Circadian Clock Component Rev-erb α Regulates Diurnal Rhythm of UDP-Glucuronosyltransferase 1a9 and Drug Glucuronidation in Mice

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ABSTRACT

UDP-glucuronosyltransferases (UGTs) are a family of phase II enzymes that play an important role in metabolism and elimination of numerous endo- and xenobiotics. Here, we aimed to characterize diurnal rhythm of Ugt1a9 in mouse liver and to determine the molecular mechanisms underlying the rhythmicity. Hepatic Ugt1a9 mRNA and protein displayed robust diurnal rhythms in wild-type mice with peak levels at zeitgeber time (ZT) 6. Rhythmicity in Ugt1a9 expression was confirmed using synchronized Hepa-1c1c7 cells. We observed time-varying glucuronidation (ZT6 > ZT18) of propofol, a specific Ugt1a9 substrate, consistent with the diurnal pattern of Ugt1a9 protein. Loss of Rev-erb α (a circadian clock component) downregulated the Ugt1a9 expression and blunted its rhythm in mouse liver. Accordingly, propofol glucuronidation was reduced and its dosing time dependency was lost in Rev-erb α ^{−/−} mice. Dec2 (a transcription factor) was screened to be the potential intermediate that mediated Rev-erb α regulation of Ugt1a9. We confirmed Rev-erb α as a negative regulator of Dec2 in mice and in Hepa-1c1c7

cells. Based on promoter analysis and luciferase reporter assays, it was found that Dec2 *trans*-repressed Ugt1a9 via direct binding to an E-box-like motif in the gene promoter. Additionally, regulation of Ugt1a9 by Rev-erb α was Dec2-dependent. In conclusion, Rev-erb α generates and regulates rhythmic Ugt1a9 through periodical inhibition of Dec2, a transcriptional repressor of Ugt1a9. Our study may have implications for understanding of circadian clock-controlled drug metabolism and of metabolism-based chronotherapeutics.

SIGNIFICANCE STATEMENT

Hepatic Ugt1a9 displays diurnal rhythmicities in expression and glucuronidation activity in mice. It is uncovered that Rev-erb α generates and regulates rhythmic Ugt1a9 through periodical inhibition of Dec2, a transcriptional repressor of Ugt1a9. The findings may have implications for understanding of circadian clock-controlled drug metabolism and of metabolism-based chronotherapeutics.

Introduction

UDP-glucuronosyltransferases (UGTs) are a family of phase II enzymes that catalyze the glucuronidation and detoxification of numerous endogenous (e.g., bilirubin, bile acids, thyroid hormone, and steroid hormones) and exogenous compounds (e.g., drugs, carcinogens, and environmental pollutants) (Strassburg et al., 2008; Sato et al., 2012). In humans, UGTs have been classified into four subfamilies, UGT1, UGT2, UGT3, and UGT8. The UGT1 gene complex locus (located on chromosome 2q37) contains 13 unique alternate first exons followed by four common exons. Each unique first exon and four common exons constitute a *UGT1* gene (Gong et al., 2001). Therefore, there are a total of 13 *UGT1* genes whose protein products (i.e., UGT1 enzymes) are main contributors to xenobiotic glucuronidation (Nakamura et al., 2008). Of

note, UGT1A9 plays an important role in metabolism of many clinical drugs, including propofol, mycophenolic acid, irinotecan, and flavopiridol (an anticancer drug) (Villeneuve et al., 2003; Mikstacki et al., 2017). Based on sequence analysis, mouse Ugt1a9 and human UGT1A9 are highly homologous enzymes that may perform similar or identical functions (Gong et al., 2001; Mackenzie et al., 2005).

Propofol (2,6-diisopropyl-phenol) is a short-acting intravenous anesthetic. Although propofol undergoes multiple metabolic pathways (such as glucuronidation, hydroxylation, and sulfation) in the body, the major route of metabolism appears to be glucuronidation reaction (C1-hydroxyl group is the site of glucuronidation) (Simons et al., 1988; Sneyd et al., 1994). Extensive metabolism and rapid clearance may partially explain why propofol has a short duration of action (Court et al., 2011). UGT1A9 is the principal enzyme responsible for glucuronidation of propofol in the liver, generating the conjugate metabolite propofol glucuronide (Liang et al., 2011). Accordingly, propofol has been widely used as a probe substrate to assay the functional activity of UGT1A9 (Court et al., 2005).

In mammals, most aspects of physiology and behaviors (e.g., the sleep/wake cycle, body temperature, heartbeat, and hormone release) are

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ABBREVIATIONS: Bmal1, brain and muscle Arnt-like protein-1; C/EBP α , CCAAT/enhancer-binding protein α ; Clock, circadian locomotor output cycles kaput; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hnf4 α , hepatocyte nuclear factor 4 α ; propofol-MP, N-methylpyridinium derivative of propofol; qPCR, quantitative polymerase chain reaction; siNC, short interfering RNA for negative control; si Rev-erb α , short interfering RNA for Rev-erb α ; siRNA, short interfering RNA; UGT, UDP-glucuronosyltransferase; UPLC-QTOF/MS, ultraperformance liquid chromatography-quadrupole time-of-flight mass spectrometry; ZT, zeitgeber time.

subjected to circadian rhythms (Reppert and Weaver, 2002; Gachon and Firsov, 2011). Circadian rhythms are generated and regulated by a hierarchical circadian clock system, in which the central clock resides in the suprachiasmatic nucleus of the hypothalamus, and peripheral clocks are present in peripheral tissues (Ralph et al., 1990). At the molecular level, the clock is composed of positive and negative components that constitute autoregulatory feedback loops. The main positive components are circadian locomotor output cycles kaput (Clock) and brain and muscle Arnt-like protein-1 (Bmal1), two basic helix-loop-helix transcription factors (Shearman et al., 2000). Clock and Bmal1 form a heterodimer that drives the transcription of *Per* and *Cry* as well as other circadian genes by binding to the promoter E-boxes (Preitner et al., 2002). Once reaching a high level, *Per* and *Cry* proteins repress the Clock-Bmal1 activity and in turn downregulate the expression of themselves and other circadian genes, thereby generating oscillations in circadian gene expression (Kume et al., 1999). The nuclear heme receptor Rev-erb α is another circadian clock component that regulates circadian rhythms via repressing the transcription and expression of Bmal1 (Preitner et al., 2002). Rev-erb α is also implicated in regulation of metabolic diseases and xenobiotic metabolism (Solt et al., 2012).

Temporal variations in drug metabolism (i.e., the dependence of drug metabolism on dosing time) have long been recognized (Busto and Sellers, 1986). Moreover, circadian metabolism and pharmacokinetics have been shown to elicit a dosing time dependency in toxicity (chronotoxicity) or in efficacy (chronotherapy) for several drugs such as acetaminophen, coumarin, and cyclophosphamide (Ozturk et al., 2017; Zhang et al., 2018a; Zhao et al., 2019). Circadian metabolism thus appears to be an essential part of drug chronotherapeutic studies. Drug-metabolizing enzymes are surely the key determinants of drug metabolism. Temporal oscillations in enzyme expression are thought to be a main source of circadian metabolism and pharmacokinetics (Lu et al., 2020). Therefore, it is of great interest to identify circadian drug-metabolizing genes (whose expression levels oscillate with the time of day) and to clarify the mechanisms underlying their rhythmic expressions (Zhang et al., 2009). In the present study, we uncover *Ugt1a9* as a circadian gene in mouse liver and identify Rev-erb α as a circadian oscillator contributing to *Ugt1a9* rhythm. Mechanistically, Rev-erb α drives rhythmic expression of *Dec2* through a *trans*-repression action. *Dec2* further generates a diurnal rhythm in *Ugt1a9* expression via periodically binding to an E-box-like motif in gene promoter and cyclically repressing gene transcription.

Materials and Methods

Materials. Propofol and propofol glucuronide were purchased from TargetMol (Shanghai, China). Anti-*Ugt1a9* and anti-*Dec2* antibodies for Western blotting were purchased from Bioss (Beijing, China). Uridine diphosphoglucuronic acid, alamechicin, D-saccharic 1,4-lactone monohydrate, 2-fluoro-1-methylpyridinium-p-toluene-sulfonate, triethylamine, and anti-Rev-erb α antibody were purchased from Sigma-Aldrich (St Louis, MO). Rabbit monoclonal glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*; ab181602) antibody was obtained from Abcam (Cambridge, MA). The transfection reagent jetPRIME was purchased from Polyplus Transfection (Illkirch, France). Fetal bovine serum (FBS) and trypsin were obtained from Hyclone (Logan, UT). Short interfering RNA (siRNA) targeting Rev-erb α and *Dec2* (named siRev-erb α and siDec2, respectively; the sequences are provided in Table 1) and siRNA for negative control (siNC) were obtained from Transheep Bio-Tech (Shanghai, China). pRL-TK vector was purchased from Promega (Madison, WI).

Animal Studies. Rev-erb $\alpha^{-/-}$ (C57BL/6 background) mice have been established and validated in our laboratory (Wang et al., 2018). All wild-type and Rev-erb $\alpha^{-/-}$ mice, receiving food and water ad libitum, were housed in a temperature- and humidity-controlled room with a 12-hour light/dark cycle (light on 7:00 AM and off at 7:00 PM). For characterization of diurnal gene expression, liver tissues were collected from wild-type and Rev-erb $\alpha^{-/-}$ mice

(male, 8–12 weeks of age, $n = 5$ per group) at each circadian time point (ZT2, ZT6, ZT10, ZT14, ZT18, and ZT22) and immediately frozen in liquid nitrogen and stored at -80°C until processing for mRNA and protein analyses. Please note that ZT is zeitgeber time in a 12-hour light/dark cycle; ZT0 represents light on and ZT12 represents light off.

For pharmacokinetic experiments, propofol (100 mg/kg) was administered to wild-type and Rev-erb $\alpha^{-/-}$ mice (male, 8–12 weeks of age) by intraperitoneal injection at ZT6 or ZT18. At predetermined time points (5, 30, 60, and 120 minutes), mice ($n = 5$ per time point) were rendered unconscious with isoflurane, and plasma was sampled via a cardiac puncture. The plasma samples were prepared by protein precipitation for ultraperformance liquid chromatography–quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS) analysis as previously described (Liu et al., 2014; Maas et al., 2017). Of note, because of its poor ionization efficiency and significant volatility, detection of propofol by liquid chromatography–mass spectrometry is difficult (Maas et al., 2017). To facilitate propofol analysis, plasma samples were treated with 2-fluoro-1-methylpyridinium-p-toluene-sulfonate (a derivatization agent) and triethylamine (a catalyst) following the published procedures (Maas et al., 2017). This derivatization step converts propofol to its N-methylpyridinium derivative (named propofol-MP) which can be readily and reliably quantified (Thieme et al., 2009; Maas et al., 2017).

Plasmid Construction. Mouse Rev-erb α (GenBank accession number: NM_145434.4), *Dec2* (GenBank accession number: NM_024469.2), and CCAAT/enhancer-binding protein α (*C/EBP α* ; GenBank accession number: NM_001287514.1) genes were synthesized and cloned into the expression vector pcDNA3.1 (Biowit Technologies, Shenzhen, China). *Ugt1a9*-Luc and *Dec2*-Luc (–2000/+100 bp) reporters were prepared and cloned into the pGL4.17 vector (Kaile Bio-Tech, Guangzhou, China). After verification by DNA sequencing, the plasmids were transformed into *Escherichia coli* JM109 and extracted using EasyPure HiPure Plasmid MiniPrep kit (TransGen Biotech, Beijing, China).

Cell Culture and Transfection. Hepa-1c1c7 and HepG2 cells were cultured in RPMI 1640 medium (Gibco, Thermo Fisher Scientific) supplemented with 10% FBS in a 5% CO₂ humidified atmosphere at 37°C. HepG2 and Hepa-1c1c7 cells were seeded in 6-well or 12-well plate. Cells were transfected with overexpression plasmid (Rev-erb α or REV-ERB α plasmid, 1 to 2 μg per well) or siRNA (siRev-erb α or short hairpin RNA targeting REV-ERB α , 25–50 μM) or control (blank pcDNA3.1 for overexpression and siNC or short hairpin RNA for knockdown) using the jetPRIME transfection reagent (Polyplus Transfection). After 24 or 48 hours, cells were collected for quantitative polymerase chain reaction (qPCR) or Western blotting analyses. Twenty-four hours' treatment was sufficient to induce mRNA changes in tested genes, whereas longer treatment time of 48 hours was needed to induce changes in gene expression at the protein level.

Serum Shock Experiments. Serum shock experiments were performed to induce circadian gene expression in cultured Hepa-1c1c7 cells as previously described (Matsunaga et al., 2012). In brief, Hepa-1c1c7 cells were cultured in RPMI 1640 medium (Gibco, Thermo Fisher Scientific) supplemented with 10% FBS in a 35-mm culture dish for 24 hours. This was followed by incubation in serum-free medium for 12 hours. Thereafter, 50% FBS was added for 2 hours, and the culture medium was changed back to serum-free medium. Cells were collected for RNA extraction at 0, 4, 8, 12, 16, 20, and 24 hours after serum shock. The *Ugt1a9* mRNA levels were measured by qPCR assays.

qPCR Assay. Total RNA was extracted from mouse livers or cultured cells using RNAiso Plus reagent (Takara Bio Inc., Shiga, Japan). cDNAs were synthesized from total RNA using a PrimeScript RT Master Mix (Takara Bio Inc.). qPCR reactions were performed with GoTaq qPCR Master Mix (Promega). Amplification procedures have been described in our previous publication (Guo et al., 2018). Mouse *Ppib* (peptidylprolyl isomerase B) or human *GAPDH* gene was used as an internal control. The relative changes in gene expression were determined using the $2^{-\Delta\Delta\text{CT}}$ method. All primer sequences are summarized in Table 1.

Western Blotting. Mouse livers or cells were lysed in radio-immunoprecipitation assay buffer (with 1 mM phenylmethylsulfonyl fluoride). Protein concentrations were determined using a BCA assay kit (Beyotime Biotechnology, China). Protein samples (40 μg) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The membrane was incubated with the primary antibody overnight at 4°C after being blocked with 5% nonfat milk in Tris-buffered saline/Tween 20 for 1 hour and then incubated with the secondary antibody for 1 hour at room

TABLE 1
Oligonucleotides used in this study

	Forward (5'-3' sequence)	Reverse (5'-3' sequence)
<i>qPCR</i>		
<i>mRev-erbα</i>	TTTTTCGCCGGAGCATCCAA	ATCTCGCAAGCATCCGTTG
<i>mDec2</i>	CCCTCATTTGCAAGAGAGACAG	AGGTATCCTTGGTATCGTCTCG
<i>mUgt1a9</i>	CAATCCCTCAGAGCATCA	GCCACTGTCCCTGTCAAA
<i>mPpib</i>	TCCACACCCTTTTCCGGTCC	CAAAAGGAAGACGACGGAGC
<i>hGAPDH</i>	CATGAGAAGTATGACAACAGCCT	TAGTCCTTCCACGATACCAAAGT
<i>hUGT1A9</i>	CTCATATATTCTTGTCTTTTG	GGAAAGCACAAAGTACGAAG
	GTTCCTTTTG	AGTACGAAG
<i>siRNA</i>		
<i>siRev-erbα</i>	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
<i>siDec2</i>	ACGACACCAAGGAUACCUA	UAGGUAUCCUUGGUGUCGU
<i>siNC</i>	CGAUUAGUCUAUACGUUCUCUGAG	CUCAGGAGAACGUAUAGACUAAUCG

h, human; m, mouse; Ppib, peptidylprolyl isomerase B; siDec2, siRNA targeting Dec2.

temperature. Protein bands were visualized with enhanced chemiluminescence using the Omega LumG Imaging System (Aplegen, Pleasanton, CA) and analyzed by the Quantity One software. Relative protein levels were normalized to Gapdh.

Glucuronidation Assay. The livers were collected from the wild-type and *Rev-erb α ^{-/-}* mice at ZT6 and ZT18. Liver microsomes were prepared by sequential ultracentrifugation, first at 9000g for 10 minutes and then at 100,000g for 1 hour (Zhao et al., 2019). The incubation mixture contained liver microsomes (2 mg/ml), magnesium chloride (0.88 mM), saccharolactone (4.4 mM), alamethicin (22 μ g/ml), uridine diphosphoglucuronic acid (3.5 mM), and propofol (100 μ M) in 50 mM potassium phosphate (pH 7.4). After 30 minutes (under a linear condition), the metabolic reaction was terminated by adding 200 μ l ice-cold water/acetonitrile (50:50, v/v) (containing an internal standard). The resulting mixture was centrifuged at 2000g for 10 minutes, and the supernatant was subjected to UPLC-QTOF/MS analysis.

Luciferase Reporter Assay. HepG2 cells were seeded in 48-well plates. After 24 hours, cells were transfected with 200 ng Ugt1a9-Luc (firefly) reporter plasmid, pRL-TK vector (an internal control with renilla luciferase gene, 10 ng), and overexpression plasmid (Dec2, Rev-erb α , or C/EBP α plasmid) or blank pcDNA3.1 (control). After 48 hours, cells were lysed in the passive lysis buffer to determine the luciferase activities using the Dual-Luciferase Reporter Assay System and GloMax 20/20 Luminometer (Promega).

UPLC-QTOF/MS Analysis. Propofol-MP and propofol glucuronide were quantified using an UPLC-QTOF/MS system (Waters, Milford, MA) and a BEH C18 column (2.1 $\times$ 50 mm, 2.6 μ m; Waters). The mobile phase was 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The flow rate was set at 0.3 ml/min. The gradient elution program was 20% B at 0–2 minutes, 20%–70% B at 2–4 minutes, and 70%–20% B at 4–5 minutes. The mass spectrometer was operated at the positive and negative ion full scan mode for propofol-MP and propofol glucuronide, respectively. The capillary, sampling cone, and extraction cone voltages were set at 3000, 30, and 4 V, respectively. The source and desolvation temperature were 100°C and 350°C, respectively. Peak areas of propofol-MP and propofol glucuronide were recorded with exact masses of *m/z* 270.22 $\pm$ 0.05 and 353.16 $\pm$ 0.05 Da, respectively. The calibration curves for propofol-MP and propofol glucuronide were linear ($r^2 > 0.99$) over a wide concentration range (i.e., 0.3906–12.5 μ g/ml for propofol-MP and 0.7813–12.5 μ g/ml for propofol glucuronide). The limit of detection (defined as a signal/noise ratio of 3:1) was 100 pg/ml for both propofol-MP and propofol glucuronide, and the limit of quantitation (defined as a signal/noise ratio of 10:1) was 500 pg/ml for both compounds.

Statistical Analysis. Data are presented as means $\pm$ S.D. Student's *t* test was used to test for statistical differences between two groups. One-way or two-way ANOVA followed by Bonferroni's post hoc test was used for multiple group comparisons. The level of significance was set at $P < 0.05$.

Results

Rev-erb α Ablation Downregulates Ugt1a9 Expression in Mouse Liver. To assess the regulatory effect of Rev-erb α (a clock component

whose expression oscillates greatly according to the time-of-day) on Ugt1a9, we assayed the expression of Ugt1a9 in the livers of *Rev-erb α ^{-/-}* and wild-type mice collected at six different circadian time points. Hepatic *Ugt1a9* mRNA displayed a robust diurnal rhythm in wild-type mice with a peak level at ZT6 (Fig. 1A). Rev-erb α ablation downregulated *Ugt1a9* mRNA and blunted its rhythm in mouse liver (Fig. 1A). Likewise, Ugt1a9 protein was rhythmically expressed (peaking at ZT6) in the liver of wild-type mice, but its expression was reduced (and the rhythm was dampened) in *Rev-erb α ^{-/-}* mice (Fig. 1B). We also observed temporal oscillations in *Ugt1a9* mRNA in synchronized (or serum-shocked) Hepa-1c1c7 cells (Fig. 1C). Cultured cells with a serum shock have been widely used to identify oscillating (rhythmic) genes regulated by circadian clock (Balsalobre et al., 1998). Overall, these data supported Rev-erb α as a circadian regulator of Ugt1a9.

Rev-erb α Ablation Reduces Glucuronidation of Propofol in Mice. We next tested whether downregulation of Ugt1a9 expression by Rev-erb α would result in alterations in drug metabolism (glucuronidation) using propofol as a probe substrate. Glucuronidation of propofol is believed to be mainly catalyzed by Ugt1a9, generating a glucuronidated metabolite called propofol glucuronide (Fig. 2A) (Ethell et al., 2002). According to in vitro metabolism assays, glucuronidation of propofol in wild-type liver microsomes was more extensive at ZT6 than at ZT18, consistent with the diurnal pattern of Ugt1a9 protein (Fig. 2B). However, glucuronidation of propofol at both time points was reduced in liver microsomes of *Rev-erb α ^{-/-}* mice, and the time dependency was lost (Fig. 2B). Furthermore, we determined the metabolism and pharmacokinetics of propofol in wild-type and *Rev-erb α ^{-/-}* mice after drug administration at ZT6 or ZT18. Neither dosing time nor genotype showed an influence on the pharmacokinetic profile of propofol (Fig. 2C; Table 2). In contrast, dosing at ZT6 was associated with more extensive formation of propofol glucuronide in wild-type mice as compared with ZT18 dosing (Fig. 2D; Table 2). Glucuronide formation under two dosing conditions was reduced in *Rev-erb α ^{-/-}* mice, and the time dependency was lost (Fig. 2D; Table 2). Taken together, these data indicated that Rev-erb α regulated circadian metabolism of propofol mediated by Ugt1a9.

Rev-erb α Regulates Ugt1a9 Expression in Hepa-1c1c7 Cells. The regulatory effects of Rev-erb α on Ugt1a9 were additionally assessed using mouse hepatoma Hepa-1c1c7 cells. Overexpression of Rev-erb α led to a significant increase in *Ugt1a9* mRNA (Fig. 3A). Knockdown of Rev-erb α (by siRNA) resulted in a reduction in *Ugt1a9* mRNA (Fig. 3B). In line with the mRNA changes, overexpression and knockdown of Rev-erb α caused an increase and a decrease in Ugt1a9 protein expression, respectively (Fig. 3C). Similarly, positive regulation

Fig. 1. *Rev-erba* ablation downregulates *Ugt1a9* expression in mouse liver. Diurnal rhythms of *Ugt1a9* mRNA (A) and protein (B) in the livers of wild-type and *Rev-erba*^{-/-} mice. (C) Temporal mRNA expression of *Ugt1a9* gene in Hepa-1c1c7 cells after serum treatment. Data are presented as means $\pm$ S.D. ($n = 5$). For (A and B), statistics analysis were performed with two-way ANOVA and Bonferroni post hoc test ($*P < 0.05$ for group comparisons at individual time points). For (C), statistics analysis was performed with one-way ANOVA ($*P < 0.05$). Rel, relative; WT, wild-type.

effects of REV-ERB α on UGT1A9 were observed in human hepatoma HepG2 cells (Fig. 4). All these data confirmed *Rev-erba*/REV-ERB α as a positive regulator of *Ugt1a9*/UGT1A9.

Rev-erba Ablation Upregulates Dec2 Expression in Mouse Liver. Since *Rev-erba* functions as a transcriptional repressor, it was reasoned that a negative regulator (as a mediator) was involved in positive regulation of *Ugt1a9* by *Rev-erba*. E4bp4, Dec2, and Shp are three known transcriptional repressors of drug-metabolizing enzymes and are also known targets of *Rev-erba* (Hamaguchi et al., 2004; Noshiro et al., 2007; Zhang et al., 2018a). We thus tested whether these three repressors regulate *Ugt1a9* transcription. According to luciferase reporter assays, Dec2 significantly inhibited the transcription of *Ugt1a9*, whereas neither E4bp4 nor Shp showed any effects (Fig. 5A). Thereby, Dec2 may be involved in *Rev-erba* regulation of *Ugt1a9*.

We further examined the mRNA and protein expression of Dec2 in the livers of wild-type and *Rev-erba*^{-/-} mice. Hepatic *Dec2* mRNA

exhibited a robust diurnal rhythm in wild-type mice, peaking at ZT6 (Fig. 5B). However, *Dec2* mRNA was elevated and its rhythm was dampened (reflected by a decreased peak-to-valley ratio) in *Rev-erba*^{-/-} mice (Fig. 5B). Likewise, Dec2 protein was rhythmically expressed in the liver of wild-type mice with a peak value at ZT14 (Fig. 5C). *Rev-erba* ablation upregulated the protein level of Dec2 and blunted its rhythm (Fig. 5C). It was noted that diurnal Dec2 protein was phase-shifted about 8 hours relative to its mRNA rhythm (Fig. 5, B and C). This was most likely accounted for by a significant delay in the translation of mRNA to protein product as noted previously (Dong et al., 2010; Narumi et al., 2016). Taken together, these data clearly indicated *Rev-erba* as a negative regulator of Dec2.

Rev-erba Negatively Regulates Dec2 Expression in Hepa-1c1c7 Cells. The regulatory effects of *Rev-erba* on Dec2 were also investigated using Hepa-1c1c7 cells. Overexpression of *Rev-erba* led to a significant decrease in *Dec2* mRNA (Fig. 6A). Knockdown of *Rev-erba*

Fig. 2. *Rev-erba* ablation reduces glucuronidation of propofol in mice. (A) Chemical structures of propofol and propofol glucuronide. (B) Time-dependent glucuronidation of propofol in liver microsomes of wild-type and *Rev-erba*^{-/-} mice. Statistics analysis were performed with two-way ANOVA and Bonferroni post hoc test ($*P < 0.05$). (C) Plasma propofol concentration-time profile in wild-type and *Rev-erba*^{-/-} mice after drug treatment (100 mg/kg, i.p.) at ZT6 and ZT18. (D) Plasma propofol glucuronide concentration-time profile in wild-type and *Rev-erba*^{-/-} mice after drug treatment (100 mg/kg, i.p.) at ZT6 and ZT18. Data are presented as means $\pm$ S.D. ($n = 5$). WT, wild-type.

TABLE 2
Pharmacokinetic parameters for propofol and its metabolite propofol glucuronide

Propofol (100 mg/kg, i.p.) was administered to wild-type and *Rev-erb α ^{-/-}* mice at ZT6 or ZT18.

Parameter	Unit	ZT6		ZT18	
		Wild type	<i>Rev-erbα^{-/-}</i>	Wild type	<i>Rev-erbα^{-/-}</i>
Propofol					
AUC _{0-2 h}	$\mu\text{g/ml}\cdot\text{h}$	2.38 $\pm$ 0.25	2.80 $\pm$ 0.23	2.65 $\pm$ 0.28	2.74 $\pm$ 0.29
MRT	h	1.44 $\pm$ 0.13	1.53 $\pm$ 0.12	1.04 $\pm$ 0.04	1.43 $\pm$ 0.37
V _{ss}	l/kg	21.9 $\pm$ 0.88	24.6 $\pm$ 1.57	17.0 $\pm$ 1.18	26.4 $\pm$ 3.11
CL	l/h per kilogram	16.4 $\pm$ 0.70	12.8 $\pm$ 0.04	16.0 $\pm$ 0.87	15.0 $\pm$ 2.12
Propofol glucuronide					
AUC _{0-2 h}	$\mu\text{g/ml}\cdot\text{h}$	5.13 $\pm$ 0.31	2.86 $\pm$ 0.29*	3.85 $\pm$ 0.18 [#]	2.90 $\pm$ 0.15*
Metabolism ratio ^a		2.35	1.02	1.53	1.17

AUC, area under the curve; CL, clearance; MRT, mean residence time; V_{ss}, steady-state volume of distribution.

^aThe ratio of metabolite to propofol AUC.

* $P < 0.05$ vs. wild-type; [#] $P < 0.05$ vs. ZT6 (wild-type).

(by siRNA) resulted in an elevation in *Dec2* mRNA (Fig. 6B). In line with the mRNA changes, overexpression and knockdown of *Rev-erb α* caused a decrease and an increase in *Dec2* protein expression, respectively (Fig. 6C). Therefore, these cell-based experiments confirmed *Rev-erb α* as a negative regulator of *Dec2*.

Transcriptional Regulation of *Ugt1a9* by *Dec2*. An E-box-like element (a specific *Dec2*-binding motif) was found at the position of -872/-867 bp in the proximal *Ugt1a9* promoter based on sequence analysis (jaspar.genereg.net) (Fig. 7A). To test whether *Dec2* acts on *Ugt1a9* gene transcription, we performed transient transcriptional assays using an *Ugt1a9* reporter construct (i.e., *Ugt1a9-Luc*, -2000/+100 bp). Cotransfection of *Ugt1a9-Luc* reporter with *Dec2* resulted in a significant decrease in the transcriptional activity, and the inhibitory effects of *Dec2* were dose-dependent (Fig. 7B). However, the *Dec2* effects on *Ugt1a9* transcription were lost when the E-box-like element was truncated or mutated (Fig. 7C). These data suggested that *Dec2* trans-repressed *Ugt1a9* through direct binding to an E-box-like element in gene promoter.

Furthermore, we examined the mediating role of *Dec2* in *Rev-erb α* regulation of *Ugt1a9* by performing luciferase reporter assays. As expected, *Rev-erb α* significantly induced the promoter activity of *Ugt1a9* reporter (Fig. 7D). However, the induction effect was attenuated in *Dec2*-deficient cells, supporting a role of *Dec2* in mediating *Rev-erb α* regulation of *Ugt1a9* (Fig. 7D). On the other hand, *DEC2* has been previously reported to repress the expression of *CYP2D6* (a drug-metabolizing enzyme) via inhibiting *C/EBP α* -mediated transactivation (Matsunaga et al., 2012). However, we observed no effects of mouse *C/EBP α* on *Ugt1a9* transcription (Fig. 7E), thereby excluding a role of *C/EBP α* in the regulation of *Ugt1a9* by the *Rev-erb α /Dec2* axis.

Discussion

To our knowledge, it is a previously unreported discovery that *Ugt1a9* is a rhythmic gene and *Rev-erb α* participates in regulation of *Ugt1a9*

Fig. 3. *Rev-erb α* regulates *Ugt1a9* expression in Hepa-1c1c7 cells. (A) Effects of *Rev-erb α* overexpression on *Ugt1a9* mRNA expression. (B) Effects of *Rev-erb α* knockdown on *Ugt1a9* mRNA expression. (C) Effects of *Rev-erb α* overexpression and knockdown on *Ugt1a9* protein expression. Cells were transfected with *Rev-erb α* expression plasmid or siRev-erb α for 24 or 48 hours. * $P < 0.05$ (*t* test). Rel, relative.

Fig. 4. REV-ERB α regulates UGT1A9 expression in HepG2 cells. (A) Effects of REV-ERB α overexpression on UGT1A9 mRNA expression. (B) Effects of REV-ERB α knockdown on UGT1A9 mRNA expression. (C) Effects of REV-ERB α overexpression and knockdown on UGT1A9 protein expression. Cells were transfected with REV-ERB α expression plasmid or shREV-ERB α for 24 or 48 hours. * $P < 0.05$ (t test). Rel, relative; shREV-ERB α , short hairpin RNA targeting REV-ERB α ; shRNA, short hairpin RNA.

rhythm. Rhythmicity in *Ugt1a9* mRNA is translated to a temporal variation in Ugt1a9 protein that is associated with dosing time dependency in in vivo glucuronidation of propofol, a Ugt1a9 substrate (Figs. 1 and 2). There are several lines of evidence supporting rhythmic regulation of Ugt1a9 by Rev-erb α . First, Rev-erb α ablation down-regulates Ugt1a9 expression and blunts its rhythmicity in mouse liver (Fig. 1). Second, Rev-erb α positively regulates Ugt1a9 expression in Hepa-1c1c7 cells (Fig. 3). Third, the diurnal pattern of Rev-erb α protein

parallels with that of *Ugt1a9* mRNA (Fig. 1) (Zhang et al., 2019). Mechanistically, Rev-erb α drives rhythmic expression of Dec2 through a *trans*-repression action (Figs. 5 and 6). Dec2 further generates a diurnal rhythm in Ugt1a9 expression via periodically binding to an E-box-like motif in gene promoter and cyclically repressing gene transcription (Fig. 7).

Diurnal rhythmicity in drug-processing genes is generally driven by the molecular components of the circadian clock through transcriptional

Fig. 5. Rev-erb α ablation upregulates Dec2 expression in mouse liver. (A) A screen of Ugt1a9 repressors using luciferase reporter assays. (B) qPCR analyses of circadian Dec2 expression in the livers of wild-type and Rev-erb α ^{-/-} mice. (C) Western blotting analyses of circadian Dec2 expression in the livers of wild-type and Rev-erb α ^{-/-} mice. Data are means $\pm$ S.D. ($n = 5$). For (A), statistics analysis was performed with t test (* $P < 0.05$). For (B and C), statistics analyses were performed with two-way ANOVA and Bonferroni post hoc test (* $P < 0.05$ for group comparisons at individual time points). Rel, relative; RLU, relative luciferase unit; WT, wild-type.

Fig. 6. Rev-erb α regulates Dec2 expression in Hepa-1c1c7 cells. (A) Effects of Rev-erb α overexpression on Dec2 mRNA expression. (B) Effects of Rev-erb α knockdown on Dec2 mRNA expression. (C) Effects of Rev-erb α overexpression and knockdown on Dec2 protein expression. Cells were transfected with Rev-erb α expression plasmid or siRev-erb α for 24 or 48 hours. * $P < 0.05$ (t test). Rel, relative.

actions on the *cis*-elements E-box, D-box, and ROR responsive element (RORE) or REV-ERB responsive element (RevRE) (so-called “direct mechanisms”) (Murakami et al., 2008; Zhang et al., 2009; Chen et al., 2019). By contrast, the current study supports indirect regulation of drug-processing genes via clock-controlled cycling transcriptional factors. The rhythm of cycling transcriptional factors can be propagated to their downstream target genes. Such indirect mechanisms for

generation of rhythmic enzymes have been also noted previously in the literature. For instance, Rev-erb α regulates Cyp7a1 through repression of the nuclear receptor liver receptor homolog-1 (Zhang et al., 2018b). The PAR basic leucine zipper transcription factors drive the circadian rhythm of Cyp2b10 via the cycling constitutive androstane receptor (Noshiro et al., 1990). Therefore, the molecular clock directly or indirectly (depending on the exact gene) generates and regulates circadian rhythms in drug-processing genes, thereby modulating drug metabolism and disposition.

Contrasting with the fact that Rev-erb α functions as a transcriptional repressor, it positively regulates Ugt1a9 expression. We thus predicted that a negative regulator (as an intermediate) was involved in Rev-erb α regulation of Ugt1a9. A survey of the literature identified three candidates (i.e., Dec2, E4bp4, and Shp), which are known targets of Rev-erb α and transcriptional repressors of drug-processing genes. Based on luciferase reporter assays, Dec2 inhibited the transcription of Ugt1a9, whereas E4bp4 and Shp had no effects (Fig. 5A). Furthermore, negative regulation of Dec2 by Rev-erb α was validated in mice in vivo and in Hepa-1c1c7 cells (Figs. 5 and 6). It was therefore reasonable to propose that Dec2 mediated the positive regulatory effects of Rev-erb α on Ugt1a9. It is noteworthy that Dec2 rhythm may not generated solely by Rev-erb α because the rhythmicity in Dec2 was not completely abolished in Rev-erb α ^{-/-} mice (Fig. 5). Additional circadian clock genes such as Bmal1 are potential contributors to the oscillation of Dec2 expression (Sato et al., 2018).

It is noteworthy that Dec2 is a known indirect target of Rev-erb α (Hamaguchi et al., 2004; Zhang et al., 2018a). Regulation of Dec2 by Rev-erb α is mediated by Bmal1, which is the direct target of Rev-erb α and drives the transcription of Dec2 (Guillaumond et al., 2005). This is supported by the facts that Bmal1 induces promoter activity of Dec2-Luc reporter and that Rev-erb α attenuates the induction effects of Bmal1 (data not shown). Hepatocyte nuclear factor 4 α (Hnf4 α) is shown to be a hepatic activator of Ugt1a9 (Barbier et al., 2005). We recently revealed that Hnf4 α is an oscillating gene that is under the control of Bmal1 (Lin et al., 2019). Therefore, there is a possibility that Hnf4 α may contribute to the diurnal rhythm of Ugt1a9. However, we argue that the contribution of Hnf4 α is minor and secondary because 1) hepatic Ugt1a9 expression in Rev-erb α ^{-/-} mice displays a weak fluctuation (Fig. 1) and 2) Hnf4 α rhythm should be abolished in Rev-erb α ^{-/-} mice

Fig. 7. Transcriptional regulation of Ugt1a9 by Dec2. (A) Schematic representation of the E-box-like element in Ugt1a9 promoter. (B) Dec2 dose-dependently inhibits the promoter activity of Ugt1a9-Luc reporter. Statistics analysis was performed with one-way ANOVA and Bonferroni post hoc test (* $P < 0.05$). (C) Dec2 effects on Ugt1a9 transcription are lost when the E-box-like element was truncated or mutated. (D) Induction effect of Rev-erb α on Ugt1a9-Luc reporter is attenuated in Dec2-deficient cells. (E) Effects of mouse C/EBP α on Ugt1a9 transcription. RLU, relative luciferase unit; siDec2, siRNA targeting Dec2.

because of loss of rhythm in its circadian regulator Bmal1 (Solt et al., 2012).

Propofol has been widely used to probe the functional activity of UGT1A9 (Court, 2005; Kiang et al., 2005; Mukai et al., 2015). The present study also used propofol as a specific substrate to measure dosing time-dependent Ugt1a9 activity (Fig. 2). We observed time-varying glucuronidation of propofol (ZT6 > ZT18) both in microsomal metabolism in vitro and in mice in vivo, consistent with the diurnal pattern of Ugt1a9 protein expression (Figs. 1 and 2). Additionally, propofol glucuronidation was reduced and its time dependency was lost in *Rev-erba*^{-/-} mice, parallel changes with those in Ugt1a9 expression (Figs. 1 and 2). By contrast, the pharmacokinetic curve of the parent compound propofol was both dosing time- and genotype-independent (Fig. 2C). This is probably because Ugt1a9-mediated glucuronidation accounts for a moderate portion of total propofol clearance and temporal variations in Ugt1a9 activity are not sufficient to elicit significant changes in the pharmacokinetic curve of the parent compound (Sneyd et al., 1994; Guitton et al., 1998).

Based on sampling at a 4-hour interval, we found that both Ugt1a9 mRNA and protein peaked at ZT6 in the liver of wild-type mice (Fig. 1). However, this does not mean that there is no phase delay in Ugt1a9 protein as compared with the mRNA. We argue that the phase difference might be relatively small (<2 hours) and that this was masked by sampling at a relatively large interval (4 hours). Little phase delay has been also noted for some drug-processing proteins (e.g., Ugt1a1, Fmo5, and Mrp2) and circadian clock factors (e.g., Dec1, D-site-binding protein, Tef, E4bp4, Per2, and Ror1) (Narumi et al., 2016; Chen et al., 2019; Wang et al., 2019; Yu et al., 2019). For these proteins, their levels are governed mainly by mRNA abundance rather than other processes such as posttranslational modification and degradation (Narumi et al., 2016).

UGT1A9 is the orthologous gene of *Ugt1a9* in humans. We have provided in vitro evidence that human REV-ERB α positively regulates UGT1A9 expression (Fig. 4), consistent with the action of mouse Rev-erba on Ugt1a9. It is noteworthy that E-box-like element (−1938/−1932 bp) is found in the proximal promoter of human UGT1A9. There is a possibility that human UGT1A9 is rhythmically expressed and regulated by the REV-ERB α /DEC2 axis. However, whether human REV-ERB α regulates diurnal expression of UGT1A9 awaits further investigations.

In summary, the phase II enzyme Ugt1a9 displays diurnal rhythms at the levels of mRNA, protein, and enzymatic activity in mouse liver. Ugt1a9 rhythm is generated through transcriptional regulation by the Rev-erba/Dec2 axis. These findings may have implications for understanding of circadian clock-controlled drug metabolism and of metabolism-based chronotherapeutics.

Authorship Contributions

Participated in research design: Chen, Zhang, Wu.

Conducted experiments: Xu, Chen.

Performed data analysis: Xu, Chen, Yu, Zhang, Wu.

Wrote the manuscript: Xu, Wu.

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