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Title Page

Liver Receptor Homolog 1 (LRH-1) Regulates Organic Anion Transporter 2(OAT2) and Docetaxel Pharmacokinetics

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Running Title Page

Running Title: LRH-1 regulation of OAT2 and Pharmacokinetics

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Non-standard abbreviations:

Oat2/OAT2, mouse/human organic anion transporter 2; Lrh-1/LRH-1, mouse/human liver receptor homolog-1; PGE2, Prostaglandin E2; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; siRNA, short interfering RNA; siNC, short interfering RNA for negative control; siLrh-1, short interfering RNA for Lrh-1; EMSA, Electrophoretic mobility shift assay; ChIP, Chromatin immunoprecipitation assay; UPLC-QTOF/MS, Ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry.

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Abstract

Organic anion transporter 2 (OAT2/SLC22A7) is an uptake transporter that plays an important role in drug disposition. Here, we investigate a potential role of liver receptor homolog-1 (Lrh-1) in regulation of Oat2 and docetaxel pharmacokinetics. Hepatoma cells (Hepa1-6 and HepG2 cells) were transfected with Lrh-1/LRH-1 expression vector or siRNA. The relative mRNA and protein levels of Oat2/OAT2 in the cells or livers of Lrh-1$^{hep-/-}$ mice were determined by qPCR and Western blotting, respectively. Transcriptional regulation of Oat2/OAT2 by Lrh-1/LRH-1 was investigated using luciferase reporter, mobility shift, and chromatin immunoprecipitation (ChIP) assays. Pharmacokinetic studies were performed with wild-type (Lrh-1$^{fl/fl}$) and Lrh-1$^{hep-/-}$ mice after intraperitoneal injection of docetaxel. Overexpression of Lrh-1 in Hepa1-6 cells led to significant increases in Oat2 mRNA and protein. Consistently, Lrh-1 knockdown caused decreases in Oat2 mRNA and protein, as well as reduced cellular uptake of PGE2, a prototypical substrate of Oat2. Similarly, an activation effect of LRH-1 on OAT2 expression was observed in HepG2 cells. In addition, the levels of Oat2 mRNA and protein were markedly reduced in Lrh-1$^{hep-/-}$ mice. Lrh-1/LRH-1 induced the transcription of Oat2/OAT2 in luciferase reporter assays. Truncation analysis revealed a potential Lrh-1 response element (-716- to -702-bp) in Oat2 promoter. Direct binding of Lrh-1 to this response element was confirmed by mobility shift and ChIP assays. Furthermore, systemic exposure of docetaxel was up-regulated in Lrh-1$^{hep-/-}$ mice due to reduced hepatic uptake. In conclusion, Lrh-1 transcriptionally regulates Oat2, thereby impacting tissue uptake and pharmacokinetics of Oat2 substrates.

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Introduction

Docetaxel is a chemotherapeutic agent approved for the treatment of various types of cancers including breast cancer, non-small cell lung cancer, squamous cell carcinoma of the head and neck, gastric adenocarcinoma, and androgen-independent metastatic prostate cancer (Connolly et al., 2011). The pharmacokinetic profile of docetaxel is subjected to a large inter-individual variability, with up to 10-fold differences in drug clearance even in patients with normal hepatic function (Baker et al., 2006). The causes of this variability are probably multifactorial, including the factors affecting transport and metabolism of docetaxel. The membrane transporter systems have been shown to modulate the pharmacokinetics of docetaxel (Shirakawa et al., 1999; Hopper-Borge et al., 2004; Ehrlichova et al., 2005; de Graan et al., 2012). In particular, hepatic uptake of docetaxel is mediated by several members of solute carrier (SLC) family transporters including the organic anion transporter 2 (OAT2) (Franke et al., 2010; de Graan et al., 2012).

OAT2 is an uptake transporter also known as the member 7 of the solute carrier 22A family (SLC22A7). OAT2 is highly expressed in the liver and kidney, thus plays an important role in determining pharmacokinetics and drug efficacy (Sekine et al., 19998; Kobayashi et al., 2005). The substrates of OAT2 include anti-HIV drugs (e.g., acyclovir), anti-cancer drugs (e.g., docetaxel and methotrexate), antibiotics (e.g., erythromycin), and anti-inflammatories (e.g., salicylate) (Baker et al., 2006; Sun et al., 2001; Kobayashi et al., 2005; Burger et al., 2011; Cheng et al., 2012). OAT2 expression is reported to be a critical determinant to individual response to 5-FU based chemotherapy (Nishino et al., 2013). It is also involved in transport of many endogenous

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compounds such as uric acid, creatinine and prostaglandin E2 (PGE2) (Anzai et al., 2006; Sato et al., 2010; Shen et al., 2015). Genetic deficiency of OAT2 has been associated with hyperuricemia disorders (Anzai et al., 2007).

Liver receptor homolog-1 (LRH-1/NR5A2) is a nuclear receptor significantly expressed in various tissues including the drug-eliminating organs liver, kidney, and intestine (Fayard et al., 2004). LRH-1 receptor plays a critical role in regulation of metabolic enzymes and transporters (Kobayashi et al., 2005). For instance, LRH-1 promotes the expression of CYP7A1/CYP8B1 (two bile acid-synthesizing enzymes) and CYP19A1 (a critical enzyme for conversion of androgen to estrogen) (del Castillo-Olivares et al., 2000; Lu et al., 2000; Chand et al., 2011). It activates the transcription of many ABC transporters such as the bile salt export pump (BSEP), multidrug resistance protein 3 (MRP3), and ABCG5/G8 (Freeman et al., 2004). Therefore, LRH-1 is regarded as a key regulator of drug-detoxifying genes.

Due to important roles of OAT2 in biology and pharmacology, the regulatory mechanisms for its expression are of particular interest. Previous studies have shown that hepatocyte nuclear factor-1 alpha (HNF-1α) and HNF-4α stimulate the expression of OAT2 by binding to a DR-1 site in the promoter (Popowski et al., 2005; Maher et al., 2006), whereas several receptors [including farnesoid X receptor (FXR), constitutive androstane receptor (CAR), and pregnane X receptor (PXR)] down-regulate OAT2 via a direct or indirect mechanism (Jigorel et al., 2006; Shen et al., 2015). However, it remains unclear whether and how LRH-1 regulates OAT2 expression.

In this study, we unravel a critical role for Lrh-1 receptor in regulation of Oat2 and docetaxel

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pharmacokinetics. We first showed that Lrh-1/LRH-1 induced the mRNA and protein expression of Oat2/OAT2 in hepatoma cells. Accordingly, down-regulation of Oat2 by Lrh-1 silencing led to significant decreases in cellular uptake of PGE2 and docetaxel, two known substrates of Oat2. Through a combination of promoter analysis, mobility shift, and chromatin immunoprecipitation (ChIP) assays, we demonstrated that Lrh-1 trans-activated Oat2 by its specific binding to -716 to-702 bp region within the gene promoter. Furthermore, conditional deletion of hepatic Lrh-1 in mice (Lrh-$1^{hep-/-}$ mice) led to marked down-regulation of Oat2 and altered docetaxel pharmacokinetics.

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Materials and Methods

Materials

Rabbit polyclonal LRH-1 (AP21181C, 1:1000 dilution) antibody for Western blot analysis was purchased from Abgent (San Diego, CA, USA). Rabbit polyclonal OAT2 (ab186476, 1:1000 dilution) antibody, rabbit monoclonal GAPDH (ab181602, 1:5000 dilution) antibody and mouse monoclonal LRH-1 (ab41901) antibody for ChIP assay were obtained from Abcam (Cambridge, MA). Prostaglandin E2 (PGE2) was purchased from Tocris (Bristol, UK). Docetaxel was obtained from Shanghai TAUTO Biotech (Shanghai, China). The transfection reagent jetPRIME™ was purchased from Polyplus Transfection (Illkirch, France). Dulbecco's modified Eagle's medium (DMEM, high glucose) was purchased from Sigma-Aldrich (St Louis, MO). Fetal bovine serum (FBS) and trypsin were obtained from Hyclone (Logan, UT).

Cell lines and cell culture

Hepa1-6 cells (CRL-1830, ATCC), HepG2 cells (HB-8065, ATCC) and HEK-293T cells (CRL-11268, ATCC) were grown at 37℃ in a humidified atmosphere (5% CO_2) in plastic culture flasks (Falcon 3112, Becton Dickinson). The growth medium was Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone). The medium was changed every 2 days and the culture was split every 5 days.

Animal studies

Lrh-1 floxed mice (kindly gifted from Dr. Bruce Murphy, University of Montreal, Canada) were

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crossed with serum albumin (Alb)-Cre mice (Model Animal Research Center of Nanjing University, China) and then further intercrossed to generate liver-specific Lrh-1 knockout (named Lrh-$1^{hep-/-}$ mice) and wild-type (Lrh-$1^{fl/fl}$) mice on a pure C57BL/6J background. All mice, receiving food and water ad libitum, were housed in a temperature-controlled room with a 12-h light/ 12-h dark cycle. All mice were fasted overnight prior to each study. For the first set of study, wild-type and Lrh-$1^{hep-/-}$ mice (8-10 weeks of age, male, n = 5 per group) were sacrificed, and the livers were isolated, snap-frozen, and stored at -80℃ until processed for mRNA and protein analyses.

For the second set of pharmacokinetic study, two groups of male mice (wild-type and Lrh-$1^{hep-/-}$) were intraperitoneally injected (i.p.) with a single dose of docetaxel (10 mg/kg). Six mice were sacrificed at each time point to collect blood and livers. Blood was centrifuged at 5000 g for 10 min to obtain the plasma sample. The plasma and liver samples were processed for drug quantification as described in our previous publication (Zhang et al., 2017). Drug quantification was performed using the UPLC-QTOF/MS system (Waters, Milford, MA). Pharmacokinetic data were analyzed using the Bailer's approach as previously described (Zhou et al., 2008) with Microsoft Excel Visual Basic. All experimental procedures were approved by Jinan University Institutional Animal Care and Use committee and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Plasmid construction and cell transfection

Mouse Lrh-1 (GenBank accession number: NM_030676.3) and human LRH-1 (GenBank

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accession number: NM_205860.2) gene (full length) were synthesized and cloned into the EcoRI and HindIII sites of the expression vector pcDNA3.1(−) (Invitrogen, Carlsbad, CA). ~2-kb mouse Oat2 proximal promoter (−2000/+89; +1 indicates the transcription initiation site) and human OAT2 proximal promoter (−2000/+28) were synthesized and cloned into the NheI and HindIII sites of the blank pGL4.11 vector (Promega, Madison, WI), respectively. Shorter promoter sequences (−1.1, −0.4, and −0.15kb) were prepared by PCR reactions from the −2000/+89 Oat2 promoter plasmid using primers containing a NheI or a HindIII restriction enzyme site (Table 1). The obtained fragments were cloned into the pGL4.11 plasmid. The siRNAs for Lrh-1 (siLrh-1) and negative control (siNC) were obtained from Transheep (Transheep, Shanghai, China) (siRNA sequences are provided in Table 1). All constructs were verified by DNA sequencing. After transformed into E. Coli JM109 cells, plasmids were isolated and purified using the EasyPure HiPure Plasmid MiniPrep kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Cell transfection was performed using the jetPRIME™ transfection reagent according to the instruction manual.

qPCR Assay

Total RNA from mouse liver samples or cells was isolated using the TRIzol method. cDNAs were synthesized from total RNA with SuperScript II reverse transcriptase (Invitrogen) and random hexamer primers (Roche, Basel, Switzerland). qPCR reactions were performed following our published procedures (Zhang et al., 2017). All primer sequences are summarized in Table 1.

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Luciferase reporter assay

Luciferase reporter assay was performed following our published procedures with minor modifications (Lu et al., 2017). In brief, HEK-293T cells were seeded in 48-well plates (Corning Life Sciences, Acton, MA) at a density of 5×10^4 cells/well. On next day, cells were transfected with 250 ng of Oat2 luciferase (firefly) reporter plasmid, 25 ng of pRL-TK vector (an internal control with renilla luciferase gene), and a fixed amount (125, 250 or 500 ng) of Lrh-1 expression vector. A control experiment was performed in the absence of Lrh-1 expression vector. For Lrh-1 silencing experiments, siRNA plasmid (50nM) was co-transfected with the reporter plasmid. Cell transfection was performed using the jetPRIME™ transfection reagent (Polyplus Transfection, Illkirch, FRANCE). After 24-h transfection, cells were harvested in the passive lysis buffer. The cell lysate was assayed for luciferase activities using the Dual-Luciferase Reporter Assay System and GloMax 20/20 luminometer (Promega). Data were analyzed exactly as previously described (Lu et al., 2017).

Western blotting

Western blotting were performed as previously described (Lu et al., 2017). In brief, mouse liver or whole-cell (40 ug) lysates were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel, followed by transferring to a polyvinylidenefluoride membrane. The membrane was incubated with the primary antibody overnight at 4℃, and then with the secondary antibody for 1 h at room temperature. Proteins were visualized with enhanced chemiluminescence and analyzed by the Quantity One software. Relative protein levels were normalized to Gapdh.

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Electrophoretic mobility shift assay (EMSA) and ChIP assay

EMSA and ChIP assays were performed following our published procedures with minor modifications (Lu et al., 2017). For EMSA, the nuclear extracts of Hepa1-6 cells were incubated with the probes (Table 1). For ChIP, mouse liver was fixed in 1% formaldehyde and digested with micrococcal nuclease. The sheared chromatin was immunoprecipitated overnight with anti-Lrh-1 (Abcam, Cambridge, MA), or normal mouse IgG (control) at 4℃. The purified DNAs were analyzed by qPCR with indicated primers (Table 1).

Cellular uptake study

Hepa1-6 cells were seeded into 6-well plates and cultured in DMEM (10% FBS). 12-h later, cells were transfected with siLrh-1-3 plasmid (50 nM) or an equal amount of siNC. 36-h after transfection, the cells were used for cellular uptake experiments. Cells were incubated with PGE2 (a known endogenous substrate for Oat2) or docetaxel (a known drug substrate for Oat2, at a series of concentrations) dissolved in HBSS solution. At each time point, the incubation solution was aspirated. After washing with ice-cold PBS twice, the cells were disrupted and solubilized with 0.4 ml 50% methanol. After centrifugation (4℃) at 13000 g for 15 min, the supernatant was collected and subjected to UPLC-QTOF/MS analysis.

Quantification of PGE2 and Docetaxel

PGE2 and docetaxel were quantified using an UPLC-QTOF/MS system (Waters, Milford, MA) and a BEH C18 column (2.1 × 50 mm, 2.6 μm; Waters, Milford, MA). The mobile phase was 0.1% formic acid (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The flow rate

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was set at 0.3 ml/min. The gradient elution program was 10% B at 0 to 1 min, 10 to 95% B at 1 to 4.5min, and 95 to 10% B at 4.5 to 5 min. Mass spectrometer was operated at the negative ion full scan mode for PGE2, whereas docetaxel was analyzed at the positive ion mode. The capillary, sampling cone, and extraction cone voltages were set at 3000, 25, and 4 V, respectively. The source and desolvation temperature were 120℃ and 400℃, respectively. Peak areas of PGE2 and docetaxel were recorded with extract masses of *m/z* 351.22 ± 0.05 Da and 808.33±0.05 Da, respectively. The calibration curves for PGE2 and docetaxel were linear ($r^2 > 0.999$) over the entire concentration range (i.e., 3–1000 ng/ml for PGE2 and 10–2000 ng/ml for docetaxel). The limits of detection (LOD, defined as a signal/noise ratio of 3:1) was 30 pg/mL for both PGE2 and docetaxel, and the limit of quantitation (LOQ, defined as a signal/noise ratio of 10:1) was 100 pg/mL for both compounds. Interday/intraday variabilities of PGE2 and docetaxel were less than 5% and 3%, respectively.

Statistical analysis

Data are presented as mean ±SD (standard deviation). Pharmacokinetic parameters were analyzed for statistical significance using the normal hypothesis test (z-test). All other data were analyzed for statistical significance using Student's t test. The level of significance was set at $p < 0.05$ (*).

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Results

Lrh-1/LRH-1 regulates Oat2/OAT2 expression in hepatoma cells

We first generated an expression vector carrying mouse full-length Lrh-1. Transfection of this vector into mouse hepatoma Hepa1-6 cells led to overexpression of Lrh-1 (a 144-fold increase in mRNA and a 2.8-fold increase in protein level) (Figures 1A/B). Interestingly, overexpression of Lrh-1 resulted in a marked increase (2.2-fold, $p < 0.001$) in Oat2 mRNA (Figure 1A). Accordingly, the Oat2 protein level was significantly increased (1.8-fold, $p < 0.001$) (Figure 1B).

Next, we tried to examine the effects of Lrh-1 down-regulation on Oat2 expression. Four different siRNA fragments (i.e.,siLrh-1-1, siLrh-1-2, siLrh-1-3, siLrh-1-4) were designed and synthesized for Lrh-1 (Table 1). Of four siRNAs, siLrh-1-3 was the most efficient one in silencing Lrh-1 (Figure C) thus were used for further experiments. Use of this siRNA caused an effective down-regulation of Lrh-1 expression (an 80% reduction in mRNA and a 47% decrease in protein level) (Figures 1D/E). Consistent with its activation effect on Oat2, Lrh-1 knockdown led a significant reduction in Oat2 mRNA (62%, $p < 0.001$) and protein level (55%, $p < 0.001$) (Figures 1D/E). In addition, silencing of Lrh-1 led to markedly reduced (77%, $p < 0.001$) uptake of PGE2, a prototypical endogenous substrate of Oat2, into Hepa1-6 cells (Figure 1F).

Furthermore, we assessed the regulatory potential of human LRH-1 on OAT2 expression using HepG2 cells. Overexpression of full-length LRH-1 led to a significant increase in mRNA level of OAT2 (Figure 2A). Consistently, mRNA expression of OAT2 was significantly decreased in LRH-1 knockdown cells (Figure 2B). Taken together, these data suggested that Lrh-1/LRH-1

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was a positive regulator of Oat2/OAT2 expression.

Conditional deletion of hepatic Lrh-1 down-regulates Oat2 in mice

We showed relatively high expression of Lrh-1 protein in the livers of wild-type mice (Figure 3A). We further generated liver-specific Lrh-1 knockout mice (named Lrh-$1^{hep-/-}$ mice) by intercrossing Lrh-1 floxed and Alb-Cre mice (Figure 3B). PCR analysis of genomic DNA from the tails of Lrh-$1^{hep-/-}$ mice showed recombination of loxP sites and Alb-Cre recombinase (Figure 3B). We also confirmed the Lrh-1 was absent in the genetic mice (Figure 3C). Hepatic deletion of Lrh-1 led to significant decreases in Oat2 mRNA, and the mRNA level of Cyp8b1, a known LRH-1 target gene (Sablin et al., 2003) (Figure 3C). Consistent with the mRNA change, the Oat2 protein was markedly reduced in the liver (Figure 3D).

Lrh-1/LRH-1 is a transcriptional activator of Oat2/OAT2

An Oat2 luciferase reporter (with -2.0 kb proximal promoter) was constructed to investigate a possible role of Lrh-1 in transcriptional regulation of Oat2. We observed an activation effect of Lrh-1 on Oat2 transcription (Figure 4A). The extent of activation was positively correlated with the transfected amounts of Lrh-1 (Figure 4A). By contrast, knockdown of Lrh-1 resulted in a decreased promoter activity (Figure 4A). These results suggested that Lrh-1 was a transcriptional activator of Oat2.

Sequence analysis of Oat2 promoter using JASPAR algorithm (jaspar.genereg.net) revealed four potential Lrh-1 binding sites (i.e., A, B, C, and D-sites in Figure 4B). Accordingly, four shorter (truncated) promoter constructs (i.e., -1.1, -0.4, -0.15 and 0.03 kb) with the deletion of one or more

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potential binding sites were generated (Figure 4B). Lrh-1 greatly enhanced the transcriptional activities (a 7 to 8-fold increase, $p < 0.001$) of both -2.0 kb and -1.1 kb constructs (Figure 4B). However, transcriptional activation by Lrh-1 was diminished for other promoter constructs (i.e., -0.4, -0.15 and 0.02 kb) (Figure 4B). The data suggested that the core regulatory element responsible for Oat2 promoter activity was located between −1.1 and −0.4 kb (i.e., the B-site) (Figure 4B). In a similar manner, an LRH-1 response element (at position -746) within the human OAT2 promoter was identified (Figure 4C).

Next, EMSA assay was performed to confirm binding of Lrh-1 to the B-site (-716/-702 bp) using a biotin-labeled dimerized oligonucleotide (i.e., −722 to −696 bp of Oat2 promoter). The Lrh-1 binding site of Cyp7a1 (Cyp7a1-LrhRE, the sequence is provided in Table 1) was used in a control experiment. As expected, Cyp7a1-LrhRE generated a DNA-protein complex that was diminished upon addition of unlabeled probe (Figure 5A). Interestingly, the Oat2 probe was able to form a complex with Lrh-1 protein (Figure 5A). Formation of this complex was inhibited by unlabeled probe but unaffected in the presence of mutated probe (the sequence is provided in Table 1) (Figure 5A). The results strongly indicated that Lrh-1 protein bound directly to the B-site of Oat2 promoter. To confirm the interaction of Lrh-1 with Oat2 promoter *in vivo*, ChIP assays were performed using mouse liver samples. We observed significant recruitment of Lrh-1 to the B-site (Figure 5B). Overall, these data indicated that Lrh-1 activated the transcription of Oat2 through its specific binding to the B-site (i.e., the -716- to -702-bp region).

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Lrh-1 knockdown led to reduced uptake of docetaxel to Hepa1-6 cells

Docetaxel is a known substrate of Oat2 (Baker et al., 2009). Knockdown of Lrh-1 by siRNA led to a significant decrease in uptake of docetaxel (20 μM) into Hepa1-6 cells over a 2 h period (Figure 6A). Reduced cellular uptake of docetaxel was also observed at a wide range of dosing concentrations (10-80 μM) (Figure 6B).

Hepatic deletion of Lrh-1 alters docetaxel pharmacokinetics in mice

Pharmacokinetic studies were performed with wild-type (Lrh-$1^{fl/fl}$) and Lrh-$1^{hep-/-}$ mice after intraperitoneal injection of docetaxel (10mg/kg). The plasma docetaxel concentration versus time curve was significantly altered in Lrh-$1^{hep-/-}$ mice (Figure 7A). Lrh-$1^{hep-/-}$ mice showed increased C_{max} and AUC (i.e., the area under the curve, representing the systemic exposure of docetaxel) values (Table 2). By contrast, the liver docetaxel concentrations were lowered in Lrh-$1^{hep-/-}$ mice at the time points of ≤ 2 h (Figure 7B). Accordingly, the AUC value for the liver concentration-time profile was significantly decreased (Table 2). These data indicated that Lrh-1 regulated docetaxel pharmacokinetics via modulation of Oat2-mediated hepatic uptake of the drug (Figure 7C).

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Discussion

This study for the first time demonstrated that the nuclear receptor Lrh-1/LRH-1 regulated Oat2/OAT2 expression in mice *in vivo* and in mouse and human hepatoma cells. Through a combination of luciferase reporter, mobility shift, and ChIP assays, it was shown that Lrh-1/LRH-1 trans-activated Oat2/OAT2 through its direct binding to an Lrh-1/LRH-1 response element (-716- to -702-bp for mice and -746 to -732-bp for humans) within the gene promoter (Figures 4 & 5). Additionally, genetic deletion of hepatic Lrh-1 altered the pharmacokinetics of docetaxel, an Oat2 substrate, in mice. Alteration in docetaxel pharmacokinetics resulted from reduced drug uptake to the liver due to down-regulated Oat2 expression as the expressions of Oatp transporters (also participate in hepatic uptake of docetaxel, Sprowl and Sparreboom, 2014) were unaffected (Supplementary Figure 1). Collectively, Lrh-1 transcriptionally regulated Oat2, thereby impacting tissue uptake and pharmacokinetics of Oat2 substrates (Figure 7C).

In addition to the chemotherapeutic agents such as docetaxel, the OAT2 transporter participates in the excretion of many other types of drugs including non-steroidal anti-inflammatory drugs, angiotensin-converting enzyme inhibitors, and antibiotics (Russel et al., 2002; Marada et al., 2015; Cutler et al., 2011). Genetic deficiency of OAT2 has been linked to the alterations (e.g., reduced urinary excretion and higher level of circulating drug) in disposition of these drugs (Russel et al., 2002). Therefore, identification of LRH-1 as a novel pharmacokinetic determinant of OAT2 substrates assume great importance because this knowledge will facilitate a

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better understanding of varied pharmacokinetics and possibly pharmacodynamics of OAT2 substrates.

The finding that Oat2 was transcriptionally activated by Lrh-1 was consistent with previous microarray data which show hepatic Oat2 is markedly reduced in Lrh-1 deficient mice (Stein et al., 2014). Moreover, expression control of an uptake transporter such as Oat2 by Lrh-1 suggested an important role of this nuclear receptor in drug absorption and disposition. This was evidenced by the fact that down-regulated Lrh-1 was associated with decreased cellular uptake of PGE2 and docetaxel as well as altered docetaxel pharmacokinetics (Figures 1F, 6 & 7). Therefore, Lrh-1 expression may be a contributing factor to individual variations in pharmacokinetics /efficacy of Oat2 substrates. On the other hand, a growing number of Lrh-1 ligands are being identified (Lee et al., 2011; Whitby et al., 2011; Benod et al., 2013). Ligand modulation of LRH-1 activity would also alter the transport and disposition of OAT2 substrates, thereby accounting for drug-drug interactions.

Our data suggested a potential role of LRH-1 in maintenance of body homeostasis because its target gene OAT2 is known to transport many endogenous substances (e.g., glutamate, uric acid, creatinine, and PGE2) with important physiological functions (Shen et al., 2015; Xu et al., 2016). This is also supported by the fact that Lrh-1$^{hep-/-}$ mice show a marked decrease in hepatic glutamate content compared with Lrh-1$^{hep+/+}$ mice (Xu et al., 2016). Therefore, LRH-1 may be targeted to modulate the levels of endogenous substances for prevention and treatment of certain diseases. For

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instance, modulation of LRH-1 activity may change the glutamate level that would be exploited to alleviate glutamate excitotoxicity in the central nervous system. Also, the serum level of uric acid may be modified via LRH-1 activation to manage various diseases such as gout, hypertension, and cardiovascular diseases (Sato et al., 2010).

LRH-1 has been reported to serve as a competence factor that enhances the regulatory effects of other nuclear receptors (Lu et al., 2000; Luo et al., 2001). We have tried to determine whether Lrh-1 acts as a competence factor for HNF4α because HNF4α is a known transcriptional activator of OAT2 (Popowski et al., 2005). Interestingly, Lrh-1 and Hnf4α showed an additive effect in the induction of Oat2 promoter activity (Supplementary Figure 2), suggesting independent activation of Oat2 transcription. On the other hand, the binding site in Oat2 promoter for Lrh-1 is located at -746/-733 bp, whereas Hnf4α binds to a DR-1 site (329/-317 bp). As for Oat2, the Lrh-1 binding site does not occur in conjunction with Hnf4α binding site, supporting independent regulation of Oat2 by Lrh-1 and Hnf4α. Therefore, in addition to acting as a competence factor, LRH-1 can independently regulate gene expression.

It was noteworthy that we were unable to determine the kinetic parameters for uptake transport of docetaxel into Hepa1-6 cells (Figure 6B). This was because the rates of transport were nearly linear to the test concentrations (10-80 μM). Attempts to obtaining transport rates at high concentrations of more than 100 μM failed due to limited aqueous drug solubility (about 100 μM). Nevertheless, pairwise comparisons of cellular transport at different time points and different drug

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concentrations consistently indicated Lrh-1-dependent uptake of docetaxel (Figure 6).

This study focused on the assessment of the impact of hepatic Lrh-1 on drug pharmacokinetics because this protein is abundantly expressed in the liver and the liver is the major drug-eliminating organ (Figure 3A). However, since both Lrh-1 and Oat2 is also expressed in the kidney and intestine, the other two major drug-eliminating organs (Figure 3) (Kusuhara and Sugiyama, 2002), there is a high possibility that Lrh-1 will have impact on renal and/or intestinal disposition of Oat2 substrates. Nevertheless, whether renal and intestinal Lrh-1 regulate Oat2 expression and pharmacokinetics warrants further investigations.

We provided *in vitro* and *in vivo* evidence that mouse Lrh-1 trans-activates hepatic Oat2 to alter drug pharmacokinetics. By contrast, relatively limited data (i.e., those generated from HepG2 cells and luciferase promoter assays; Figures 2 & 4) are available here supporting the regulation of human OAT2 by LRH-1. It was acknowledged that the specific DNA region (-746 to -732-bp) for LRH-1 binding to human OAT2 needs a further validation with mobility shift and/or ChIP assays (Figure 4). Nevertheless, the ultimate question whether LRH-1 regulates OAT2 and drug pharmacokinetics in humans as its counterpart did in mice awaits further explorations.

In summary, Lrh-1/LRH-1 up-regulated Oat2/OAT2 at the mRNA, protein and activity levels in hepatoma cells. Consistently, hepatic deletion of Lrh-1 down-regulates Oat2 expression and hepatic docetaxel uptake, but increased the system exposure of docetaxel. Through a combination of luciferase reporter, mobility shift, and ChIP assays, we showed that Lrh-1 trans-

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activated Oat2 by its specific binding to -716/-702 bp promoter region. Taken together, Lrh-1 transcriptionally regulates Oat2, thereby impacting tissue uptake and pharmacokinetics of Oat2 substrates.

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Author Contributions

Participated in research design: Yu, Zhang and Wu.

Conducted experiments: Yu, Zhang and Guo.

Performed data analysis: Yu, Zhang and Wu.

Wrote the manuscript: Yu and Wu.

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Footnotes

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FY and TZ contributed equally to this work.

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Legends for Figures

Figure 1 **Lrh-1 regulates Oat2 expression and activity in Hepa1-6 cells.** Hepa1-6 cells were seeded onto 6-well plates for 12-h, and then transfected with 2 μg Lrh-1 expression plasmid or 50 nM siLrh-1. After 24-h transfection, Oat2 mRNA and protein was quantified by qPCR and Western blotting, respectively. **(A)** Effects of Lrh-1 overexpression on Oat2 mRNA. **(B)** Effects of Lrh-1 overexpression on Oat2 protein. **(C)** Lrh-1 gene silencing efficiency of four siRNA. **(D)** Effects of Lrh-1 knockdown on Oat2 mRNA. **(E)** Effects of Lrh-1 knockdown on Oat2 protein. **(F)** Effects of Lrh-1 knockdown on cellular uptake of PGE2 at different time points. After 36-h transfection of siLrh-1, the cells were incubated with HBSS solution containing 5 μM PGE2 for 0.5, 1 or 2 h. Intracellular levels of PGE2 were quantified by UPLC-QTOF/MS. Data are presented as mean ± SD (n = 6). *p < 0.05 (t test).

Figure 2 **LRH-1 regulates OAT2 expression in HepG2 cells. (A)** Effects of LRH-1 overexpression on OAT2 mRNA. HepG2 cells were seeded onto 6-well plates for 12-h, and then transfected with 2 μg LRH-1 expression vector or equal amount of pcDNA3.1 vector. After 24-h transfection, OAT2 mRNA was quantified by qPCR from total RNA. **(B)** Effects of LRH-1 knockdown on OAT2 mRNA. HepG2 cells were seeded onto 6-well plates for 12-h, and then transfected with 50 nM siLRH-1 or equal amount of siNC. After 24-h transfection, OAT2 mRNA was quantified by qPCR from total RNA. Data are presented as mean ± SD (n = 6). *p < 0.05 (t test).

Figure 3 **Hepatic deletion of Lrh-1 down-regulates Oat2 expression in mice. (A)** Relative protein levels of Lrh-1 in various tissues of wild-type (Lrh-$1^{flox/flox}$) mice. **(B)** PCR analysis of genomic DNA from the tails of wild-type (WT) and Lrh-$1^{hep-/-}$ mice. The sizes of PCR products corresponding the flox and Alb-Cre are indicated. **(C)** qPCR analysis of Oat2 in the livers from WT and Lrh-$1^{hep-/-}$ mice. **(D)** Western blotting of Oat2 in the livers from WT and Lrh-$1^{hep-/-}$ mice. Data are presented as mean ± SD (n = 5). *p < 0.05 (t test).

Figure 4 **Lrh-1/LRH-1 is a transcriptional activator of Oat2/OAT2.** HEK293T cells were

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co-transfected with Lrh-1 expression plasmid or with siLrh-1 for 24-h. **(A)** Effects of Lrh-1 overexpression or knockdown on transcriptional activity of Oat2 promoter. **(B)** Lrh-1 stimulates mouse Oat2 promoter activity by binding to the -716/-702 region (B-site). **(C)** LRH-1 stimulates human OAT2 promoter activity by binding to the -746/-732 region (B-site). Potential Lrh-1/LRH-1 binding elements in Oat2/OAT2 promoter region were predicted from JASPAR algorithm (jaspar.genereg.net). Capitals indicate the putative binding motifs for Lrh-1/LRH-1. Data are mean ± SD (n =6). *p < 0.05 (t test).

Figure 5 **Interactions of Lrh-1 with its response element (B-site) in the Oat2 promoter. (A)** EMSA assay showing formation of a DNA-protein complex between Lrh-1 and the Oat2 B site. EMSA was performed with labeled Oat2 probe or labeled Cyp7a1 probe in the presence of nuclear extracts from Hepa1-6 cells. Competitive EMSAs on labeled Oat2 probe and labeled Cyp7a1 probe were performed by adding 50-fold molar excess of indicated mutant consensus Oat2 or Cyp7a1 oligonucleotides. **(B)** ChIP assay showing recruitment of Lrh-1 to Oat2 promoter. ChIP assay was performed using an anti-LRH-1 antibody, and qPCR was performed using primers specific for Oat2 or Cyp7a1. The promoter region surrounding -716/-702 and the primer sequences for the ChIP-PCR are provided in Table 1. Data are mean ± SD (n =6). *p < 0.05 (t test).

Figure 6 **Lrh-1 knockdown led to reduced uptake of docetaxel into Hepa1-6 cells.** Hepa1-6 cells were seeded onto 6-well for 12-h, and then transfected with 50 nM siLrh-1 or equal amount of siNC. After 36-h transfection, the cells were incubated with docetaxel. **(A)** Effects of Lrh-1 knockdown on uptake of docetaxel at different time points. (B) Effects of Lrh-1 knockdown on uptake of docetaxel with different dosing concentrations. Data are mean ± SD (n =6). *p < 0.05 (t test).

Figure 7 **Hepatic deletion of Lrh-1 alters docetaxel pharmacokinetics in mice. (A)** Plasma concentrations of docetaxel in wild-type (Lrh-$1^{flox/flox}$) and Lrh-$1^{hep-/-}$ mice at different time points after docetaxel treatment (10 mg/kg, i.p., n = 6). **(B)** Liver concentrations of docetaxel in Lrh-$1^{flox/flox}$ and Lrh-$1^{hep-/-}$ mice at different time points after docetaxel treatment (10 mg/kg, i.p., n = 6). **(C)** Proposed regulatory model elucidating a critical role of LRH-1 in regulation of OAT2 expression and docetaxel pharmacokinetics.

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Table 1. Oligonucleotides used in this study

	forward (5'to 3')	reverse (5'to 3')
qRT-PCR		
hLRH-1	CTTTGTCCCGTGTGTGGAGAT	GTCGGCCCTTACAGCTTCTA
hOAT2	AGCCTCCGTCAGCTATGTAAT	CATCGCCAGTCCCGTATCA
hGAPDH	CATGAGAAGTATGACAACAGCCT	AGTCCTTCCACGATACCAAAGT
mLrh-1	GAACTGTCCAAAACCAAAAAAGG	CTTCCAGCTTCATCCCAAC
mOat2	CAACTGCGGAATCTGGTGCT	ATCAGGCAGGGCACAATGATG
mOatp1b2	CCTGAGAAGTGTCCGCATAAC	GTCCGAGTGGCAGTAAGAAAG
mOatp1a1	GTGCATACCTAGCCAAATCACT	CCAGGCCCATAACCACACATC
mOatp1a4	GCTTTTCCAAGATCAAGGCATTT	CGTGGGGATACCGAATTGTCT
mCyp8b1	GCCTTCAAGTATGATCGGTTCCT	GATCTTCTTGCCCGACTTGTAGA
mPpib	TCCACACCCTTTTCCGGTCC	CAAAAGGAAGACGACGGAGC
Reporter Plasmid		
hOAT2(-1100/+28)	CGCTTTCTCAGGGATCCCAGCCTGGAG	CCTCAAAGCCCATGCTGCTCACCCA
hOAT2(-300/+28)	GGCCCCAGGCCCCAGGGGCCTCCTCTT	CCTCAAAGCCCATGCTGCTCACCCA
hOAT2(-100/+28)	AGACAGGTAGAGGTCCCTGGAGAC	CCTCAAAGCCCATGCTGCTCACCCA
mOat2(-1100/+89)	CCGCTCGAGAATTTTCTCAGAAATGCAAA	CCCAAGCTTAGCCACCCACCTTGTGCAGCA
mOat2(-400/+89)	CCGCTCGAGTGAGTCAAACTGTGCTGTGCTT	CCCAAGCTTAGCCACCCACCTTGTGCAGCA
mOat2(-150/+89)	CCGCTCGAGGGAATGGTGGCCTTTCCCCTTAG	CCCAAGCTTAGCCACCCACCTTGTGCAGCA
EMSA		
Cyp7a1	GGACTTAGTTCAAGGCCAGATAATGCT	AGCATTATCTGGCCTTGAACTAAGTCC
Oat2	TCTTCCGAAGGCCCTGAGTTCCATTCC	GGAATGGAACTCAGGGCCTTCGGAAGA
Oat2(mutant)	TCTTCCACCTGTATGCGACCGCATTCC	GGAATGCGGTCGCATACAGGTGGAAGA
CHIP		
Cyp7a1	GCTTATCGACTATTGCAGCTCTCT	CTGGCCTTGAACTAAGTCCATCT
Oat2	AAAGAGGCAGACTGGACT	GTTGCTGGGAATGGAACT
siRNA		
siLrh-1-1	GCAUGGACUUACAGCUAUUTT	AAUAGCUGUAAGUCCAUGCTT
siLrh-1-2	CCUUGUCAUGCUGCCCAAATT	UUUGGGCAGCAUGACAAGGTT
siLrh-1-3	GCAGAAGAAAGCCCUCAUUTT	AAUGAGGGCUUUCUUCUGCTT
siLrh-1-4	CCAACCCUAUGGUCACUUUTT	AAAGUGACCAUAGGGUUGGTT
siLRH-1	AAGGATCCATCTTCCTGGTTA	TAACCAGGAAGATGGATCCTT

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Table 2. Pharmacokinetic parameters for the docetaxel concentration-time profiles

Parameter	Unit	WT	Lrh-1$^{hep-/-}$
		Plasma	
AUC	μg/ml*h	4.75±0.21	6.81±0.32*
MRT	h	1.86±0.09	1.42±0.02*
V_{ss}	L/kg	3.07±0.44	1.79±0.28*
CL	L/h/kg	2.13±0.30	1.41±0.05*
		Liver	
AUC	μg/ml*h	7.61±0.22	5.98±1.01*

*p<0.05 versus WT group. WT, wild-type (Lrh-1$^{fl/fl}$).

Figure 1

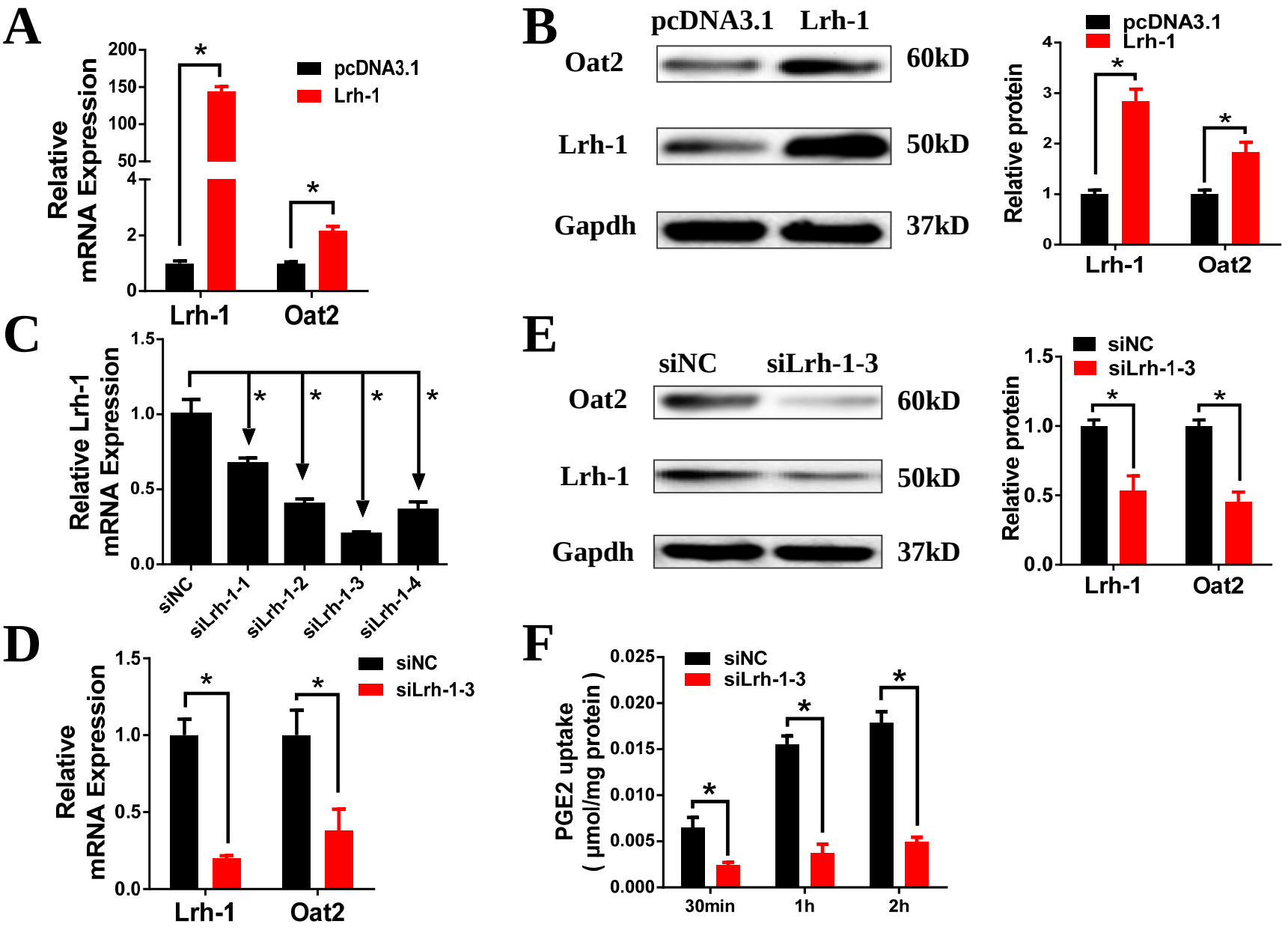


Figure 2

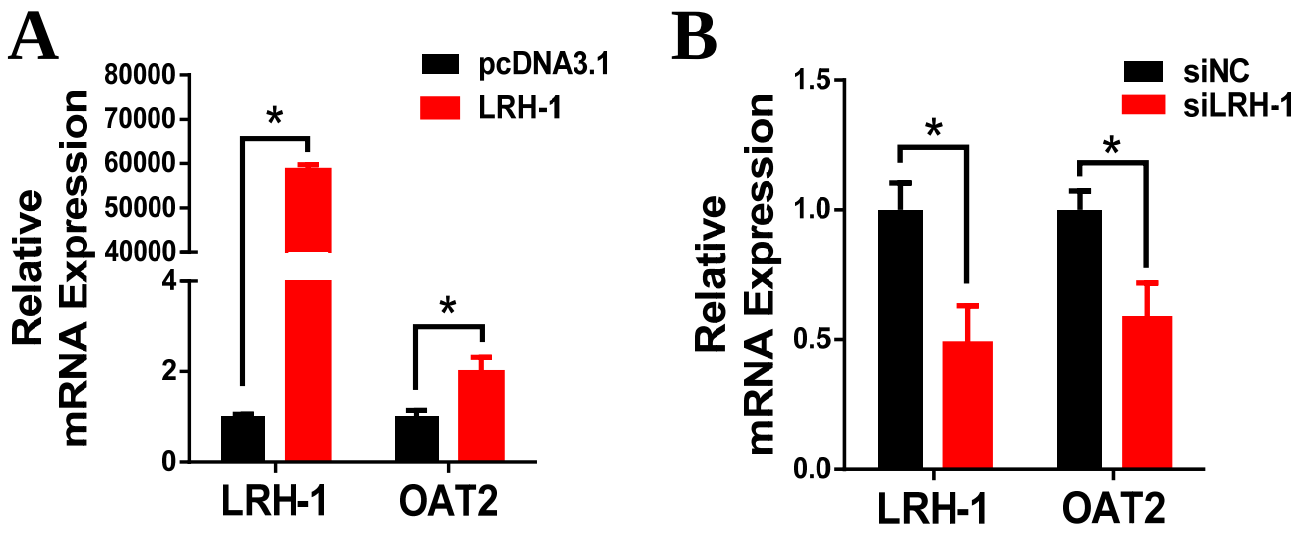
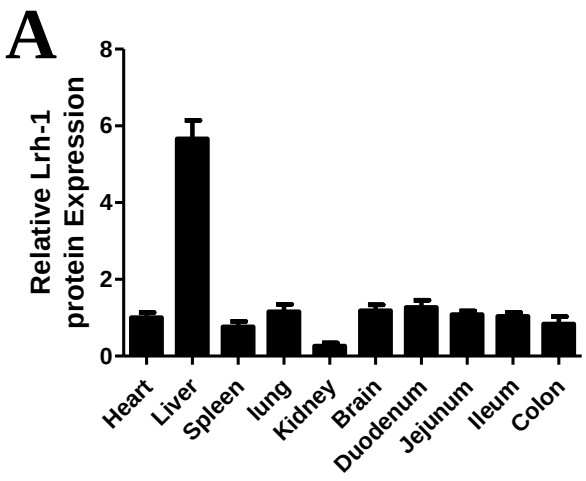
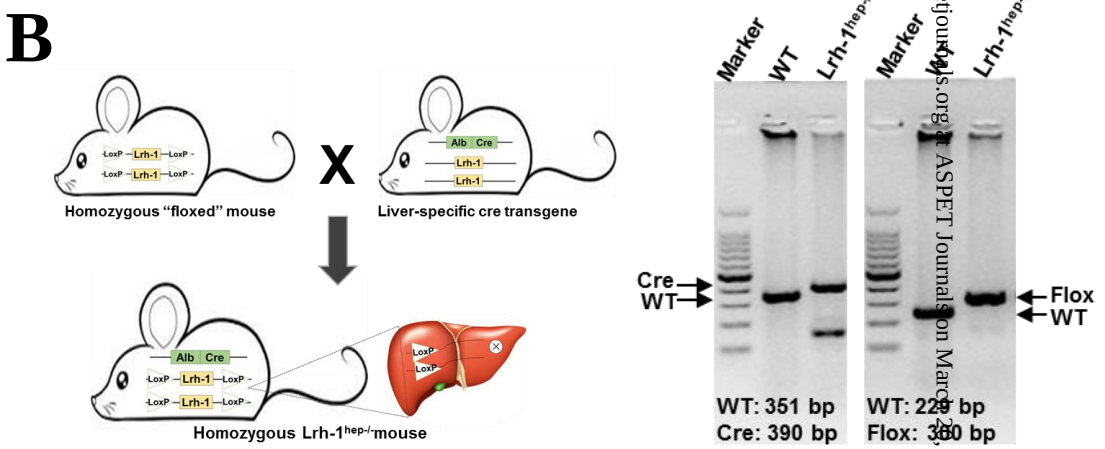
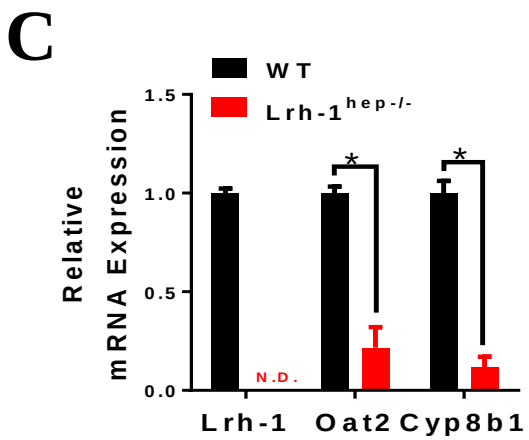


Figure 3







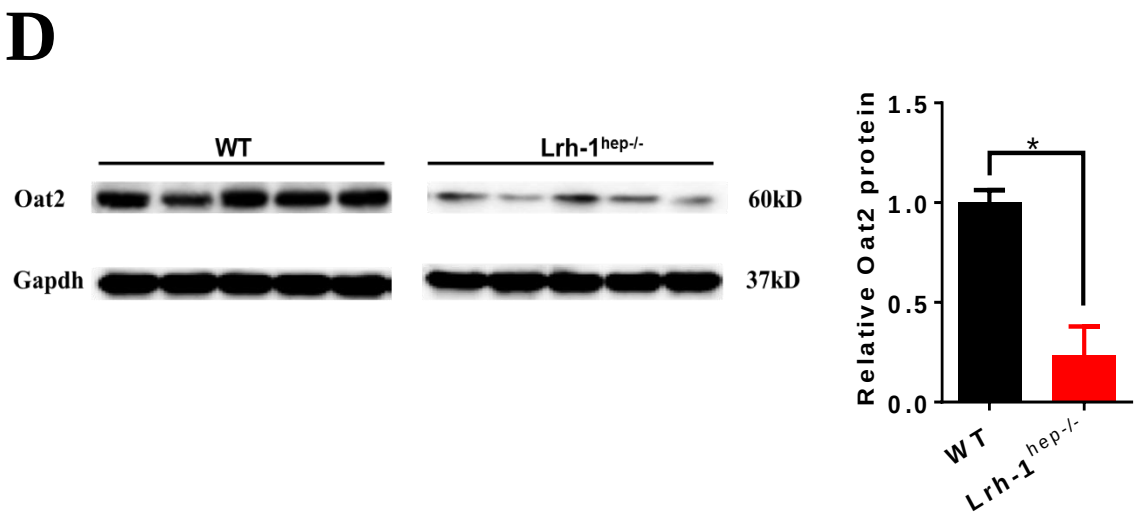


Figure 4

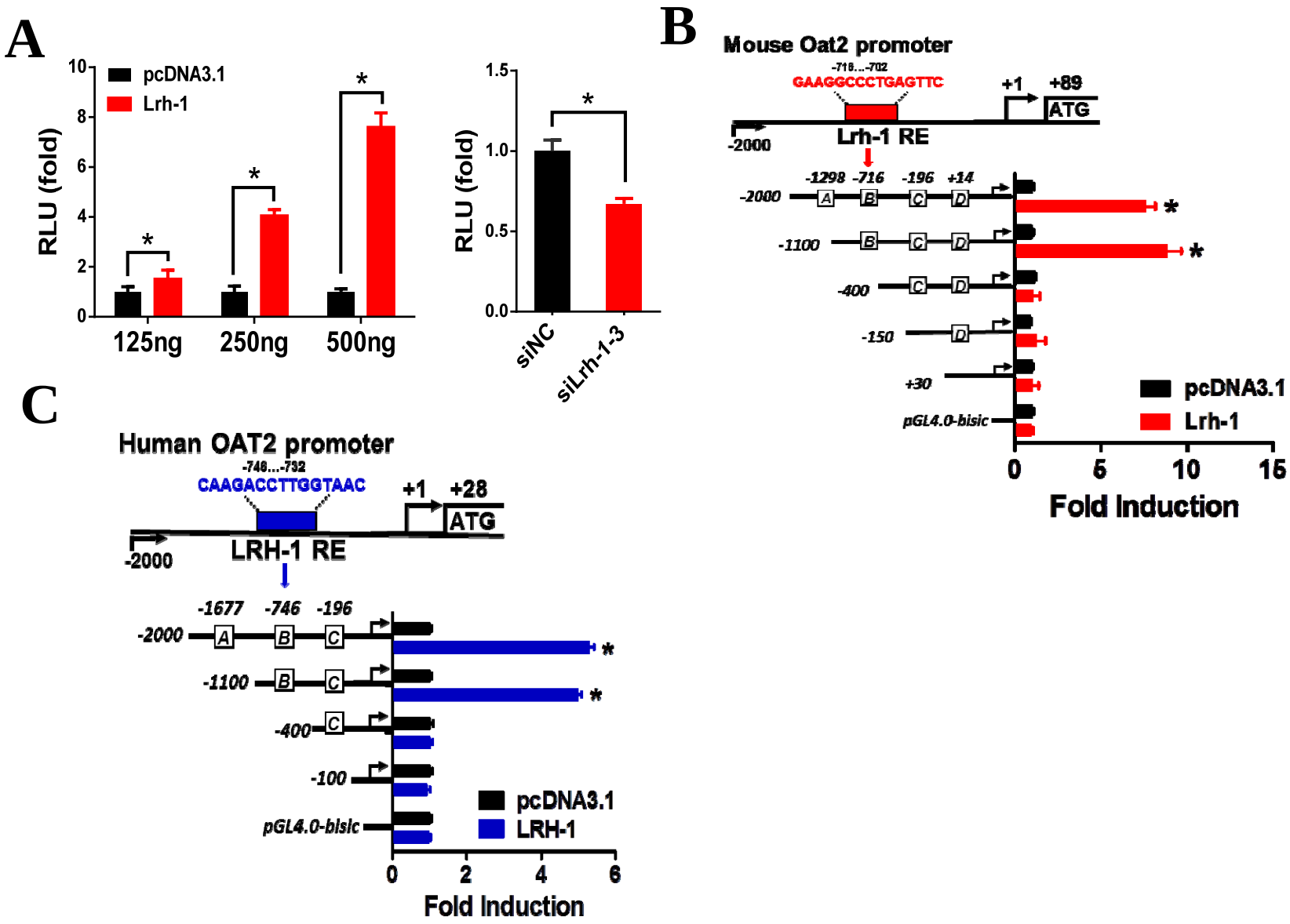


Figure 5

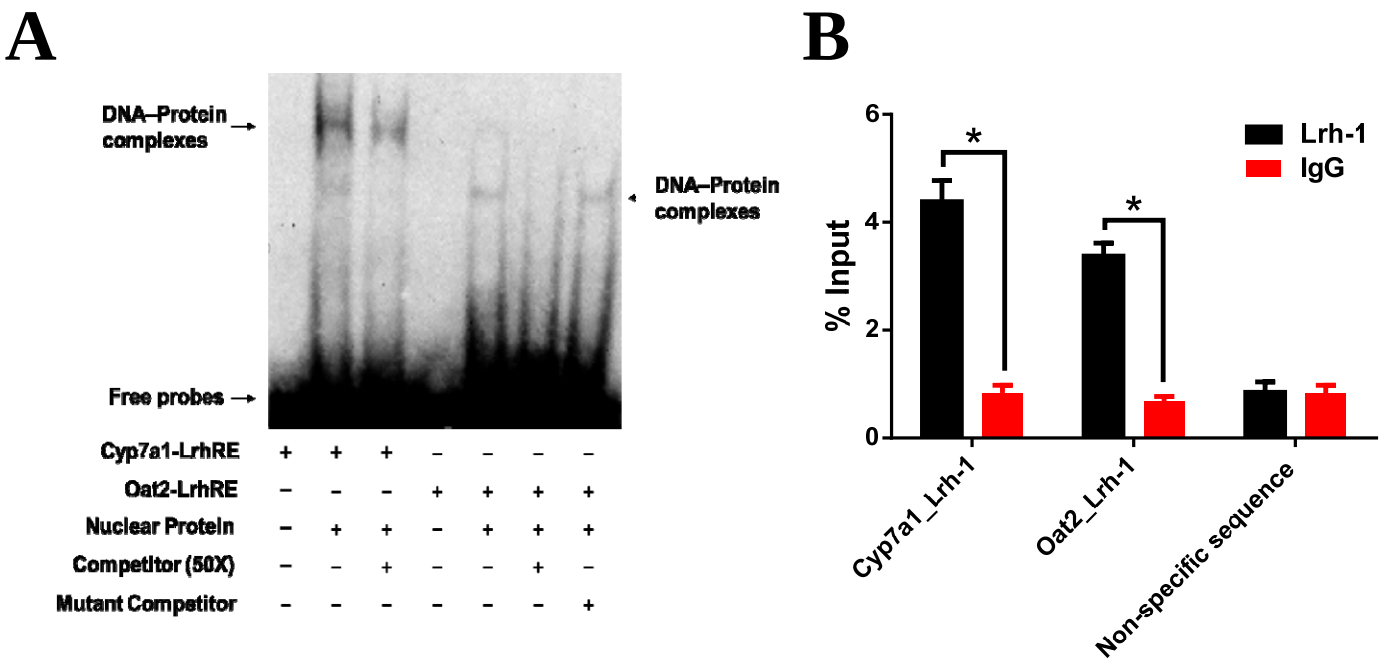


Figure 6

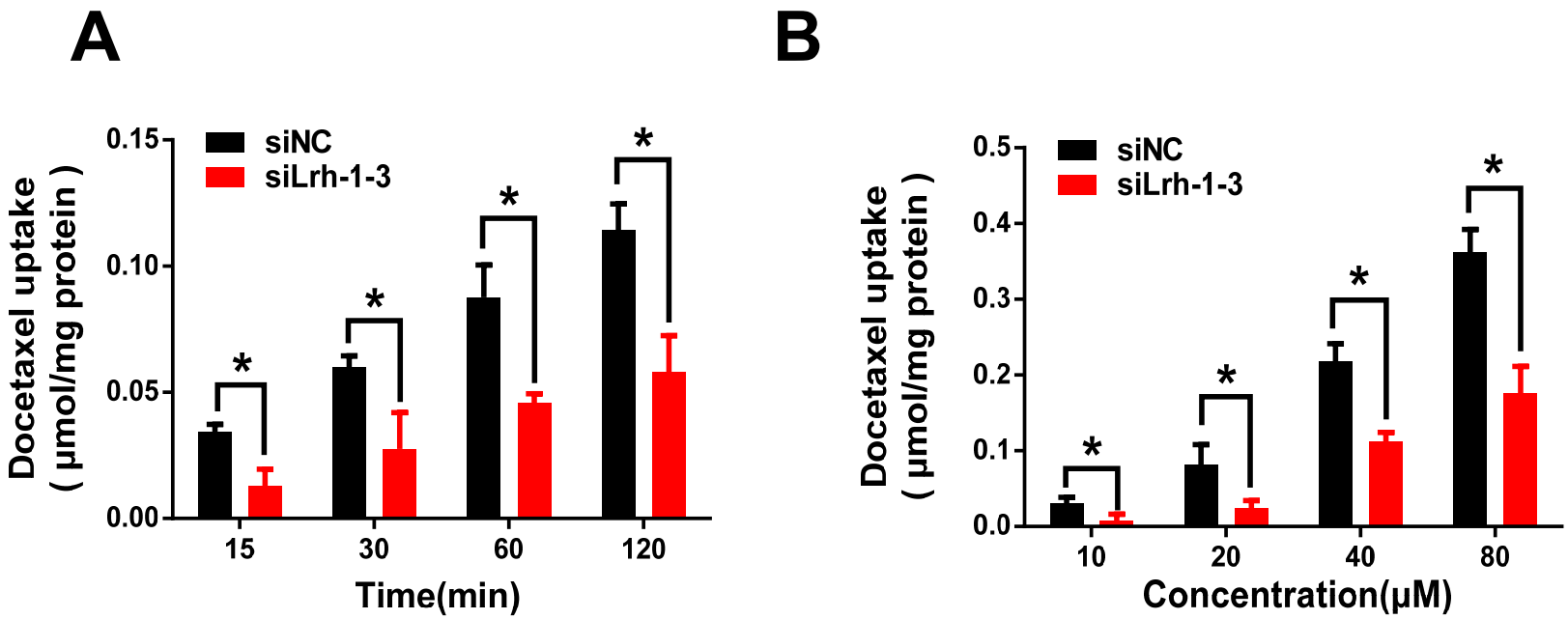


Figure 7