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Knockdown of LncRNAs HNF1 α -AS1 and HNF4 α -AS1 Alters Susceptibility of Acetaminophen-induced Cytotoxicity in HepaRG Cells

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Running Title: HNF1 α -AS1 and HNF4 α -AS1 in APAP-induced cytotoxicity

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ABBREVIATIONS

ALI, acetaminophen-induced liver injury; ANOVA, analysis of variance; APAP, acetaminophen; BSA, bovine serum albumin; CAR, constitutive androstane receptor; CYPs, cytochrome P450s; CYP1A2, cytochrome P450 family 1 subfamily A member 2; CYP2E1, cytochrome P450 family 2 subfamily E member 1; CYP3A4, cytochrome P450 family 3 subfamily A member 4; DHR123, dihydrorhodamine 123; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSTP1, glutathione S-transferase pi 1; GSTT1, glutathione S-transferase theta 1; HNF1 α , hepatocyte nuclear factor 1 α ; HNF1 α -AS1, HNF1 α antisense RNA 1; HNF4 α , hepatocyte nuclear factor 4 α ; HNF4 α -AS1, HNF4 α antisense RNA 1; LDH, lactate dehydrogenase; lncRNAs, long non-coding RNAs; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAPQI, *N*-acetyl-*p*-benzoquinone imine; PBS, phosphate buffered saline; PXR, pregnane X receptor; ROS, reactive oxygen species; RT-PCR, real-time PCR; SD, standard deviation; shRNA, small hairpin RNA; SULT1A1, sulfotransferase 1A1; UGT1A1, UDP glucuronosyltransferase family 1 subfamily A member 1; UGT1A9, UDP glucuronosyltransferase family 1 subfamily A member 9

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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) even death due to accumulation of *N*-acetyl-*p*-benzoquinone imine (NAPQI), the toxic metabolite of APAP generated by cytochrome P450s (CYPs). Long non-coding RNAs HNF1 α antisense RNA 1 (HNF1 α -AS1) and HNF4 α antisense RNA 1 (HNF4 α -AS1) are regulatory RNAs involved in the regulation of CYPs expression in both mRNA and protein levels. This study aims to determine the impact of HNF1 α -AS1 and HNF4 α -AS1 on AILI. Small hairpin RNAs (shRNAs) were used to knockdown HNF1 α -AS1 and HNF4 α -AS1 in HepaRG cells. Knockdown of these lncRNAs altered APAP-induced cytotoxicity indicated by MTT and LDH assays. Specifically, HNF1 α -AS1 knockdown decreased APAP toxicity with increased cell viability and decreased LDH release, whereas HNF4 α -AS1 knockdown exacerbated APAP toxicity with opposite effects in the MTT and LDH assays. Alterations on gene expression by knockdown of HNF1 α -AS1 and HNF4 α -AS1 were examined in several APAP metabolic pathways, including CYP1A2, 2E1, 3A4, UGT1A1, 1A9, SULT1A1, GSTP1, and GSTT1. Knockdown of HNF1 α -AS1 decreased mRNA expression of CYP1A2, 2E1, and 3A4 by 0.71-fold, 0.35-fold, and 0.31-fold, respectively, while knockdown of HNF4 α -AS1 induced mRNAs of CYP1A2, 2E1 and 3A4 by 1.3-fold, 1.95-fold, and 1.9 fold, respectively. The changes were also observed in protein levels. Knockdown of HNF1 α -AS1 and HNF4 α -AS1 had limited effects on the mRNA expression of UGT1A1, 1A9, SULT1A1, GSTP1, and GSTT1. Altogether, our study suggests that HNF1 α -AS1 and HNF4 α -AS1 affected AILI mainly through alterations of CYP-mediated APAP biotransformation in HepaRG cells, indicating an important role of the lncRNAs in AILI.

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Significance Statement

The current research identified two lncRNAs, HNF1 α -AS1 and HNF4 α -AS1, which were able to affect susceptibility of AILI in HepaRG cells, possibly through regulating the expression of APAP-metabolizing P450 enzymes. This discovery added new factors, lncRNAs, which can be used to predict P450-mediated drug metabolism and drug-induced toxicity.

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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 4,000 mg per day 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, 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 (CYPs), 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 CYPs to NAPQI has been regarded as a critical step in the development of AILI (Laine et al., 2009).

CYPs 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 CYPs are critical factors in the maintenance of human health and therapeutic efficacy of drugs. However, great inter-individual variability has been observed in CYP expression and functions as well as CYP-mediated drug

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metabolism (Tracy et al., 2016). Several CYP subfamily members, including CYP1A2, 2E1, and 3A4, have been proven to mediate biotransformation of APAP to NAPQI, which are important mediators for predicting ALI (Tonge et al., 1998).

Multiple regulatory factors and mechanisms are involved in the regulation of CYP 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 CYP enzymes are able to alter APAP metabolism and ALI outcome (Court et al., 2017). However, these studies mainly focus on the roles of genetic polymorphisms in the CYP genes, which can only account for a small portion of the inter-individual differences in expression of CYPs and 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 non-coding genes with a length of more than 200 nucleotides (Cabili et al., 2011). Recent studies have shown that the overwhelmingly abundant lncRNAs, comparing to coding RNAs, in human and other species have important functions in multiple physiological 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 CYP-mediated drug metabolism and the toxicological consequences on CYP-generated metabolites are still not fully understood.

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Neighborhood antisense lncRNAs are a common phenomenon in multiple living organisms, including human (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 1 α (HNF1 α) antisense RNA 1 (HNF1 α -AS1) and HNF4 α antisense RNA 1 (HNF4 α -AS1) are neighborhood antisense lncRNAs of the transcription factors HNF1 α and HNF4 α , respectively. These two lncRNAs have been reported to regulate mRNA levels of several CYPs (including CYP1A2, 2E1, and 3A4) in opposing manners in *in vitro* models (Chen et al., 2018; Wang et al., 2019a). Based on this observation, we hypothesize that the lncRNAs HNF1 α -AS1 and HNF4 α -AS1 have opposite effects modulating AILI via alterations in APAP metabolism by CYPs. To test the hypothesis, transfection of small hairpin RNA (shRNA) containing vectors were used to knock down HNF1 α -AS1 and HNF4 α -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) assay, 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.

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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). William's E Medium, collagen I coated T-25 flasks, collagen I coated 12-well plates, collagen I coated chamber slides, Glutamax™ supplement, Opti-MEM medium, Lipofectamine™ stem transfection reagent, MTT, Pierce LDH Cytotoxicity Assay Kit, and DHR123 were obtained from Thermo Fisher Scientific (Carlsbad, CA). A shRNA negative control and shRNAs targeting HNF1 α -AS1 or HNF4 α -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 firstly cultured in a HepaRG growth medium (William's E Medium supplied with Glutamax™ and growth additives) for two weeks until cells became fully confluent. Cells were then kept in a mixture of HepaRG growth medium and HepaRG differentiation medium (William's 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

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were fully differentiated. Cells were incubated in a humidified cell incubator at 37°C with 5% CO₂.

shRNA Transfection. To generate lncRNA loss-of-function HepaRG models, plasmid vector-containing shRNAs with different targets were designed and purchased from GeneCopoeia (Rockville, MD, USA). 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 6-well plates with a concentration of ~50,000 cells/well. Plasmid transfection was performed when the cells reached to ~90% confluence. Liposomes were prepared by mixing 1 µg shRNA-containing vector in 50 µL Opti-MEM medium with 5 µl Lipofectamine™ stem transfection reagent in 50 µl 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/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 h.

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 h in a 37°C incubator. After incubation, the remaining solution was removed carefully from the plates and 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve formed crystal. The plates

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were then agitated on an orbital shaker for 15 min for completed dissolution. Absorbance of solution was then measured at 570 nm with a spectrophotometric plate reader. Cell viability was calculated as percentage of the control group.

LDH Assay. Cell damage was measured by the LDH release assay according to the manufacturer's protocol. Same as the MTT assay above, one set of cells serving as a positive control was added with 10 μ L lysis buffer after APAP treatment, while another set of cells was added with 10 μ L sterile water. Cells were then incubated for 45 min in a cell incubator. After incubation, 50 μ L supernatant of each sample was transferred into a new 96-well flat bottom plate. A reaction mixture (50 μ L) was then added to each sample followed by gentle mixing. Plates were then incubated at room temperature for 30 min protected from light. After a final incubation, 50 μ L of the stop solution were 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, shHNF1 α -AS1, or shHNF4 α -AS1) were seeded into collagen-I coated chamber slides with a density of 100,000 cells/well and incubated overnight for attachment. Cells were then treated with 10 mM of APAP in a 500- μ L culturing medium for 24 h. After the treatment, the medium was removed and cells were rinsed once with PBS. Diluted DHR123 solution (5 μ M) was then added to each well and plates were incubated for 45 min with protection from light. After incubation, the DHR123 solution was replaced with

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PBS. Images of fluorescent stained cells at $\times 400$ magnification were taken using an EVOS Fluorescence Microscope (Thermo Fisher Scientific, Carlsbad, CA). Quantification of fluorescence signals was performed with ImageJ software (NIH).

RNA Isolation and Quantitative Real-Time PCR (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 Nano Drop spectrophotometer (Nano Drop Technologies, Wilmington, DE) at 260 nm and RNA integrity was evaluated using an Agilent 2,200 Tape Station (Agilent Technologies, Santa Clara, CA). One μg 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, Hercules, CA) with the primer sequences shown in Supplemental Table S1. RNA or mRNA levels of GAPDH, HNF1 α -AS1, CYP1A2, 2E1, 3A4, SULT1A1, UGT1A1, 1A9, GSTP1, and GSTT1 were measured using an iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA). RNA level of HNF4 α -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 $2^{-\Delta\Delta\text{Ct}}$ method.

Protein Sample Preparation and Western Blotting. Cell lysates were prepared from HepaRG cells cultured in a collagen-I coated T-25 flask with a RIPA buffer (supplied with protease inhibitor cocktail). Protein concentrations were determined using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA). Eighty μg of protein was loaded and run on a polyacrylamide gel using a Mini-PROTEAN Tetra System (Bio-Rad Laboratories, Hercules, CA). Proteins were then transferred onto PVDF membranes and blocked in 5%

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bovine serum albumin (BSA) for 1 h. After blocking, membranes were incubated with primary antibodies diluted in 2.5% BSA (anti-GAPDH 1:4,000, anti-CYP1A2 1:1,000, and anti-CYP2E1 1:1,000) overnight. Then membranes were incubated in an anti-rabbit IgG antibody (1:5,000) diluted in 2.5% BSA. Protein bands were visualized using a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA).

Statistical Analysis. The data are shown as mean \pm SD (standard deviation). 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, USA). Differences were regarded as statistically significant if $p < 0.05$.

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Results

Impact on APAP-induced Cytotoxicity by Knockdown of HNF1 α -AS1 and HNF4 α -AS1. To study the roles of the lncRNAs, HNF1 α -AS1 and HNF4 α -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 HNF1 α -AS1 and HNF4 α -AS1 by shRNA transfection in HepaRG cells yielded a decrease in their RNA levels to 0.41-fold (95% confidence interval CI = 0.24 to 0.58, *** p <0.001) and 0.46-fold (CI = 0.23 to 0.71, ** p <0.01) comparing to their control groups (cells transfected with shNC), respectively, which is indicative of successful knockdown (Fig. 1A and 1B). Cells with stable lncRNA knockdown were then treated with different concentrations of APAP for the assessment of cytotoxicity.

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 h. Cell viability was normalized to the control group (cells transfected with shNC receiving no APAP treatment). As shown in Fig. 2A, knockdown of HNF1 α -AS1 and HNF4 α -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 HNF1 α -AS1 led to increases in cell viability when treated with APAP at concentrations of 10 or 30 mM, while the opposite was observed in HNF4 α -AS1 knockdown cells, where 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

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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 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 HNF1 α -AS1 and HNF4 α -AS1 when not challenged with APAP. Increases in LDH release were observed in HNF4 α -AS1 knockdown cells treated with 10 or 30 mM APAP. By contrast, HNF1 α -AS1 knockdown cells treated with the same concentrations of APAP had lower LDH values in comparison to 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 ALI. Fig. 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 HNF4 α -AS1 knockdown had 1.64-fold (CI =1.36 to 2.02, ** p <0.01) higher fluorescent intensity comparing to the control group (cells transfected with shNC receiving 10 mM APAP treatment), while cells with HNF1 α -AS1 knockdown led to a 0.47-fold (CI =0.36 to 0.59, * p <0.05) lower fluorescent intensity. Notably, only metabolically active hepatocyte-like cells, which express CYP

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enzymes, were stained positively by DHR123, while no fluorescence was detected in cholangiocyte-like cells in the cultures of HepaRG cells.

This observation indicates that the production of ROS co-localizes to cells where CYP-mediated bioactivation of APAP occurs. The cells with HNF4 α -AS1 knockdown showed an observable brighter green fluorescence compared to 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 HNF1 α -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 HNF1 α -AS1 or HNF4 α -AS1 altered cell susceptibility to APAP cytotoxicity. The absence of HNF4 α -AS1 increased susceptibility to APAP-induced cytotoxicity, while deletion of HNF1 α -AS1 afforded tolerance to APAP cytotoxicity.

Impact on Metabolic Pathways of APAP by Knockdown of HNF4 α -AS1 and HNF1 α -AS1. To determine how knockdown of HNF1 α -AS1 and HNF4 α -AS1 affects APAP-induced cytotoxicity, the enzymes involved in the metabolic 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 CYP-mediated bioactivation of APAP. The major CYPs involved in this process are CYP1A2, 2E1, and 3A4. Several phase II enzymes, including SULT1A1, UGT1A1, and 1A9, are also able to biotransform APAP, forming non-toxic APAP-conjugates. Furthermore, NAPQI can be detoxified by reacting with cellular glutathione, mediated by GSTP1 and GSTT1. The mRNA levels of these genes were

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measured in HNF4 α -AS1 and HNF1 α -AS1 knockdown as well as control shNC HepaRG cells. As shown in Fig. 5A, knockdown of HNF1 α -AS1 and HNF4 α -AS1 affected the mRNA levels of all selected APAP-metabolizing CYP genes. Knockdown of HNF1 α -AS1 repressed mRNA levels of all CYP genes examined. Specifically, HNF1 α -AS1 knockdown repressed mRNA levels of CYP1A2 to 0.71-fold (CI = 0.61 to 0.81, * p >0.05), CYP2E1 to 0.35-fold (CI = 0.31 to 0.39, ** p <0.01), and CYP3A4 to 0.31-fold (CI = 0.0052 to 0.61, * p <0.05) comparing to the control group (cells transfected with shNC). By contrast, knockdown of HNF4 α -AS1 induced mRNA levels of CYP1A2 by 1.3-fold (CI = 0.38 to 2.22, p >0.05), CYP2E1 by 1.95-fold (CI = 1.27 to 2.63, *** p <0.001), and CYP3A4 by 1.9-fold (CI = 1.00 to 2.81, ** p <0.01) comparing to 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 CYPs is similar to that of mRNA expression. Collectively, these data indicate that lncRNAs HNF1 α -AS1 and HNF4 α -AS1 are involved in the regulation of functional activity of biotransformation pathways for APAP, ultimately impacting APAP-induced cytotoxicity.

HNF4 α -AS1 knockdown produced no changes in mRNA levels of selected phase II enzymes, including SULT1A1, UGT1A1, and 1A9 (Fig. 6A). The mRNA levels of SULT1A1 and UGT1A9 was decreased by knockdown of HNF1 α -AS1 to 0.64-fold (CI = 0.48 to 0.80, * p <0.05) and 0.72-fold (CI = 0.59 to 0.85, * p <0.05) comparing to the control group, respectively, while no changes in the mRNA level of UGT1A1 was 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 HNF1 α -AS1 and HNF4 α -AS1 were detected (Fig. 6B), considering the expression level of GSTP1 in

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HepaRG cells is very low (C_q 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 CYP enzymes by knockdown of HNF1 α -AS1 and HNF4 α -AS1 are the most likely mechanisms for the differential susceptibility to APAP-induced cytotoxicity in HepaRG cells.

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Discussion

Regulation of transcription factors including HNF1 α and HNF4 α , either by gene edition techniques or endogenous 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 HNF1 α -AS1 and HNF4 α -AS1 also affect APAP-induced cytotoxicity independently. The manipulation of expression of HNF1 α -AS1 and HNF4 α -AS1 alone is unable to affect expression of HNF1 α and HNF4 α as described in our previous studies (Chen et al., 2018; Wang et al., 2019b), but is able to alter the mRNA and protein levels of APAP-metabolizing CYP enzymes and downstream APAP-induced cytotoxicity in HepaRG cells. These results suggest several critical features of the lncRNAs in the regulation of CYP expression and functions. Firstly, these lncRNAs work as downstream factors to regulate the CYPs together with the transcription factors of HNF1 α and HNF4 α . Indeed, several studies have suggested that the RNA levels of lncRNAs HNF1 α -AS1 and HNF4 α -AS1 are controlled by HNF1 α and HNF4 α (Chen et al., 2018; Ding et al., 2018; Wang et al., 2019a). In addition to directly binding to the CYP genes, the transcription factors HNF1 α and HNF4 α may also regulate CYP expression indirectly through their neighborhood lncRNAs. Secondly, the lncRNAs have distinct functions in the regulation of their target genes. As showed here and in our previous studies, knockdown of HNF1 α -AS1 and HNF4 α -AS1 generated opposing effects on the expression of several CYPs at both the mRNA and protein levels, which correlated well with contrasting effects on APAP-induced cytotoxicity. This phenomenon indicates that the lncRNAs HNF1 α -AS1 and HNF4 α -AS1 may be involved in a dynamic interrelated regulation of CYPs, where both upregulation

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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 CYPs by activation of the pregnane X receptor (PXR) or constitutive androstane receptor (CAR) (Burk et al., 2004). However, how altered expression of CYPs returns to normal basal levels and whether negative feedback loops contribute to the regulation of CYP genes remain largely unknown. The roles of lncRNAs HNF1 α -AS1 and HNF4 α -AS1, which have opposing effects on the regulation of CYP expression in controlling CYP functions under different physiological 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 HNF1 α -AS1 or HNF4 α -AS1 (Chen et al., 2018). These lncRNAs may regulate expression of CYPs 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 signaling, decoys, guides, and scaffolds (Wang and Chang, 2011). Identifying what molecules are able to interact with lncRNAs is a critical step to understand how lncRNAs perform their regulatory functions. One study has showed that HNF1 α -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 α OS1, a neighborhood antisense lncRNA of mouse *Hnf1 α* gene, has been shown to interact with enhancer of zeste homolog 2 (EZH2) in mouse liver by RNA immunoprecipitation

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sequencing (Wang et al., 2018). EZH2 is a catalytic subunit of the polycomb repressive complex 2, which mediates the formation of tri-methylation at histone H3 lysine 27 (Plath et al., 2003; Cifuentes-Rojas et al., 2014). This evidence suggests that lncRNAs HNF1 α -AS1 and HNF4 α -AS1 may regulate CYP expression through multiple mechanisms by interacting with different types of molecules. Identification of molecules, mainly miRNAs or proteins, binding to HNF1 α -AS1 and HNF4 α -AS1, is one of our current research interests to uncover the molecular mechanisms of HNF1 α -AS1- and HNF4 α -AS1-mediated regulation of CYP 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 HNF1 α -AS1 and HNF4 α -AS1 is important for APAP-induced cytotoxicity. Notably, in the correlation study performed by Wang and her colleagues using human liver samples, results do not only show that expression levels of HNF1 α -AS1 are positively correlated to several CYPs, but also pointed out that HNF1 α -AS1 is expressed at different levels among individuals (Wang et al., 2019a). Besides the well-known factors that regulate CYP-mediated drug metabolism, which have been shown to impact clinical outcomes of drug treatment, expression and function of CYP-regulating lncRNAs should be also counted as an additional factor (Pinto and Dolan, 2011; Tang and Chen, 2015).

Multiple mechanisms are able to regulate the expression of lncRNAs. The NCBI database of human single nucleotide polymorphisms (SNPs) has listed thousands of SNPs existing in the *HNF1 α -AS1* and *HNF4 α -AS1* genes (<https://www.ncbi.nlm.nih.gov/snp/>), which might be responsible for the inter-individual

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variations in the expression of these lncRNAs. Multiple studies have suggested that lncRNAs are differentially expressed under disease conditions, including cancer, which also suggest that lncRNAs can be affected and expressed differently for a 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 HNF1 α -AS1 and HNF4 α -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 ALI. The hepatoma derived HepaRG cell line has been widely used as a new *in vitro* model in the study of liver functions due to its high expression levels of drug metabolizing enzymes and transporters (Aninat et al., 2006; Guillouzo et al., 2007). However, several limitations still exist in HepaRG cell model. The differentiated HepaRG cells are composed of both hepatocyte-like cells, which act similar to primary hepatocytes, and cholangiocyte-like cells, which act similar 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. Secondary, several SNPs have been identified in HepaRG cells, including *CYP2D6*, *OATP1B1*, and *MRP2* genes, which may lead to dysfunctions of these proteins. The human primary hepatocytes, which is 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.

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In conclusion, the present study demonstrates that lncRNAs HNF1 α -AS1 and HNF4 α -AS1 are able to alter APAP-induced cytotoxicity in HepaRG cells with opposite effects, primarily by affecting expression of the CYP 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,
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Legends for Figures

Fig. 1. Knockdown of lncRNAs HNF1 α -AS1 and HNF4 α -AS1 in HepaRG cells. HepaRG cells during a growth period were stably transfected with a negative control shRNA (shNC), shRNAs targeting HNF1 α -AS1 (shHNF1 α -AS1), or shRNAs targeting HNF4 α -AS1 (shHNF4 α -AS1). Relative expression of HNF1 α -AS1 and HNF4 α -AS1 was measured by RT-PCR. The changes of relative mRNA expression compared to the shNC controls were calculated using the $2^{-\Delta\Delta t}$ method after normalization with GAPDH. Data are shown as mean \pm SD (n = 3) and analyzed by two-tailed unpaired Student's *t* test. ***p*<0.01 and ****p*<0.001 versus shNC controls.

Fig. 2. Changes of APAP-induced cytotoxicity by knockdown of HNF1 α -AS1 and HNF4 α -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 mean \pm SD (n = 3) and analyzed by a two-way ANOVA followed by Tukey's test. **p*<0.05, ***p*<0.01, and ****p*<0.001 versus shNC controls receiving different concentrations of APAP.

Fig. 3. Changes of APAP-induced cytotoxicity viewed by DHR123 staining in HepaRG cells with knockdown of lncRNAs HNF1 α -AS1 and HNF4 α -AS1. (A) Quantification of fluorescent intensity in different group of cells after treated with 10 mM APAP. (B) The representative images are shown with green fluorescence in hepatocyte-liver cells. Bar = 200 μ m. Data are shown as mean \pm SD and analyzed by a one-way ANOVA analysis

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followed by Dunnett's test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the shNC controls receiving 10 mM APAP.

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 HNF1 α -AS1 and HNF4 α -AS1. (A) Relative mRNA expression of CYP1A2, 2E1, and 3A4 was measured by RT-PCR. The changes of relative mRNA expression compared to the shNC controls were calculated using the $2^{-\Delta\Delta t}$ method after normalization with GAPDH. Data are shown as mean \pm SD ($n = 3$) and analyzed by a one-way ANOVA analysis followed by Dunnett's test. Three separate one-way ANOVAs were run. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the shNC controls. (B) Protein expression of CYP1A2 and 2E1 was determined by Western blots. GAPDH was used as an internal control.

Fig. 6. Impact on expression of APAP-metabolizing phase II enzymes in HepaRG cells with knockdown of HNF1 α -AS1 and HNF4 α -AS1. (A) Relative mRNA expression of SULT1A1, UGT1A1, and 1A9 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 to the shNC controls were calculated using the $2^{-\Delta\Delta t}$ method after normalization with GAPDH. Data are shown as mean \pm SD ($n = 3$) and analyzed by a one-way ANOVA analysis followed by Dunnett's test. Three ANOVAs for the top panel; two for the bottom panel. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus shNC controls.

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Fig.7. Mechanistic summary of HNF1 α -AS1 and HNF4 α -AS1 in susceptibility of AILI.

Fig. 1

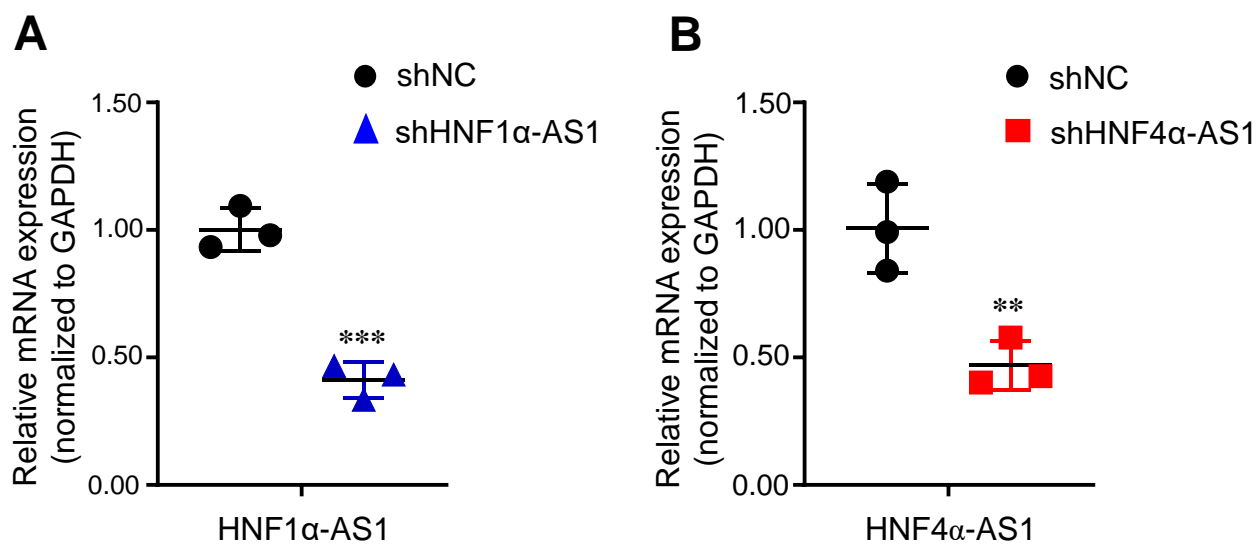
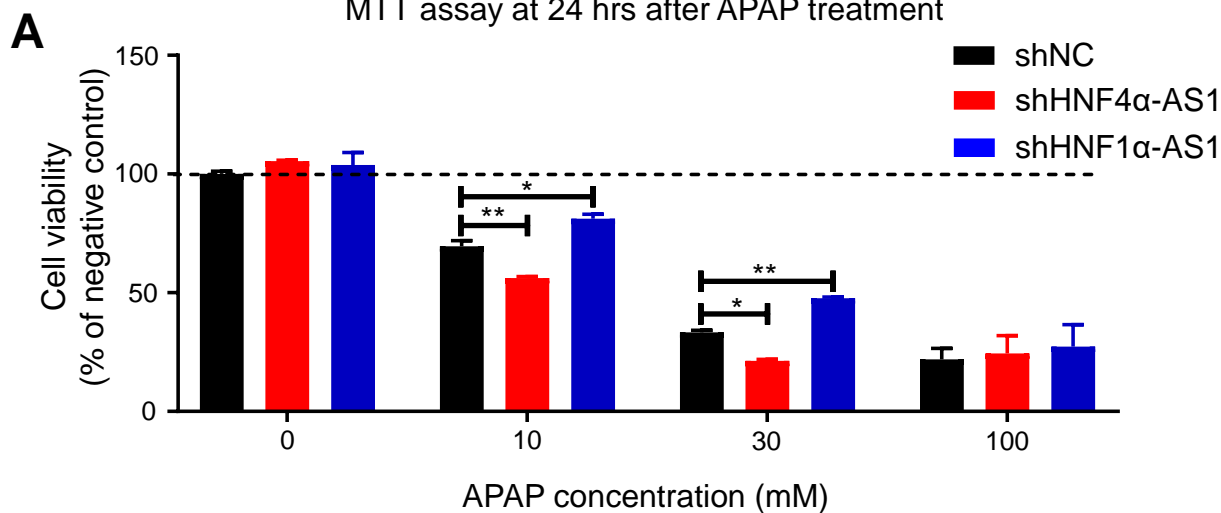


Fig. 2

MTT assay at 24 hrs after APAP treatment



LDH assay at 24 hrs after APAP treatment

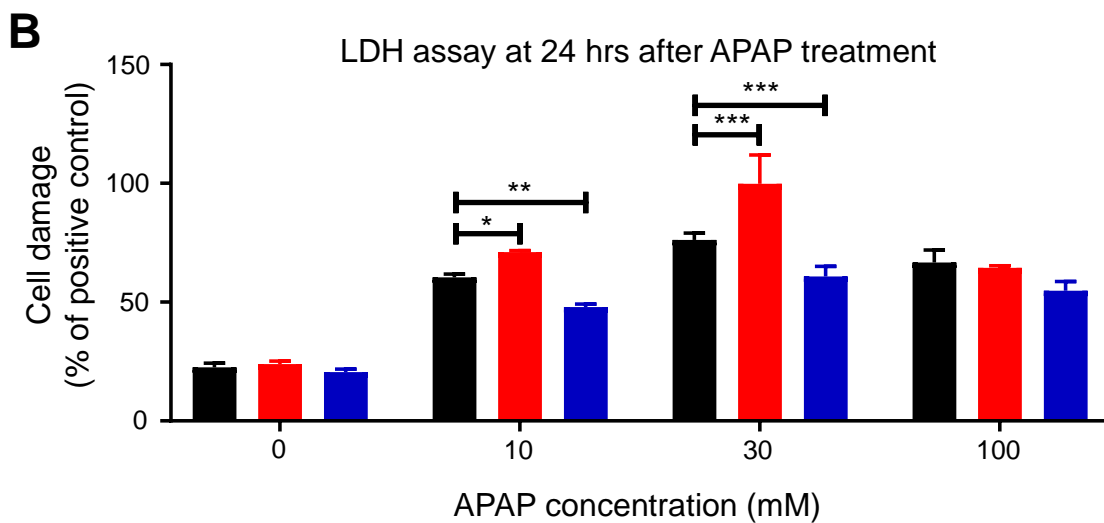


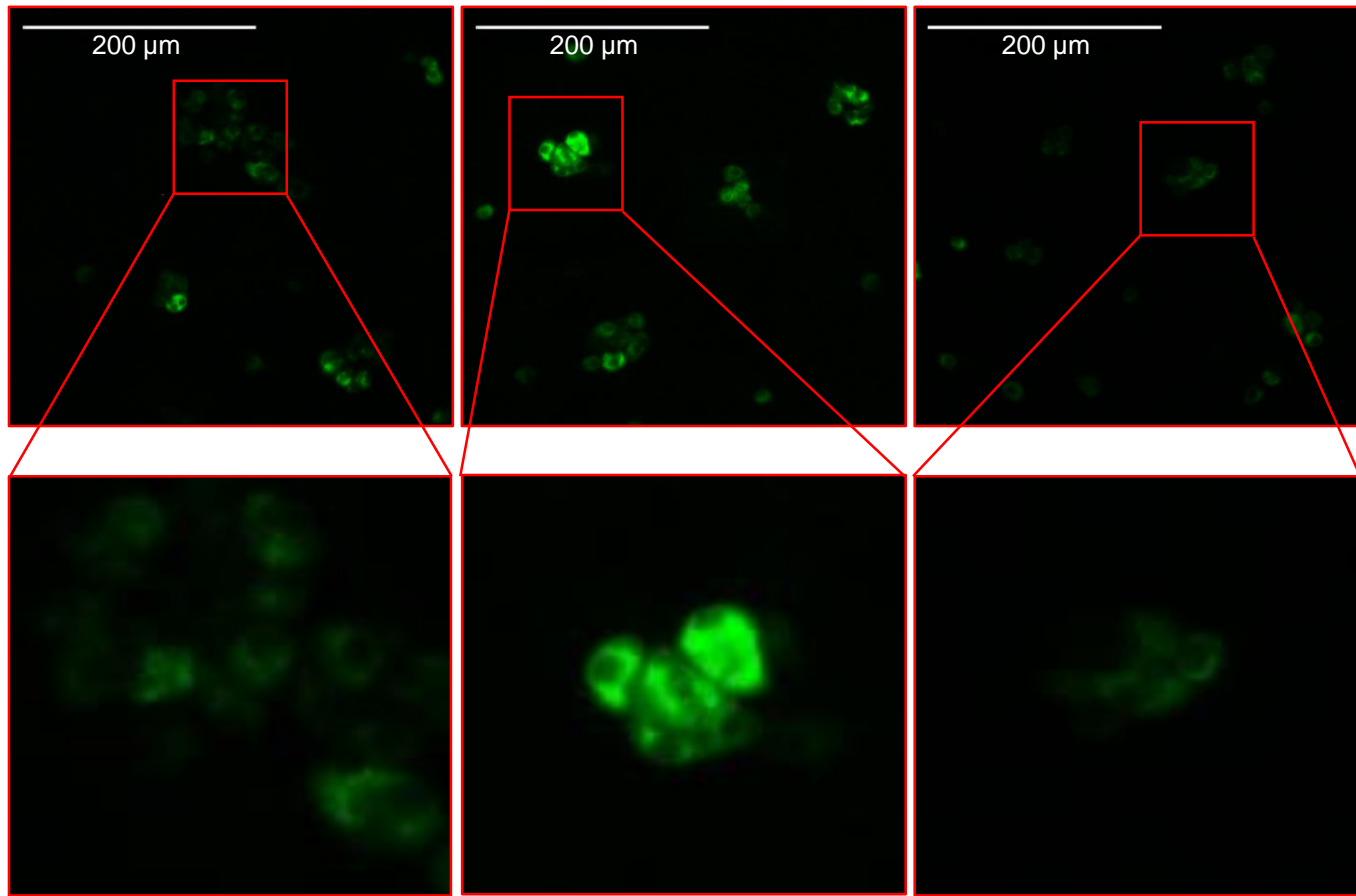
Fig. 3

A

shNC + 10 mM APAP

shHNF4 α -AS1 + 10 mM APAP

shHNF1 α -AS1 + 10 mM APAP



B

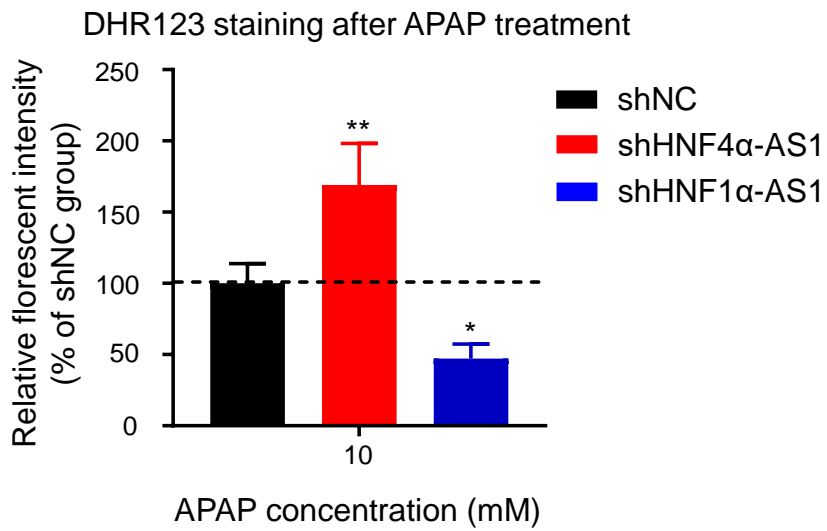


Fig. 4

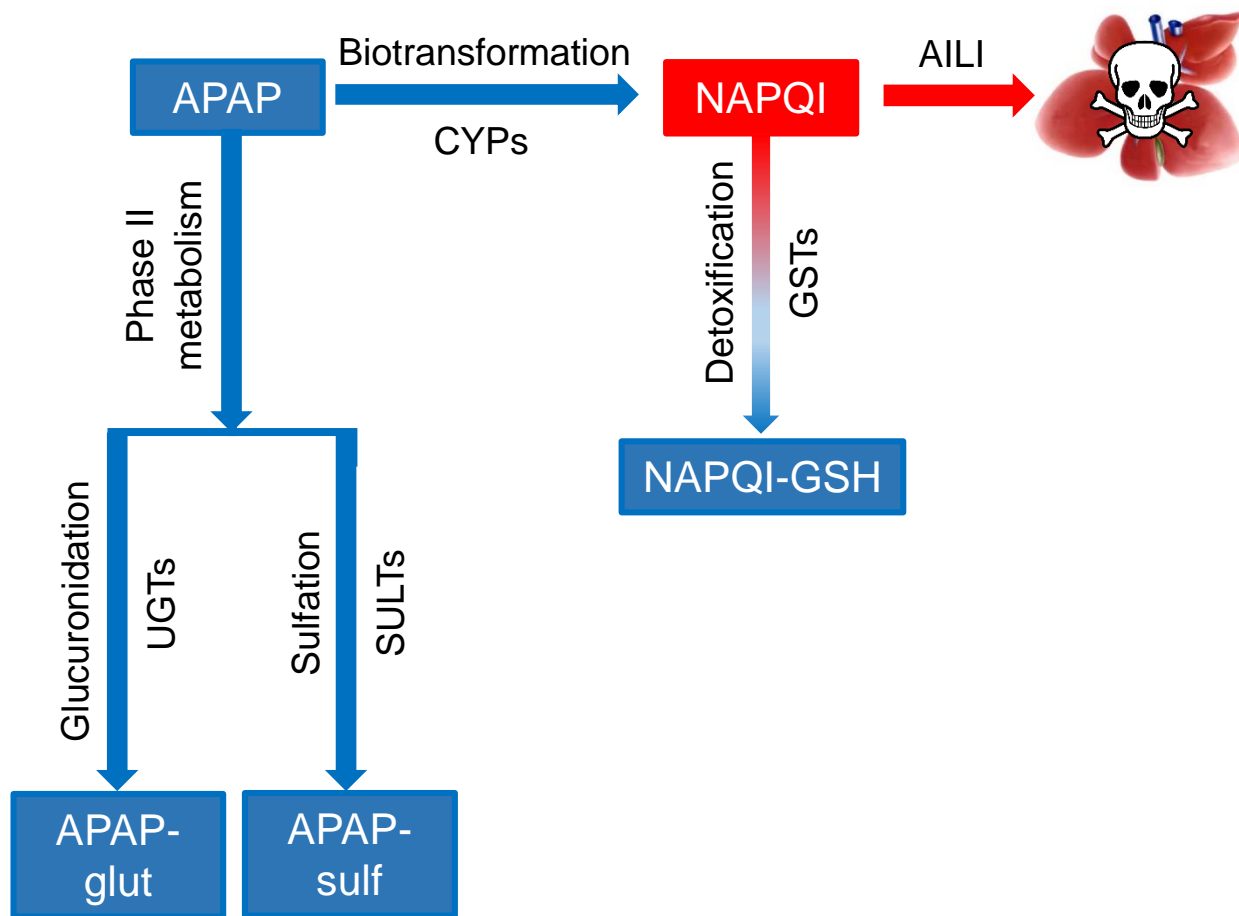
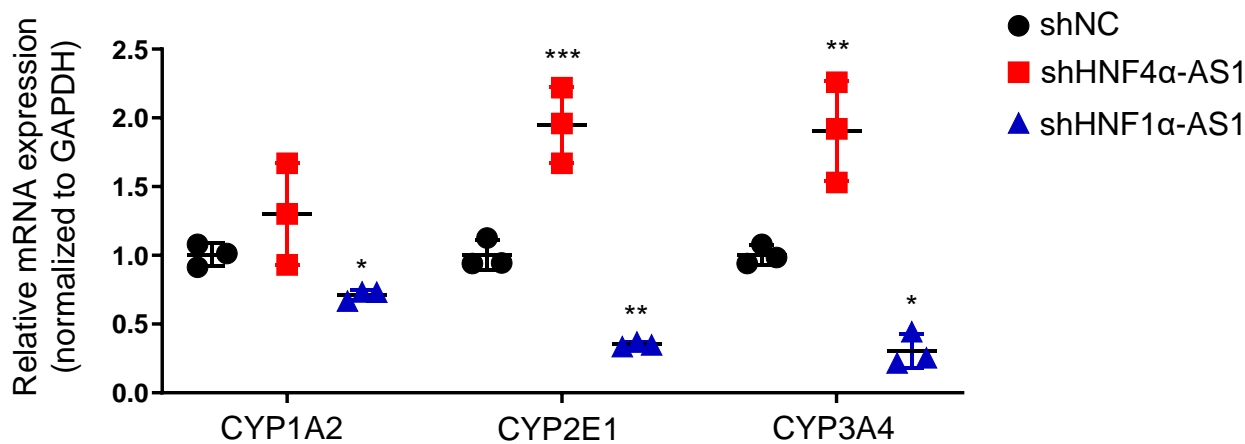


Fig. 5

A mRNA expression of APAP-metabolizing CYPs



B

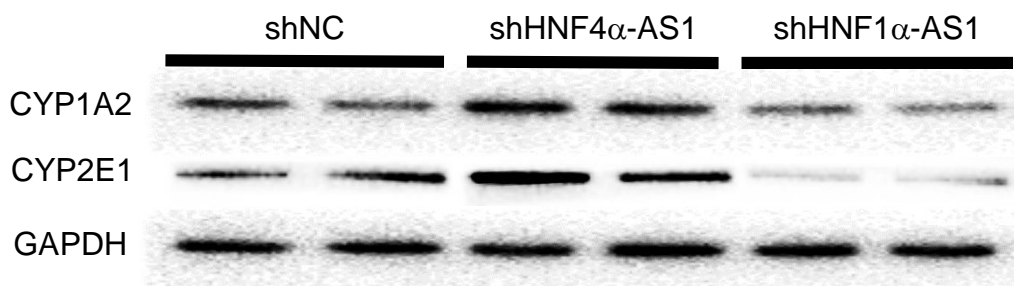


Fig. 6

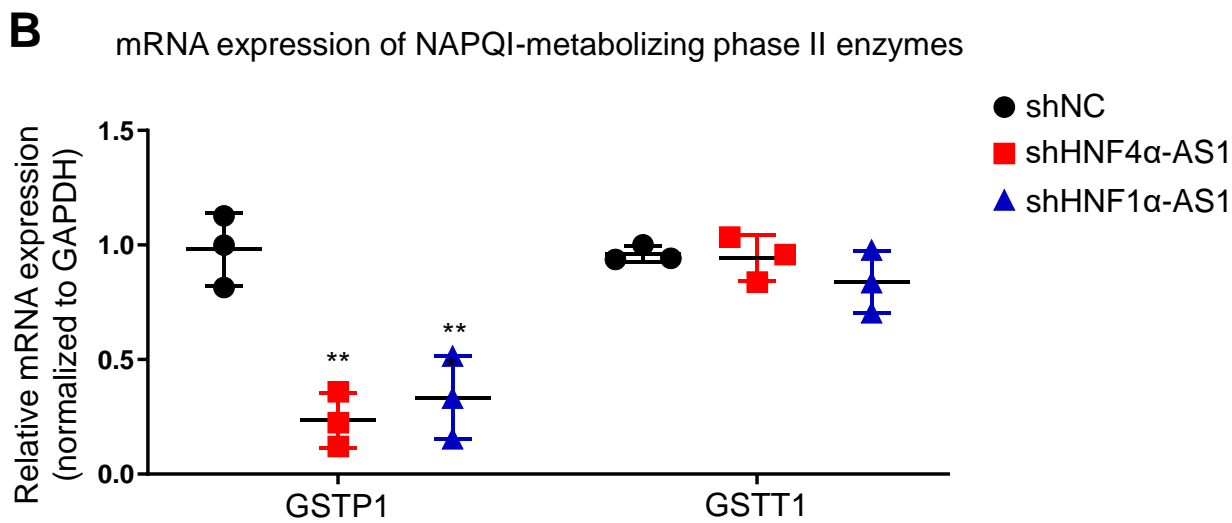
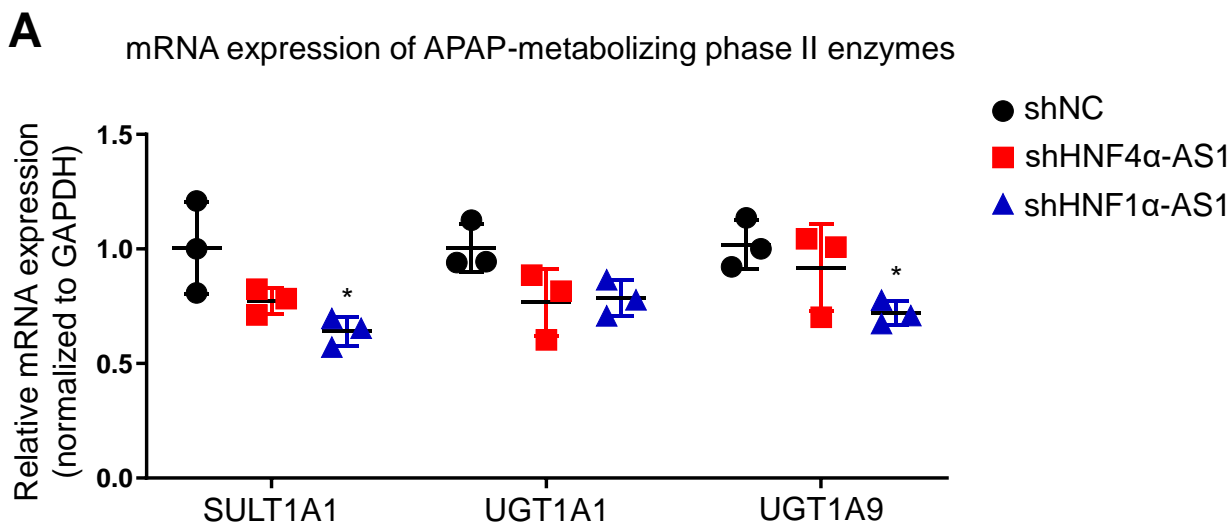
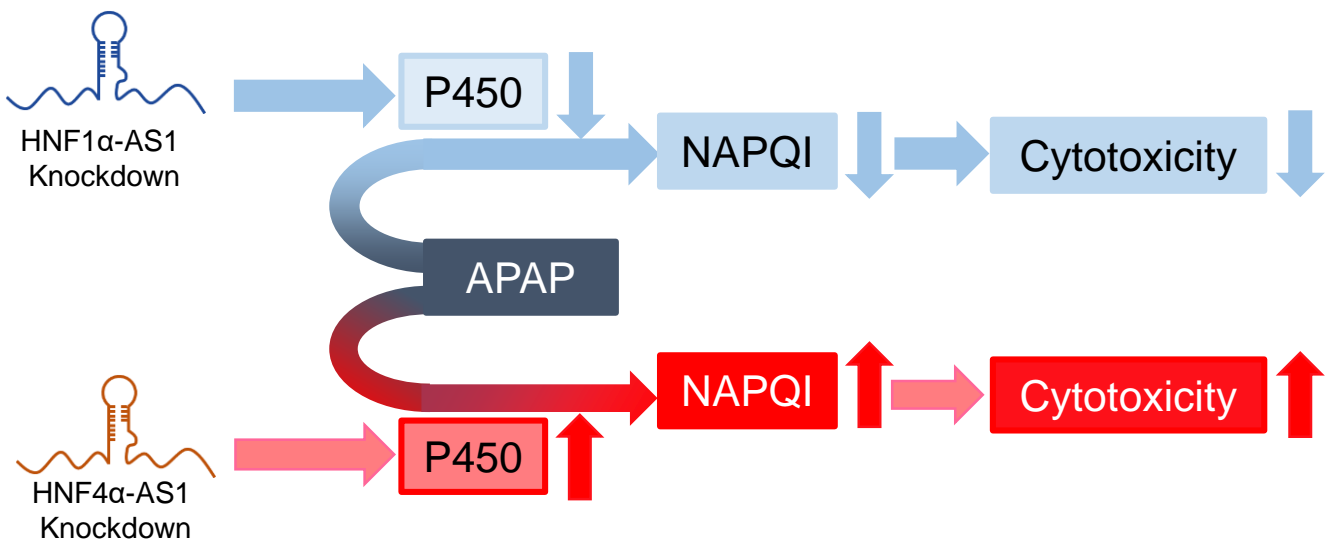


Fig. 7



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Knockdown of LncRNAs HNF1 α -AS1 and HNF4 α -AS1 Alters Susceptibility of Acetaminophen-induced Cytotoxicity in HepaRG Cells

Supplemental Table S1. List of primers used for real time PCR.

SYBR Green primer

Gene	Forward sequence	Reverse sequence
GAPDH	5'-GGGAGCCAAAAGGGTCAT-3'	5'-GAGTCCTTCCACGATACCAA-3'
HNF1 α -AS1	5'-TCAAGAAATGGTGGCTAT-3'	5'-GCTCTGAGACTGGCTGAA-3'
CYP1A2	5'-CCAACGTCATTGGTGCCATG-3'	5'-GTGATGTCCCGGACACTGTTC-3'
CYP2E1	5'-AATGGACCTACCTGGAAGGAC-3'	5'-CCTCTGGATCCGGCTCTCATT-3'
CYP3A4	5'-GATTGACTCTCAGAATTCAAAGA AACTGA-3'	5'-GGTGAGTGGCCAGTTCATACATA ATG-3'
SULT1A1	5'-AGGAGTTCATGGACCACAGC-3'	5'-TGAAGGTGGTCTTCCAGTCC-3'
UGT1A1	5'-CCTTGCCTCATCAGAATTCCTTC-3'	5'-ATTGATCCCAAAGAGAAAACCCAC-3'
UGT1A9	5'-ATTCCCAGCTGCTTGCTCTC-3'	5'-AAGCCTTGA ACTCCCGGTC-3'
GSTP1	5'-TCCCAGTGACTGTGTGTTG-3'	5'-GAAGCCCCTTTCTTTGTTCA-3'
GSTT1	5'-TCACCTTGACGGAGAGTGTG-3'	5'-CCAGGTA CT CATCCACACGG-3'

TaqMan™ primer (Life Technologies, Carlsbad, CA)

Gene	Assay ID
GAPDH	Hs99999905_m1
HNF4 α -AS1	Hs01378672_m1