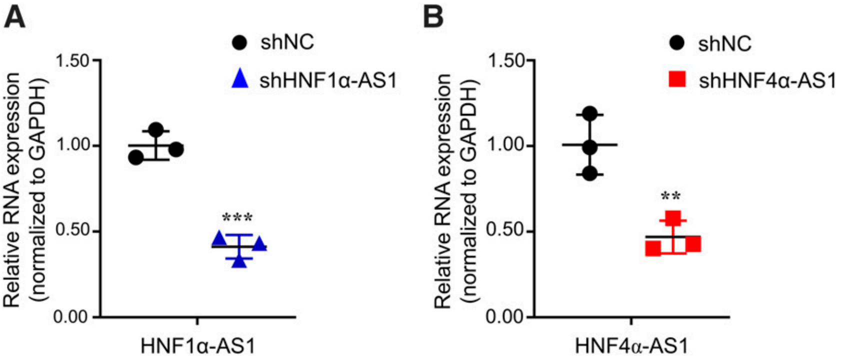

Fig. 1. Knockdown of lncRNAs HNF1α-AS1 and HNF4α-AS1 in HepaRG cells. HepaRG cells during a growth period were stably transfected with shNC, shHNF1α-AS1, or shHNF4α-AS1. (A) Relative expression of HNF1α-AS1 in HepaRG cells transfected with shNC or shHNF1α-AS1. (B) Relative expression of HNF4α-AS1 in HepaRG cells transfected with shNC or shHNF4α-AS1. Relative expression of HNF1α-AS1 and HNF4α-AS1 was measured by RT-PCR. The changes of relative mRNA expression compared with the shNC controls were calculated using the $2^{-\Delta\Delta t}$ method after normalization with GAPDH. Data are shown as means ± S.D. (n = 3) and analyzed by two-tailed unpaired Student's t test. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. shNC controls.

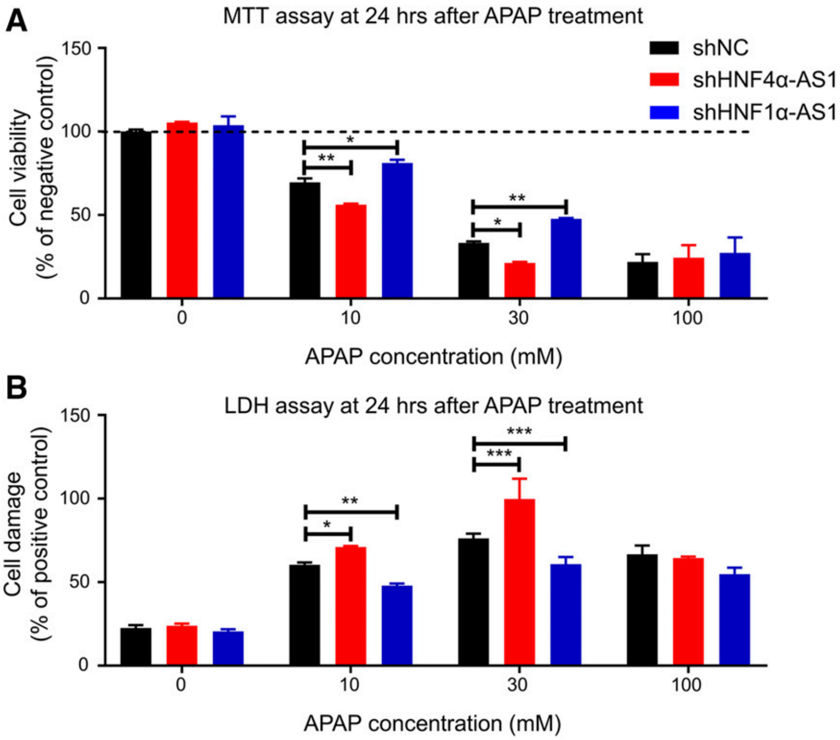

Fig. 2. Changes of APAP-induced cytotoxicity by knockdown of HNF1α-AS1 and HNF4α-AS1 in HepaRG cells. (A) Cell viability evaluated by the MTT assay after APAP treatment at concentrations of 0, 10, 30, and 100 mM. (B) Cell damage assessed by the LDH release assay after APAP treatment. Data are shown as means ± S.D. ($n = 3$) and analyzed by a two-way ANOVA followed by Tukey's test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. shNC controls receiving different concentrations of APAP.

The MTT assay was performed to test cell viability after APAP challenge. Cells were treated with different concentrations of APAP (0, 10, 30, or 100 mM) for 24 hours. Cell viability was normalized to the control group (cells transfected with shNC receiving no APAP treatment). As shown in Fig. 2A, knockdown of HNF1α-AS1 and HNF4α-AS1 did not alter cell viability in non-APAP-challenged cells. However, when treated with APAP at concentrations of 10 or 30 mM, cells with lncRNAs knockdown showed a differential susceptibility to APAP toxicity. Specifically, knockdown of HNF1α-AS1 led to increases in cell viability when treated with APAP at concentrations of 10 or 30 mM, whereas the opposite was observed in HNF4α-AS1 knockdown cells, in which the cell viability was lower at the same APAP concentrations (Fig. 2A). These results indicate that these lncRNAs play opposing roles in the susceptibility to APAP-induced cytotoxicity. At 100 mM APAP, which is a highly toxic concentration, no differences in cytotoxicity were observed, and cell viability was low among all three groups of HepaRG cells.

The LDH release assay was used for detecting the extent of cell damage. Aside from cell death, damage of living cells was another parameter to assess drug-induced cytotoxicity. As expected, Fig. 2B shows no increases in LDH release after knockdown of HNF1α-AS1 and HNF4α-AS1 when not challenged with APAP. Increases in LDH release were observed in HNF4α-AS1 knockdown cells treated with 10 or 30 mM APAP. By contrast, HNF1α-AS1 knockdown cells treated with the same concentrations of APAP had lower LDH values in comparison with shNC cells. Similar to the results of the MTT assay, no differences in LDH release values were observed at 100 mM APAP in all three groups of HepaRG cells.

Overproduction and accumulation of reactive oxygen species (ROS) and induction of oxidative stress is one of the main features for AILI. Figure 3 shows the analysis of ROS production by DHR123 staining. Under normal conditions, no fluorescence was detected in any of the groups of cells (data not shown). However, with APAP treatment, differences in fluorescent intensity were observed. Positive stained cells, indicative of ROS accumulation, were observed in all three groups of cells exposed to 10 mM APAP (Fig. 3A). Quantification results (Fig. 3B) showed that cells with HNF4α-AS1 knockdown had 1.64-fold-higher (CI = 1.36–2.02, **$P < 0.01$) fluorescent intensity compared with the control group (cells transfected with shNC receiving 10 mM APAP treatment), whereas cells with HNF1α-AS1 knockdown led to a 0.47-fold-lower (CI = 0.36–0.59, *$P < 0.05$) fluorescent intensity. Notably, only metabolically active hepatocyte-like cells, which express P450 enzymes, were stained positively by DHR123, whereas no fluorescence was detected in cholangiocyte-like cells in the cultures of HepaRG cells.

This observation indicates that the production of ROS colocalizes to cells in which P450–mediated bioactivation of APAP occurs. The cells with HNF4α-AS1 knockdown showed an observable, brighter green fluorescence compared with other groups, indicating higher levels of ROS, which is consistent with higher toxicity in those cells. By contrast, fewer positive stained cells and dimmed green fluorescence were detected in HNF1α-AS1 knockdown cells, indicating lower levels of ROS, which is also in agreement with the higher tolerance of these cells to APAP.

The results from the cytotoxicity assays performed here provided strong evidence that knockdown of lncRNA HNF1α-AS1 or HNF4α-AS1 altered cell susceptibility to APAP cytotoxicity. The absence of HNF4α-AS1 increased susceptibility to APAP-induced cytotoxicity, whereas deletion of HNF1α-AS1 afforded tolerance to APAP cytotoxicity.

Impact on Metabolic Pathways of APAP by Knockdown of HNF4α-AS1 and HNF1α-AS1. To determine how knockdown of HNF1α-AS1 and HNF4α-AS1 affects APAP-induced cytotoxicity, the enzymes involved in the metabolic

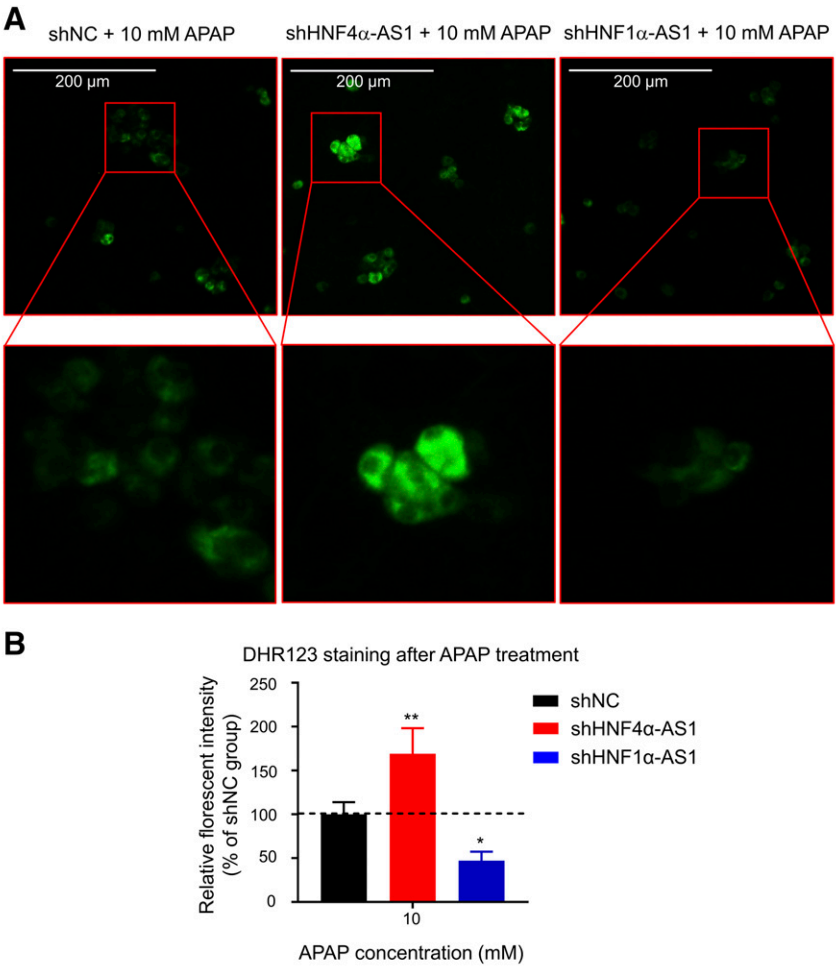

Fig. 3. Changes of APAP-induced cytotoxicity viewed by DHR123 staining in HepaRG cells with knockdown of lncRNAs HNF1α-AS1 and HNF4α-AS1. (A) Quantification of fluorescence intensity in different group of cells after treatment with 10 mM APAP. (B) The representative images are shown with green fluorescence in hepatocyte liver cells. Scale bar, 200 μM. Data are shown as means ± S.D. and analyzed by a one-way ANOVA analysis followed by Dunnett's test. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the shNC controls receiving 10 mM APAP.

pathways of APAP were examined. As shown in Fig. 4, APAP is metabolized by several phase I and II enzymes, whose combined functions ultimately determine cytotoxicity outcome. NAPQI, the toxic metabolite, is produced by P450–mediated bioactivation of APAP. The major P450s involved in this process are CYP1A2, 2E1, and 3A4. Several phase II enzymes, including SULT1A1, UGT1A1, and UGT1A9, are also able to biotransform APAP, forming nontoxic APAP conjugates. Furthermore, NAPQI can be detoxified by reacting with cellular glutathione, mediated by GSTP1 and GSTT1. The mRNA levels of these genes were measured in HNF4α-AS1 and HNF1α-AS1 knockdown as well as control shNC HepaRG cells. As shown in Fig. 5A, knockdown of HNF1α-AS1 and HNF4α-AS1 affected the mRNA levels of all selected APAP-metabolizing P450 genes. Knockdown of HNF1α-AS1 repressed mRNA levels of all P450 genes examined. Specifically, HNF1α-AS1 knockdown repressed mRNA levels of CYP1A2 to 0.71-fold (CI = 0.61–0.81, $^{*}P > 0.05$), CYP2E1 to 0.35-fold (CI = 0.31–0.39, $^{**}P < 0.01$), and CYP3A4 to 0.31-fold (CI = 0.0052–0.61, $^{*}P < 0.05$) compared with the control group (cells transfected with shNC). By contrast, knockdown of HNF4α-AS1 induced mRNA levels of CYP1A2 by 1.3-fold (CI = 0.38–2.22, $P > 0.05$), CYP2E1 by 1.95-fold (CI = 1.27–2.63, $^{***}P < 0.001$), and CYP3A4 by 1.9-fold (CI = 1.00–2.81, $^{**}P < 0.01$) compared with the control group. These changes were further confirmed by analysis of protein abundance by Western blots. As shown in Fig. 5B, the pattern of protein-level changes for P450s is similar to that of mRNA expression. Collectively, these data indicate that lncRNAs HNF1α-AS1 and HNF4α-AS1 are involved in the regulation of functional activity of biotransformation pathways for APAP, ultimately impacting APAP-induced cytotoxicity.

HNF4α-AS1 knockdown produced no changes in mRNA levels of selected phase II enzymes, including SULT1A1,

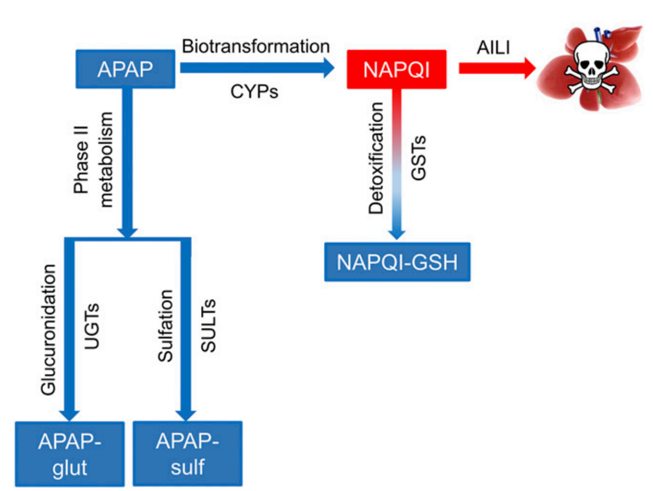

Fig. 4. Metabolic pathways of APAP in liver by phase I and II enzymes.

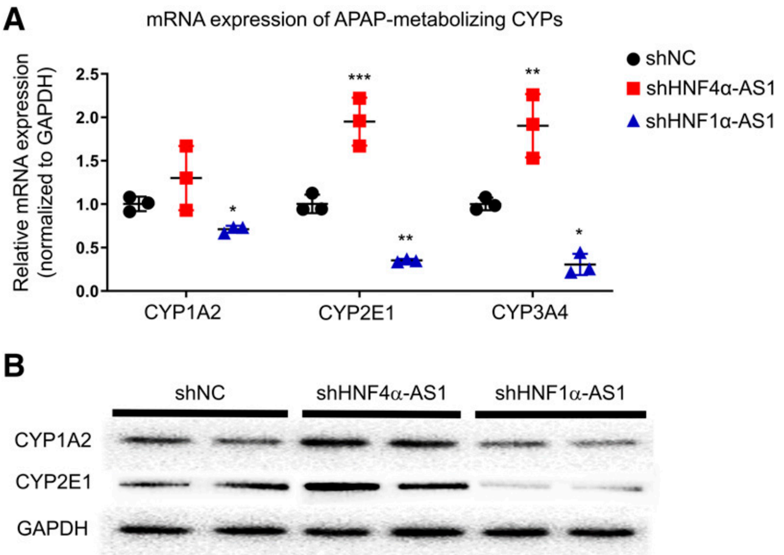

Fig. 5. Impact on expression of APAP-metabolizing phase I enzymes in HepaRG cells with knockdown of HNF1α-AS1 and HNF4α-AS1. (A) Relative mRNA expression of CYP1A2, 2E1, and 3A4 was measured by RT-PCR. The changes of relative mRNA expression compared with the shNC controls were calculated using the $2^{-\Delta\Delta t}$ method after normalization with GAPDH. Data are shown as means ± S.D. ($n = 3$) and analyzed by a one-way ANOVA analysis followed by Dunnett's test. Three separate one-way ANOVAs were run. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. the shNC controls. (B) Protein expression of CYP1A2 and 2E1 was determined by Western blots. GAPDH was used as an internal control.

UGT1A1, and 1A9 (Fig. 6A). The mRNA levels of SULT1A1 and UGT1A9 were decreased by knockdown of HNF1α-AS1 to 0.64-fold (CI = 0.48–0.80, *$P < 0.05$) and 0.72-fold (CI = 0.59–0.85, *$P < 0.05$) compared with the control group, respectively, whereas no changes in the mRNA level of UGT1A1 were found. For GSTs, the mRNA levels of GSTT1 were comparable among all three groups of cells. Even though the decreases in mRNA levels of GSTP1 by knockdown of both HNF1α-AS1 and HNF4α-AS1 were detected (Fig. 6B), considering the expression level of GSTP1 in HepaRG cells is very low (quantitation cycle value around 35, data not shown), these changes were not believed to cause major changes in APAP metabolism and cytotoxicity.

Taken together, these results indicate that the changes in mRNA and protein levels of P450 enzymes by knockdown of HNF1α-AS1 and HNF4α-AS1 are the most likely mechanisms for the differential susceptibility to APAP-induced cytotoxicity in HepaRG cells.

Discussion

Regulation of transcription factors, including HNF1α and HNF4α, either by gene edition techniques or endogenous micro RNAs (miRNAs) has been reported to affect cellular response to APAP-induced cytotoxicity (Martovetsky et al., 2013; Li et al., 2014; Yu et al., 2018). The current study also proves that the neighborhood antisense lncRNAs HNF1α-AS1 and HNF4α-AS1 also affect APAP-induced cytotoxicity independently. The manipulation of expression of HNF1α-AS1

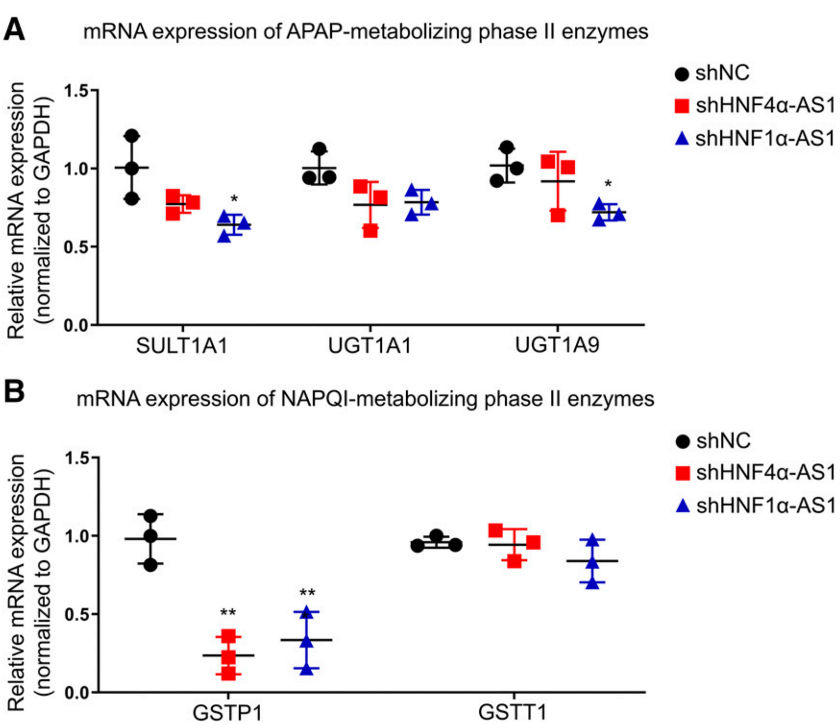

Fig. 6. Impact on expression of APAP-metabolizing phase II enzymes in HepaRG cells with knockdown of HNF1α-AS1 and HNF4α-AS1. (A) Relative mRNA expression of SULT1A1, UGT1A1, and UGT1A9 was measured by RT-PCR. (B) Relative mRNA expression of GSTP1 and GSTT1 was measured by RT-PCR. The changes of relative mRNA expression compared with the shNC controls were calculated using the $2^{-\Delta\Delta t}$ method after normalization with GAPDH. Data are shown as means ± S.D. ($n = 3$) and analyzed by a one-way ANOVA analysis followed by Dunnett's test. Three ANOVAs for the top panel and two for the bottom panel. *$P < 0.05$and **$P < 0.01$ vs. shNC controls.

and HNF4α-AS1 alone is unable to affect expression of HNF1α and HNF4α, as described in our previous studies (Chen et al., 2018; Wang et al., 2019), but is able to alter the mRNA and protein levels of APAP-metabolizing P450 enzymes and downstream APAP-induced cytotoxicity in HepaRG cells. These results suggest several critical features of the lncRNAs in the regulation of P450 expression and function. Firstly, these lncRNAs work as downstream factors to regulate the P450s together with the transcription factors of HNF1α and HNF4α. Indeed, several studies have suggested that the RNA levels of lncRNAs HNF1α-AS1 and HNF4α-AS1 are controlled by HNF1α and HNF4α (Chen et al., 2018; Ding et al., 2018; Wang et al., 2019). In addition to directly binding to the P450 genes, the transcription factors HNF1α and HNF4α may also regulate P450 expression indirectly through their neighborhood lncRNAs. Secondly, the lncRNAs have distinct functions in the regulation of their target genes. As shown here and in our previous studies, knockdown of HNF1α-AS1 and HNF4α-AS1 generated opposing effects on the expression of several P450s at both the mRNA and protein levels, which correlated well with contrasting effects on APAP-induced cytotoxicity. This phenomenon indicates that the lncRNAs HNF1α-AS1 and HNF4α-AS1 may be involved in a dynamic interrelated regulation of P450s in which both upregulation and downregulation can occur. Generally, the binding of transcription factors to their target genes promotes gene expression, leading to upregulation of gene expression, which is the case for induction of some P450s by activation of the pregnane X receptor (PXR) or constitutive androstane receptor (CAR) (Burk et al., 2004). However, how altered expression of P450s returns to normal basal levels and whether negative-feedback loops contribute to the regulation of P450 genes remain largely unknown. The roles of lncRNAs HNF1α-AS1 and HNF4α-AS1, which have opposing effects on the regulation of P450 expression in controlling P450 functions under different physiologic conditions, need to be addressed in future studies. Activation of some nuclear receptors, such as PXR and CAR, has been reported to affect AILI (Zhang et al., 2002; Cheng et al., 2009). Expression of PXR or CAR can also be affected by alterations of lncRNAs HNF1α-AS1 or HNF4α-AS1 (Chen et al., 2018). These lncRNAs may regulate expression of P450s and affect susceptibility to AILI through indirect alterations of PXR or CAR, but this assumption needs to be confirmed in a future study.

lncRNAs need cofactors to perform their functions in gene regulation. Studies have shown that lncRNAs are able to interact with other molecules, such as DNA, RNA, and proteins, to perform functions as signals, decoys, guides, and scaffolds (Wang and Chang, 2011). Identifying which molecules are able to interact with lncRNAs is a critical step to understand how lncRNAs perform their regulatory functions. One study has shown that HNF1α-AS1 is able to directly interact with miRNA and regulates proliferation and invasion of non-small-cell lung cancer cells (Zhang et al., 2018). lncRNA HNF1 homeobox A, opposite strand 1, a neighborhood antisense lncRNA of mouse *Hnf1α* gene, has been shown to interact with enhancer of zeste homolog 2 in mouse liver by RNA immunoprecipitation sequencing (Wang et al., 2018). Enhancer of zeste homolog 2 is a catalytic subunit of the polycomb repressive complex 2, which mediates the formation of trimethylation at histone H3 lysine 27 (Plath et al., 2003; Cifuentes-Rojas et al., 2014). This evidence suggests that lncRNAs HNF1α-AS1 and HNF4α-AS1 may regulate P450 expression through multiple mechanisms by interacting with different types of molecules. Identification of molecules, mainly miRNAs or proteins, that bind to HNF1α-AS1 and HNF4α-AS1 is one of our current research interests to uncover the molecular mechanisms of HNF1α-AS1– and HNF4α-AS1–mediated regulation of P450 expression.

lncRNAs should be considered as novel factors predicting drug metabolism and cytotoxicity. By generating loss-of-function cell models, the current study has suggested that expression of lncRNAs HNF1α-AS1 and HNF4α-AS1 is important for APAP-induced cytotoxicity. Notably, in the correlation study performed by Wang et al. (2019) using human liver samples, results showed not only that expression levels of HNF1α-AS1 are positively correlated to several P450s but also that HNF1α-AS1 is expressed at different levels among individuals. Besides the well known factors that regulate P450–mediated drug metabolism, which have been shown to impact clinical outcomes of drug treatment, expression and function of P450–regulating lncRNAs should also be counted as an additional factor (Pinto and Dolan, 2011; Tang and Chen, 2015).

Multiple mechanisms are able to regulate the expression of lncRNAs. The National Center for Biotechnology Information data base of human single nucleotide polymorphisms (SNPs) has listed thousands of SNPs existing in the *HNF1α-AS1* and *HNF4α-AS1* genes (https://www.ncbi.nlm.nih.gov/snp/), which might be responsible for the interindividual variations in the expression of these lncRNAs. Multiple studies have suggested that lncRNAs are differentially expressed under disease conditions, including cancer, which also suggests that lncRNAs can be affected and expressed differently for the same individual at different times or conditions (Huarte, 2015). However, no studies have been performed to determine how SNPs in these genes affect expression or function of HNF1α-AS1 and HNF4α-AS1 or their downstream-regulated genes. More future studies are urgently needed to address these knowledge gaps in their clinical relevance for drug metabolism and cytotoxicity.

In the current research project, HepaRG was used as an experimental model to study the roles of lncRNA in AILI. The hepatoma-derived HepaRG cell line has been widely used as a new in vitro model in the study of liver functions because of its high expression levels of drug metabolizing enzymes and transporters (Aninat et al., 2006; Guillouzo et al., 2007). However, several limitations still exist in the HepaRG cell model. The differentiated HepaRG cells are composed of both hepatocyte-like cells, which act similarly to primary hepatocytes, and cholangiocyte-like cells, which act similarly to epithelial cells and do not respond to APAP treatment. When harvesting samples from differentiated HepaRG cells, it is difficult to separate these two types of cells, which will ultimately affect the experimental outcomes. Second, several SNPs have been identified in HepaRG cells, including *CYP2D6*, *organic-anion-transporting polypeptide 1B1*, and *multidrug resistance-associated protein 2* genes, which may lead to dysfunction of these proteins. The human primary hepatocytes, which are regarded as the "gold standard model" for in vitro metabolism and toxicity studies, will be used in the future to validate our findings in HepaRG cells.

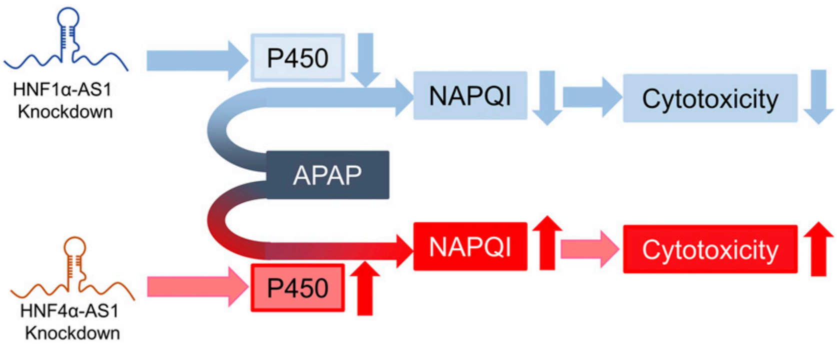

Fig. 7. Mechanistic summary of HNF1α-AS1 and HNF4α-AS1 in susceptibility of AILI.

In conclusion, the present study demonstrates that lncRNAs HNF1α-AS1 and HNF4α-AS1 are able to alter APAP-induced cytotoxicity in HepaRG cells with opposite effects, primarily by affecting expression of the P450 enzymes (Fig. 7).

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Authorship Contributions

Participated in research design: Chen, Wang, Manautou, Zhong.

Conducted experiments: Chen, Wang.

Performed data analysis: Chen, Wang, Zhong.

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

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