

# Ser100-Phosphorylated ROR $\alpha$ Orchestrates CAR and HNF4 $\alpha$ to Form Active Chromatin Complex in Response to Phenobarbital to Regulate Induction of CYP2B6<sup>[S]</sup>

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## ABSTRACT

We have previously shown that the retinoid-related orphan receptor alpha (ROR $\alpha$ ) phosphorylation plays a pivotal role in sulfotransferase 1E1 gene regulation within mouse liver. Here, we found serine 100-phosphorylated ROR $\alpha$  orchestrates constitutive androstane receptor (CAR) and hepatocyte nuclear factor 4 alpha (HNF4 $\alpha$ ) to induce CYP2B6 by phenobarbital (PB) in human primary hepatocytes (HPHs). ROR $\alpha$  knockdown using small interfering RNAs suppressed CYP2B6 mRNAs in HPH, whereas transient expression of ROR $\alpha$  in COS-1 cells activated CYP2B6 promoter activity in reporter assays. Through chromatin immunoprecipitation (IP) and gel shift assays, we found that ROR $\alpha$  in the form of phosphorylated (p-) S100 directly bound to a newly identified ROR $\alpha$  response element (ROR $\alpha$  response element on CYP2B6 promoter, –660/–649) within the CYP2B6 promoter in untreated or treated HPH. In PB-treated HPH, p-Ser100 ROR $\alpha$  was both enriched in the distal phenobarbital response element module (PBREM) and the proximal okadaic acid response element (OARE), a known HNF4 $\alpha$  binding site. Chromatin conformation capture assay revealed direct contact between the PBREM and OARE only in PB-treated HPH. Moreover, CAR preferably interacted with phosphomimetically mutated ROR $\alpha$  at Ser100 residue in co-IP assay. A gel shift

assay with a radiolabeled OARE module and nuclear extracts prepared from PB-treated mouse liver confirmed that HNF4 $\alpha$  formed a complex with Ser 100-phosphorylated ROR $\alpha$ , as shown by supershifted complexes with anti-p-Ser100 ROR $\alpha$  and anti-HNF4 $\alpha$  antibodies. Altogether, the results established that p-Ser100 ROR $\alpha$  bridging the PBREM and OARE orchestrates CAR and HNF4 $\alpha$  to form active chromatin complex during PB-induced CYP2B6 expression in human primary hepatocytes.

## SIGNIFICANCE STATEMENT

CYP2B6 is a vital enzyme for the metabolic elimination of xenobiotics, and it is prone to induction by xenobiotics, including phenobarbital via constitutive androstane receptor (CAR) and hepatocyte nuclear factor 4 alpha (HNF4 $\alpha$ ). Here, we show that retinoid-related orphan receptor alpha (ROR $\alpha$ ), through phosphorylated S100 residue, orchestrated CAR-HNF4 $\alpha$  interaction on the CYP2B6 promoter in human primary hepatocyte cultures. These results signify not only the role of ROR $\alpha$  in the molecular process of CYP2B6 induction, but it also reveals the importance of conserved phosphorylation sites within the DNA-binding domain of the receptor.

## Introduction

Cytochrome P450 2B6, or CYP2B6, is a drug-metabolizing enzyme that is mainly expressed in the liver. The enzyme is responsible for the metabolism of about 2%–10% therapeutic drugs (Hedrich et al., 2016). Several of its drug substrates, including antimalarial artemisinin (Simonsson et al., 2003), efavirenz (Robertson et al., 2008) and carbamazepine

(Oscarson et al., 2006), and nonsubstrates, such as phenobarbital (PB) and phenytoin (Wang et al., 2004), can induce the enzyme posing a potential drug-drug interaction. In fact, regulatory agencies recommend a routine clinical risk assessment for CYP2B6 induction by a new therapeutic product (Zhang et al., 2009; Fahmi et al., 2016). Thus, CYP2B6 induction as well as its polymorphism remain important parameters determining therapeutic or toxic outcomes of certain pharmaceuticals, including efavirenz and cyclophosphamide (Desta et al., 2007; Wang et al., 2013; Zanger and Klein, 2013; Hedrich et al., 2016).

Over the past few decades, the molecular mechanism of CYP2B6 induction has been extensively studied and it is well

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**ABBREVIATIONS:** 2B6, CYP2B6; 3C, chromatin conformation capture; CAR, constitutive androstane receptor; ChIP, chromatin IP; CI, confidence interval; DR1, direct repeat protein-binding motif separated by a single nucleotide; EMSA, electro mobility shift assay; ER $\alpha$ , estrogen receptor  $\alpha$ ; FXR, farnesoid X receptor; hCAR, human CAR; HNF4 $\alpha$ , hepatocyte nuclear factor 4 alpha; HPH, human primary hepatocyte; HRP, horseradish peroxidase; IP, immunoprecipitation; KO, knockout; MAP, mitogen-activated protein; mCAR, mouse CAR; mSult, mouse Sult; NR, nuclear receptor; OARE, okadaic acid response element; p-, phosphorylated; PB, phenobarbital; PBREM, PB response element module; PCR, polymerase chain reaction; ROR $\alpha$ , retinoid-related orphan receptor alpha; RORE, ROR $\alpha$  response element; RXR $\alpha$ , retinoid X receptor  $\alpha$ ; siRNA, small interfering RNA; Sult, sulfotransferase; TCPOBOP, 1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene, 3,3',5,5'-Tetrachloro-1,4-bis(pyridyloxy)benzene; WT, wild type.

understood that the constitutive androstane receptor (CAR) [nuclear receptor (NR) 1I3] mediates CYP2B6 induction by phenobarbital, a prototype CYP2B6 inducer (Sueyoshi et al., 1999; Wang and Negishi, 2003; Wang et al., 2004; Negishi, 2017). Studies revealed that two DNA elements, the distal phenobarbital response element module (PBREM) and the proximal okadaic acid response element (OARE), on the CYP2B6 regulatory region are crucial for the induction of the gene by CAR activators (Swales et al., 2005; Inoue and Negishi, 2008). In the human or rodent liver primary cells, PB induces dephosphorylation of CAR at Threonine 38, which, in turn, initiates nuclear localization and occupation of PBREM motif and subsequent gene induction (Mutoh et al., 2009). Studies have shown that hepatocyte nuclear factor 4 alpha (HNF4 $\alpha$ ), which constitutively occupies a direct repeat protein-binding motif separated by a single nucleotide (DR1) unit within the OARE motif in the CYP2B6 proximal promoter and is also a key factor for the induction of the CYP2B6 gene (Inoue and Negishi, 2009).

Ser100 of retinoid-related orphan receptor alpha (ROR $\alpha$ ) represents a highly conserved phosphorylation site between the two zinc fingers in the nuclear receptor superfamily DNA-binding domain. Amino acid sequence comparison among the receptors suggests the site is conserved in 41 out of 46 total human nuclear receptors (Negishi, 2017). In a recent publication, we found ROR $\alpha$  was phosphorylated at Ser100 in mouse liver and that phosphorylation reversed ROR $\alpha$  from suppressor of mouse sulfotransferase (Sult) 1e1 gene to coactivator (Fashe et al., 2018), showing that ROR $\alpha$  can regulate its transcriptional activity through phosphorylation/dephosphorylation and dramatically affect the DNA binding as well as protein-protein interaction properties of the receptor (Hashiguchi et al., 2016; Fashe et al., 2018). Phosphorylation of this conserved site in other nuclear receptors has also been shown (Sun et al., 2007; Sueyoshi et al., 2019). For example, phosphorylation of CAR Thr38 was shown to be a key element for its functions in gene induction mechanism by phenobarbital (Mutoh et al., 2009, 2013). Furthermore, we have observed that equivalent phosphorylation in farnesoid X receptor (FXR) Ser154 (Hashiguchi et al., 2016), retinoid X receptor  $\alpha$  (RXR $\alpha$ ) Thr167 (Sueyoshi et al., 2019), and estrogen receptor  $\alpha$  (ER $\alpha$ ) Ser216 (Shindo et al., 2013) contributes to regulation of their respective biologic activities. Thus, the conserved site of phosphorylation may endow each nuclear receptor with unidentified phases of functional regulation.

In this report, we have identified a role of ROR $\alpha$  in CYP2B6 gene induction by phenobarbital in human primary hepatocytes through phosphorylation of conserved Ser100, which enhanced ROR $\alpha$  to orchestrate CAR and HNF4 $\alpha$  interactions and CYP2B6 transcriptional activation. Previously, ROR $\alpha$  had been implicated in the regulation of several CAR target genes, including Cyp2b10, Sult1e1, and Sult2a1 in mouse liver cells (Kang et al., 2007; Fashe et al., 2018) and CYP7B1, CYP2C8, and SULT2A1 gene in human liver cells (Echchgadda et al., 2007; Wada et al., 2008; Chen et al., 2009; Ou et al., 2013). However, the modulation of ROR $\alpha$  transcriptional activity via Ser100 phosphorylation and its role in regulation of CYP2B6 expression has not been reported. Here, utilizing gene reporter assays, chromatin immunoprecipitation (ChIP), coimmunoprecipitation (co-IP), chromatin conformation capture (3C), and electromobility shift assay (EMSA), we report that Ser100-phosphorylated ROR $\alpha$  binds to a newly identified response

element, ROR $\alpha$  response element (RORE) on CYP2B6 promoter, to activate gene transcription in cultures of human primary hepatocytes and synergized CAR activation of CYP2B6 gene expression. Situated between the PBREM and OARE motifs, where CAR and HNF4 $\alpha$  bind, respectively, phosphorylated (p-) Ser100 ROR $\alpha$  enhanced CAR and HNF4 $\alpha$  to form an active chromatin complex on the CYP2B6 promoter in response to PB to induce gene expression.

## Materials and Methods

**Antibodies, Chemicals, Reagents, and Animal Treatments.** Antibodies against ROR $\alpha$  (no. PP-H3910-00) and HNF4 $\alpha$  (no. PP-H1415-00) were purchased from Perseus Proteomics Inc (Tokyo, Japan). Anti-p-Ser100 ROR $\alpha$  peptide antibody ( $\alpha$ P-Ser100 ROR $\alpha$ ) and anti-ROR $\alpha$  were custom-generated by Genescript (Piscataway, NJ). Anti-GFP-horseradish peroxidase (HRP) (no. ab66663) and anti-FLAG-HRP (no. S8592) were obtained from Abcam (Cambridge, MA) or Sigma-Aldrich (St. Louis, MO), respectively. High Capacity Archive kit, lipofectamine RNAiMAX Reagent, Power SYBR Green Master Mix (no. A25741), TaqMan Universal Master Polymerase Chain Reaction (PCR) Mix (no. 4304437), TaqMan PCR probes [no. Hs00231959\_1 for human CAR (hCAR), no. Hs00536545\_1 for human ROR $\alpha$ , no. Hs00167937\_1 for CYP2B6, no. Hs01060665\_g1 for  $\beta$ -actin, and no. Hs00609178\_1 for human G6PC] were obtained from Life Technologies Corporation (Grand Island, NY). Dual luciferase assay system and TNT Coupled Reticulocyte Lysate Systems were from Promega (Madison, WI), and phosphatase inhibitor cocktail-2 and -3 were from Sigma-Aldrich. ChIP IT Express kit was from active motif (Carlsbad, CA). Polynucleotide T4 kinase, T4 DNA ligase, and AluI were from New England Biolabs (Ipswich, MA). Small interfering RNAs (siRNAs) targeting ROR $\alpha$  or scrambled control siRNAs were from Dharmacon (Lafayette, CO). All animals were housed in a room maintained at 22°C with a 12:12-hour light/dark cycle (7:00 AM to 7:00 PM). Mice were fed ad libitum with National Institutes of Health-31 Open Formula Autoclavable diet (Zeigler). Phenobarbital (10 mg/kg) or vehicle (PBS) was administered intraperitoneally and kept for 24 hours for liver nuclear extraction and immunohistochemical analyses. All animal procedures were approved by the Animal Care and Use Committee at National Institute of Environmental Health Sciences at the National Institutes of Health and performed humanly in accordance with the Public Health Service Policy.

**Plasmids.** All reporter constructs harboring CYP2B6-promoter DNA fragments used in this study were prepared in pGL3 vector and were described in previous research works (Swales et al., 2005). RORE was deleted within -1.8 kb CYP2B6 promoter and placed in front of luciferase gene in PGL3 vector. ROR $\alpha$  plasmids were described in our previous publication (Fashe et al., 2018).

**Cell Culture and Transfection.** Human primary hepatocytes were obtained from Life Technologies Corporation and cultured in William's E Medium without phenol red (no. A1217601) supplemented with 100 nM dexamethasone and L-glutamine. COS-1 and Huh7 cells were in Dulbecco's modified Eagle's medium. All media were supplemented with fetal bovine serum and penicillin (100 U/ml) and streptomycin (100  $\mu$ /ml), and the cells were cultured at 5% CO<sub>2</sub> and 37°C. Transfections with a given plasmid were performed with FUGENE 6 per the manufacturer's instructions, whereas lipofectamine RNAiMAX Reagent was used for the transfection of siRNAs into the human primary hepatocytes.

**ChIP Assays.** The ChIP assays were carried out using active motifs ChIP IT EXPRESS kit per the manufacturer's instructions. Briefly, human primary hepatocytes were seeded in 10-cm culture dish overnight before phosphate buffer (PBS) or 1 mM PB treatment of 6 hours. Then the culture medium was washed off, and the cells were incubated in 1% formaldehyde in PBS at room temperature for 10 minutes followed by a 5-minute incubation in glycine. The cells were lysed, nuclear material was collected, and the chromatin was

sheared. Overnight immunoprecipitations were performed with anti-RORα, p-Ser100 RORα, HNF4α, or anti-RXRα antibodies, mouse normal IgG, and G protein-conjugated magnetic beads. After washing the beads, the formalin cross-linking was reversed, and PCR amplification was achieved by using primers targeting selected motifs (PBREM, RORE, and OARE) of CYP2B6 gene regulatory region (Table 1).

**3C Assays.** 3C assays were performed in human primary hepatocytes (HPHs). The cells were seeded in a 10-cm culture dish and treated with PBS or 1 mM PB for 6 hours. The cells were incubated in 2% formaldehyde in PBS for 10 minutes before a 5-minute treatment with glycine. Then the cells were harvested in a lysis buffer made of 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% NP40, and 1% Triton X in the presence of protease inhibitor cocktail and placed on ice for 10 minutes. The lysates were centrifuged, and the pellet was taken up in 440 μl distilled H<sub>2</sub>O, 60 μl Cutsmart buffer and SDS (0.3%) were added, and the mixture was incubated at 37°C and 225 rpm for 1 hour followed by addition of 2.5% Triton X-100 in the same incubation conditions for another hour. Restriction enzyme digestion and ligation were achieved by AluI and T4 DNA ligase, respectively. The formalin fixations were reversed at 65°C overnight in the presence of proteinase K (20 μg/ml), which was followed by an RNase treatment of 45 minutes at 37°C, and DNAs were extracted by QIAquick spin column per manufacturer's instructions. Primers were designed and amplifications were made using Green Taq, and amplicons were separated in a 2% agarose gel from which the expected bands were excised, and gel was extracted to confirm the identity of the amplicon by sequencing.

**Gel Shift Assays.** Gel mobility shift assays were described in our previous study (Fashe et al., 2018). Shortly, radioactive probes were generated by end-labeling double-stranded oligonucleotides (NR1/PBREM, 2B6-RORE, or DR1/OARE, Table 1) with [ $\gamma$ -<sup>32</sup>P]ATP in the presence of T4 polynucleotide kinase. Mock (pcDNA3.1), CAR, RXRα, RORα, and HNF4α proteins were in vitro translated using TNT Coupled Reticulocyte Lysate Systems per the manufacturer's instructions. One microliter of the protein mixture was mixed with the radioactive probes and incubated at room temperature for 10 minutes before electrophoresis in acrylamide gel.

**Reporter Assay.** COS-1 cells in 24-well plates were cotransfected with expression vectors for CAR, HNF4α, RORα, CAR, and RORα or HNF4α and RORα for reporter assays. Five reporter constructs harboring CYP2B6 regulatory region, termed in this study as −1.8 kb, −1.8 kb ΔPBREM, −1.8 kb ΔRORE, or −1.8 kb ΔOARE, were used in this study. Renilla luciferase plasmids were also included in the transfection mixture to normalize the luminescent activity. The relative luciferase activity was measured after 48 hours using the Dual Luciferase Assay System (Promega).

**Real-Time PCR.** Total RNAs were extracted from human primary hepatocytes treated with PBS/PB in Trizol reagent, which were reverse transcribed using a High Capacity Archive kit. The mRNAs were quantified by real-time PCR using TaqMan universal PCR master mix and probes for RORα and CYP2B6. mRNA expression of hCAR, human G6PC, CYP7B1, SULT2A1, and human β-actin was

also measured as a positive or negative control for the expression of RORα in human primary hepatocytes treated with siRNAs.

**Co-IP and Western Blot Assays.** Huh7 cells seeded in 10-cm culture dishes were transfected with expression plasmids of GFP-RORα or its S100A or S100D mutants in the presence or absence of hCAR-V5 or HNF4α-V5. The cells were lysed in a buffer composed of 20 mM Tris-HCl (pH, 7.5), 100 mM NaCl, 0.5 mM EDTA, and 1% Triton X in 10% glycerol supplemented with protease inhibitor cocktail. The cell debris was removed by centrifugation, and 250 μg protein of the lysate was mixed with anti-GFP agarose overnight, washed (3 times) with the lysis buffer, and subjected to 1× SDS/PAGE sample buffer for Western blot assay. The input (5%) and the IP samples were electrophoresed in 10% acrylamide gel at 150 V and transferred onto polyvinylidene difluoride membranes at 15 V for 1 hour. The membranes were probed with anti-V5 (HRP) or anti-GFP (HRP) antibodies for 1 hour at room temperature and visualized by C-DiGit Chemiluminescent Western Blot Scanner (LI-COR, Inc., Lincoln, NE) using WesternBright Sirius HRP substrate (Advanta Inc, CA).

**Statistical Analysis.** Multiple groups were analyzed by two-way analysis of variance with Tukey's multiple comparison test to obtain *P* values for the observed differences and 95% confidence interval (CI) for activation ratios. Two groups were compared by unpaired Student's *t* test. These statistical analyses were conducted using the software GraphPad Prism.

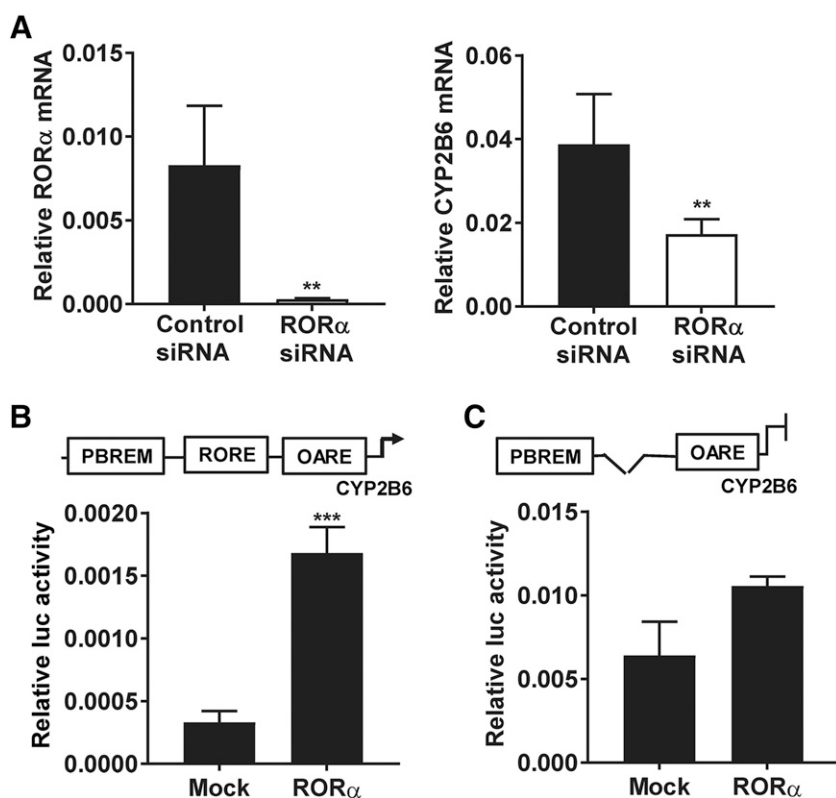
## Results

**RORα Regulates the Basal Gene Expression of CYP2B6 in Human Primary Hepatocyte Cultures.** Expression of CYP2B6 mRNAs was studied in human primary hepatocytes treated with control siRNAs or siRNAs targeting RORα for 3 days. Expression RORα mRNAs were suppressed by 96% in the cells treated with siRNAs targeting RORα compared with the control ones. In the same cultures, the basal expression of CYP2B6 was suppressed (*P* < 0.05) along with RORα mRNAs (Fig. 1A). To confirm the effect of RORα suppression in human primary hepatocytes, we measured the expression of known RORα target genes, such as SULT2A1, G6PC, and CYP7B1. As expected, the change in the expression of these genes was also statistically significant, whereas the expression of CAR mRNA, a non-RORα target gene, was not affected (Supplemental Fig. 1). This clearly suggests that RORα regulates the basal gene expression of CYP2B6 in human primary hepatocytes.

To support these observations, we performed a cell-based reporter assay. For this purpose, RORα and a −1.8 kb CYP2B6 promoter placed in front of luciferase gene in PGL3 vector were cotransfected into COS-1 cells. Supporting the above observations, RORα activated a −1.8 kb CYP2B6

TABLE 1  
Primers and oligos used in gel mobility shift (EMSA), ChIP, and 3C assays

Application/Motif	Forward Sequence (5'–3')	Reverse Sequence (5'–3')
EMSA		
NR1/PBREM	TCAGGGTCAGGAAAGTACAGTT	AACTGTACTTTCCTGACCCCTGA
2B6-RORE	TCTTGACCTCAATTGATC	GATCAATTGAGGTCAAGA
DR1/OARE	ACCTGGACTTTGATATCTC	GAGATATCAAAGTCCAGGT
ChIP		
PBREM	TTACTGTGTGTAAAGCACTTC	GACAAACAGTCCCTATTTGTAAG
2B6-RORE	CCGAGTAGCTGGGATTAAAGTACCCA	CTTCCCAACGTGCTGGGATTACAG
OARE	GGACAAATGCATGCAAGCAC	TGACCTGATGCCATCCCTTTAC
3C		
PBREM/OARE	CTGTGACAGGATGTGTGGGTG	GGCAGGGATACAGTGGTAGAG



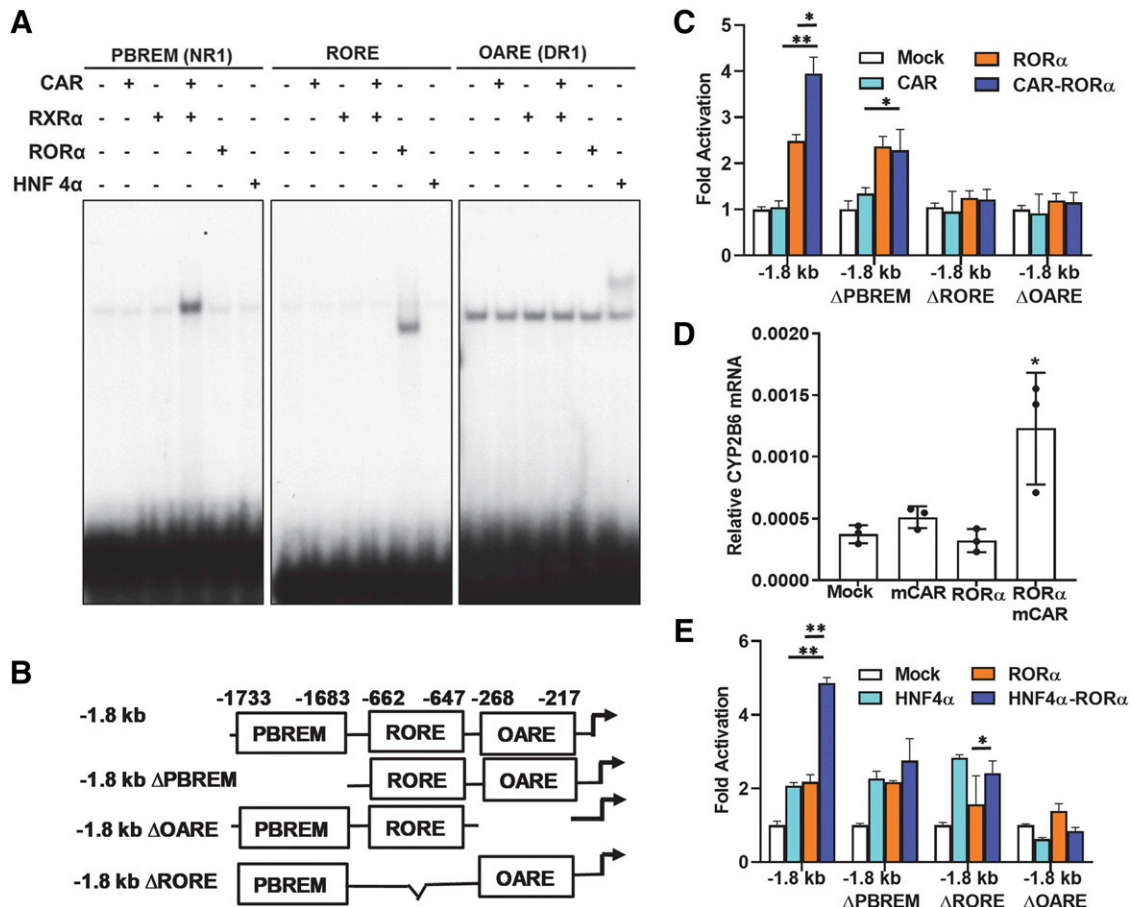
**Fig. 1.** ROR $\alpha$  regulates the basal expression of CYP2B6 in the liver cells. (A) The expression of CYP2B6 mRNAs was suppressed when ROR $\alpha$  expression was attenuated by treatment with siRNAs targeting ROR $\alpha$  ( $n = 5$ ). (B and C) ROR $\alpha$  activated CYP2B6 reporter gene. Cell-based reporter assays were performed in COS-1 cells transiently expressing ROR $\alpha$  and a luciferase reporter gene harboring  $-1.8$  kb CYP2B6 promoter ( $n = 3$ ). ROR $\alpha$  activated this  $-1.8$  kb CYP2B6 reporter but was unable to do so when the ROR $\alpha$  response element was deleted as shown in (C). Luc on vertical axes stands for luciferase. The asterisk (\*) between the groups represents statistically significant difference (\*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ). Mean and S.D. are shown for each group of treatment.

promoter in COS-1 cells (Fig. 1B). It has been reported that ROR $\alpha$  regulates its target genes by directly binding a DNA core motif AGGTCA preceded by A/T rich sequence (Giguère et al., 1995; Jetten et al., 2001). Thus, we analyzed this  $-1.8$  kb CYP2B6 gene promoter for an RORE sequence and identified an element (5'... TGACCTCAATTG...3'), termed as 2B6-RORE, located  $-660$  and  $-649$  upstream of the CYP2B6 transcription start site. To confirm whether the transcriptional activity of ROR $\alpha$  was attributed to its binding to 2B6-RORE motif, we deleted this putative RORE sequence within the  $-1.8$  kb CYP2B6 promoter ( $1.8$  kb $\Delta$ RORE) and performed a cell-based reporter assay. ROR $\alpha$  could not activate the  $-1.8$  kb $\Delta$ RORE reporter, which suggests the 2B6-RORE is the key element for the CYP2B6 gene activation by ROR $\alpha$  (Fig. 1C). Moreover, we performed a gel shift assay using a radioactive probe prepared by labeling the 2B6-RORE with [ $\gamma$ - $^{32}$ P]ATP, and an In Vitro translated ROR $\alpha$  proteins. The results indicated that ROR $\alpha$  could strongly bind to this sequence (Fig. 2A).

**ROR $\alpha$  and CAR Coactivate CYP2B6 Promoter.** Since the CYP2B6 gene is an archetypal xenobiotic-inducible gene and the nuclear receptor CAR generally spearheads the molecular process of induction of this gene, we would like to define the role of ROR $\alpha$  in the molecular mechanism of CYP2B6 gene induction. For this purpose, we performed gene reporter analysis using CYP2B6  $-1.8$  kb and its deletion mutant reporters shown in Fig. 2B. As a first experiment, ROR $\alpha$ , CAR, or both were cotransfected with  $-1.8$  kb 2B6 reporter into COS-1 cells. The results in Fig. 2C clearly showed ROR $\alpha$  and CAR synergistically activated the reporter, since CAR + ROR $\alpha$  cotransfection showed 3.97- (95% CI ratio; 3.12–4.84), 3.93- (95% CI ratio; 3.08–4.78), and 2.48-fold (95% CI ratio; 1.63–3.33) activation over mock, CAR,

or ROR $\alpha$  cotransfected reporters, respectively. We analyzed the role of each of the three DNA elements (i.e., PBREM, 2B6-RORE, and OARE) in the activation of CYP2B6 promoter by preparing reporter constructs by deleting each element within the  $-1.8$  kb CYP2B6 promoter (Fig. 2B). Interestingly, deletion of PBREM, 2B6-RORE, or OARE abolished the synergy between CAR and ROR $\alpha$ , suggesting that the three motifs were all important for the synergistic activation of the CYP2B6 gene by CAR and ROR $\alpha$  (Fig. 2C). To further confirm the coactivation of the CYP2B6 gene by CAR and ROR $\alpha$ , we transfected Huh7 cells with expression plasmids of CAR, ROR $\alpha$ , or both and treated the cells with DMSO or 1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene, 3,3',5,5'-Tetrachloro-1,4-bis(pyridyloxy)benzene (TCPOBOP) for 24 hours. We could observe that strong induction of the CYP2B6 gene by TCPOBOP only when CAR and ROR $\alpha$  were cotransfected, confirming the synergy between the two nuclear receptors (Fig. 2D). Moreover, the binding of ROR $\alpha$  onto the OARE motif was further enhanced by coexpression of CAR in Huh7 cell per ChIP assay data (Supplemental Fig. 2).

Similarly, since HNF4 $\alpha$  binds the OARE motif that was crucial for the activation of CYP2B6 promoter, we performed a similar reporter assay using the reporter constructs discussed above in COS-1 cells. The results showed ROR $\alpha$  and HNF4 $\alpha$  coactivated the CYP2B6 promoter. Deletion of PBREM did not affect the activity of either receptor, but deletion of any of the three motifs (i.e., PBREM, 2B6-RORE, and OARE) abolished the observed coactivation (Fig. 2E). In an effort to investigate the actual interaction between HNF4 $\alpha$  and ROR $\alpha$  on the CYP2B6 promoter, we also performed a ChIP assay using anti-HNF4 $\alpha$  antibody in Huh7 cells transiently expressing ROR $\alpha$ . It was observed that the binding of HNF4 $\alpha$  to



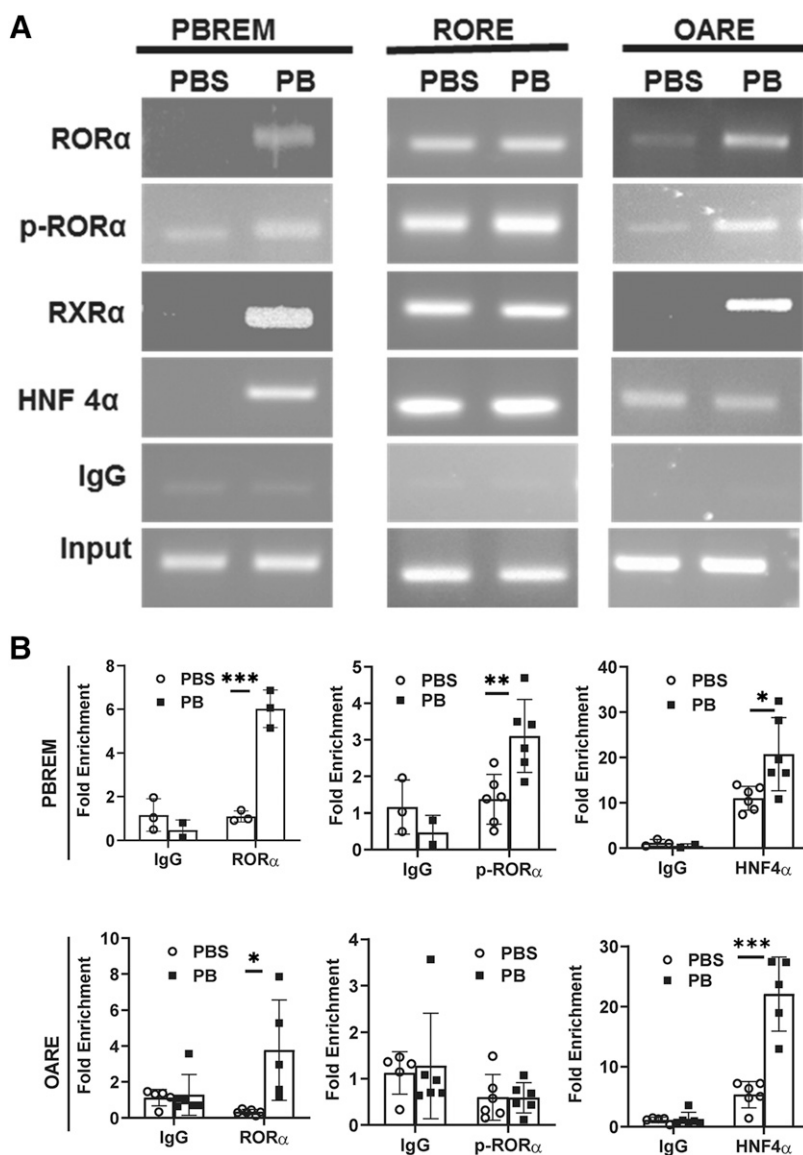
**Fig. 2.** ROR $\alpha$  coactivated CYP2B6 promoter with CAR or HNF4 $\alpha$ . (A) ROR $\alpha$  binding DNA element in CYP2B6 promoter was identified by gel shift analyses. Gel shift assay was performed as in *Materials and Methods* using 32P labeled DNA probes shown in Table 1. The assay revealed ROR $\alpha$  bound to its putative RORE in CYP2B6 promoter. CAR and HNF4 $\alpha$  bound NR1 and DR1, respectively, as previously reported (Sueyoshi et al., 1999; Inoue and Negishi, 2008). (B) Luciferase reporter constructs used in this study. Three elements (PBREM, 2B6-RORE, and OARE) in CYP2B6 gene promoter and their locations relative to transcriptional start site are shown. (C) ROR $\alpha$  and CAR coactivated CYP2B6 reporter genes in COS-1 cells. ROR $\alpha$  and CAR coactivation was observed only with the intact -1.8 kb CYP2B6 promoter. Statistical analysis was performed with values between single-factor and two-factor cotransfection. (D) ROR $\alpha$  and CAR synergistic activation of CYP2B6 gene. CAR, ROR $\alpha$ , or both were transfected into Huh7 cells, and CYP2B6 mRNA expression was measured from total RNAs. (E) ROR $\alpha$  and HNF4 $\alpha$  coactivated CYP2B6 reporter genes in COS-1 cells. HNF4 $\alpha$  and CAR coactivation was observed only with the intact -1.8 kb CYP2B6 promoter. (C–E) Statistical analysis results were shown between single-factor and two-factor cotransfection. (C–E) The asterisk (\*) between the groups represents statistically significant difference (\* $P$  < 0.05; \*\* $P$  < 0.01). Mean and S.D. are shown for each transfected group ( $n$  = 3).

the OARE motif was enhanced in cells transiently expressing ROR $\alpha$  but not CAR (Supplemental Fig. 2).

**ROR $\alpha$  Mediated the Contact between PBREM and OARE in PB-Treated Human Primary Hepatocytes.** Having established that CAR and ROR $\alpha$  coactivate the CYP2B6 promoter and also knowing that the three motifs, PBREM, 2B6-RORE, and OARE, are critical for the activation of the CYP2B6 gene promoter, we were interested to examine the dynamics of CAR, ROR $\alpha$ , and HNF4 $\alpha$  interactions with the aforementioned DNA motifs on the CYP2B6 promoter in untreated and PB-treated human primary hepatocyte cultures. For this purpose, we performed ChIP assays using anti-RXR $\alpha$ , anti-ROR $\alpha$ , anti-p-Ser100-ROR $\alpha$ , and anti-HNF4 $\alpha$  antibodies. As reported in the previous studies, anti-RXR $\alpha$ , antibody against a CAR partner, was used here as an indicator of CAR binding to PBREM (Saito et al., 2013; Hori et al., 2016). All of the evaluated antibodies immunoprecipitated 2B6-RORE chromatin region in both untreated and treated conditions. In contrast, all of the antibodies precipitated more PBREM and OARE chromatin region in PB-treated than

nontreated human primary hepatocytes (Fig. 3A). We also showed quantitative ChIP results for PBREM and OARE in Fig. 3B, and the results suggested essentially the same with Fig. 3A, excepting the marginal increase of p-Ser100 ROR $\alpha$ -precipitated band for OARE. Nonetheless, substantial amount of p-Ser100 ROR $\alpha$  bound on PBREM, RORE, and OARE in PB-treated cells. These observations suggest that p-Ser100 ROR $\alpha$  exists on 2B6-RORE, and after PB treatment, drastic DNA-protein interaction changes were introduced in the CYP2B6 promoter.

To find roles of ROR $\alpha$  phosphorylation at Ser100 in the molecular interaction between ROR $\alpha$  with CAR or HNF4 $\alpha$ , we performed co-IP assay as shown in Fig. 4, A and B. We used phosphomimetic mutant ROR $\alpha$  with Asp100 instead of Ser (ROR $\alpha$  S100D) in these co-IP experiments. CAR preferably interacted with ROR $\alpha$  S100D in Huh7 cells transiently expressing CAR and ROR $\alpha$  wild type (WT) or ROR $\alpha$  S100D (Fig. 4A). Unexpectedly, coexpression of ROR $\alpha$  mutants with HNF4 $\alpha$  in Fig. 4B suggested ROR $\alpha$  S100D destabilized HNF4 $\alpha$  protein. Although the mechanism for this HNF4 $\alpha$  destabilization



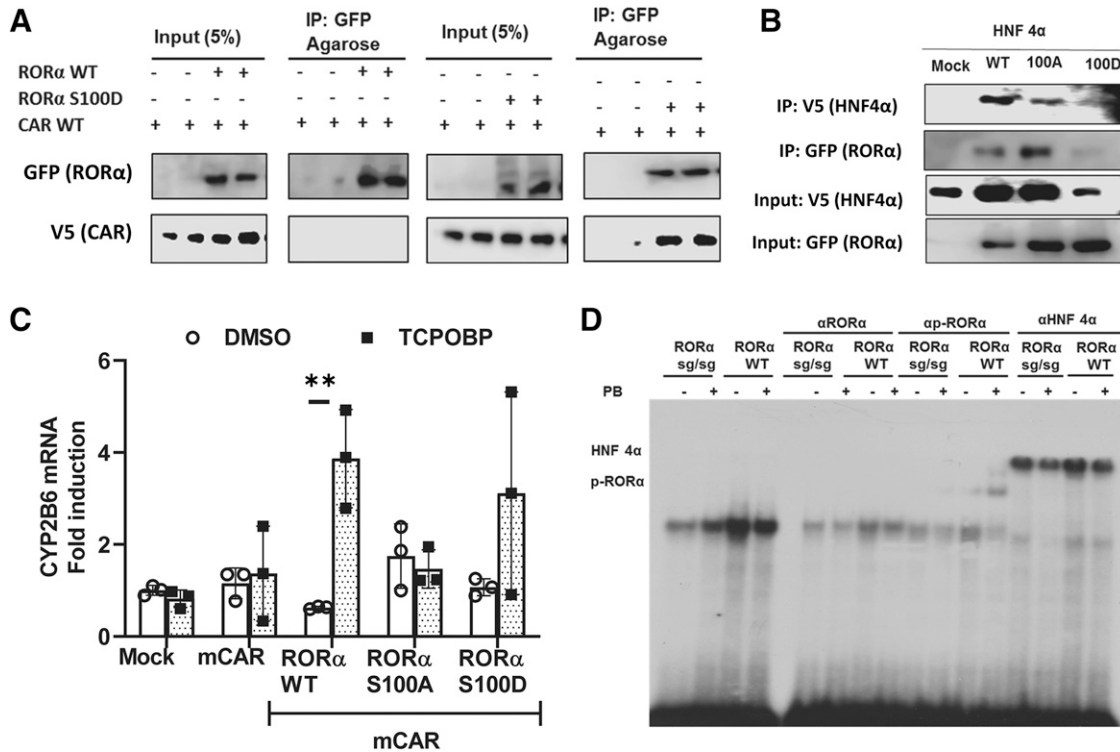
**Fig. 3.** Phenobarbital induced p-Ser100 RORα onto the PBREM and OARE motifs in human primary hepatocytes. (A) ChIP-PCR assays were performed in human primary hepatocytes using antibodies against RORα, p-Ser100 RORα, HNF4α, and RXRα to find interaction of these factors with PBREM, 2B6-RORE, and OARE. Though both RORα and p-Ser100-RORα were detected at RORE before and after PB treatment, PB initiated increase of DNA bands corresponding to RORα and p-RORα interaction with PBREM and OARE. (B) The same set of antibodies and PCR primers was used for quantitative ChIP-PCR analysis for OARE and PBREM. The asterisk (\*) between the groups represents statistically significant difference (\* $P < 0.05$ ; \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). Mean and S.D. are shown for each transfected group ( $n = 6$ ).

was not clear at this point, this suggested some molecular communication between these factors existed. Even with consideration of different amount of HNF4α in the input samples, HNF4α and RORα interaction was clearly shown in this co-IP assay (Fig. 4B). In HepG2 cells that were treated with TCPOBOP, which is a mouse CAR (mCAR)-specific ligand activator, quantitative mRNAs (real-time-quantitative PCR) analysis revealed that CYP2B6 mRNAs were induced in cells transiently expressing mCAR and RORα WT or RORα S00D but not RORα S100A, suggesting that p-Ser100 RORα was crucial for the CAR-RORα synergy (Fig. 4C).

To find S100-phosphorylated RORα in 2B6-RORE binding-protein complex, we performed EMSA assay as shown in Fig. 4D. A radioactive 2B6-OARE was incubated with nuclear extracts prepared from PBS- or PB-treated RORα WT or RORα staggerer mice (mutant mice with one exon deletion that codes a part of ligand-binding domain of RORα) liver as described in the *Materials and Methods* section. A single shifted band was observed, and the band intensity was stronger with the nuclear extracts from RORα WT than with the nuclear extracts from RORα staggerer mice livers.

Anti-HNF4α antibody supershifted the complex, and anti-RORα blocked the formation of the complex, suggesting the multiple components, including RORα and HNF4α, existed in the shifted bands. Interestingly, anti-p-Ser100 RORα supershifted the complex formed with nuclear extracts from RORα WT mice livers, suggesting that p-Ser100 RORα was also a member of the complex, and phosphorylation of this site increased by PB (Fig. 4D).

Since the data suggested that p-Ser100 RORα was an essential component of PB induction of the CYP2B6 gene in human primary hepatocytes, we aimed to confirm expression as well as the subcellular localization of p-Ser100 RORα in liver cells. First, we analyzed phosphorylation of RORα with P38 mitogen-activated protein (MAP) kinase in HepG2 cells ectopically expressing the kinase and WT RORα or RORα S100A. The results in Fig. 5A indicated protein band reacting with anti-p-Ser 100 RORα antibody clearly increased when WT RORα was cotransfected in HepG2 cells. Slight increase of the bands with same migration in RORα S100A-transfected HepG2 may represent endogenous RORα in HepG2 extracts. Thus, this Western blot analysis of the cell



**Fig. 4.** p-Ser100 ROR $\alpha$  functional interaction with CAR and HNF4 $\alpha$ . (A) ROR $\alpha$  S100D mutant interacted with CAR. Coimmunoprecipitation assay was performed in Huh7 cells transiently expressing ROR $\alpha$  WT or its phosphomimetic mutant, ROR $\alpha$  S100D, in the presence or absence of CAR. CAR preferably interacted with ROR $\alpha$  S100D. (B) HNF4 $\alpha$  protein was destabilized by coexpression with ROR $\alpha$  S100D. ROR $\alpha$  WT, ROR $\alpha$  S100A, or ROR $\alpha$  S100D were cotransfected into Huh7 cells for a co-IP assay. (C) CYP2B6 gene coactivation by CAR and ROR $\alpha$  S100D mutant. Expression plasmids of mCAR were transfected into HepG2 cells in the presence of ROR $\alpha$  WT, ROR $\alpha$  S100A, or ROR $\alpha$  S100D, and CYP2B6 mRNA expression was measured. The asterisk (\*) between the groups represents statistically significant difference (\*\* $P < 0.01$ ). Mean and S.D. are shown for each group ( $n = 3$ ). (D) HNF4 $\alpha$  and p-Ser100 ROR $\alpha$  binding to OARE. Gel shift analysis was performed as in *Materials and Methods* section using DR1 radioactive probe shown in Table 1 and mouse liver nuclear extracts. Stronger binding was observed with nuclear extracts from ROR $\alpha$  WT mice liver than with ROR $\alpha$  sg/sg, which lacks one exon in lateral organ boundaries domain of ROR $\alpha$  gene. Though anti-ROR $\alpha$  antibody blocked the gel shift, phospho-specific anti-Ser100 ROR $\alpha$  antibody produced a super shift with only WT PB-treated mice liver nuclear extracts. Anti-HNF4 $\alpha$  antibody supershifted the bands.

lysates suggested that ROR $\alpha$  S100 residue was phosphorylated by p38 (Fig. 5A).

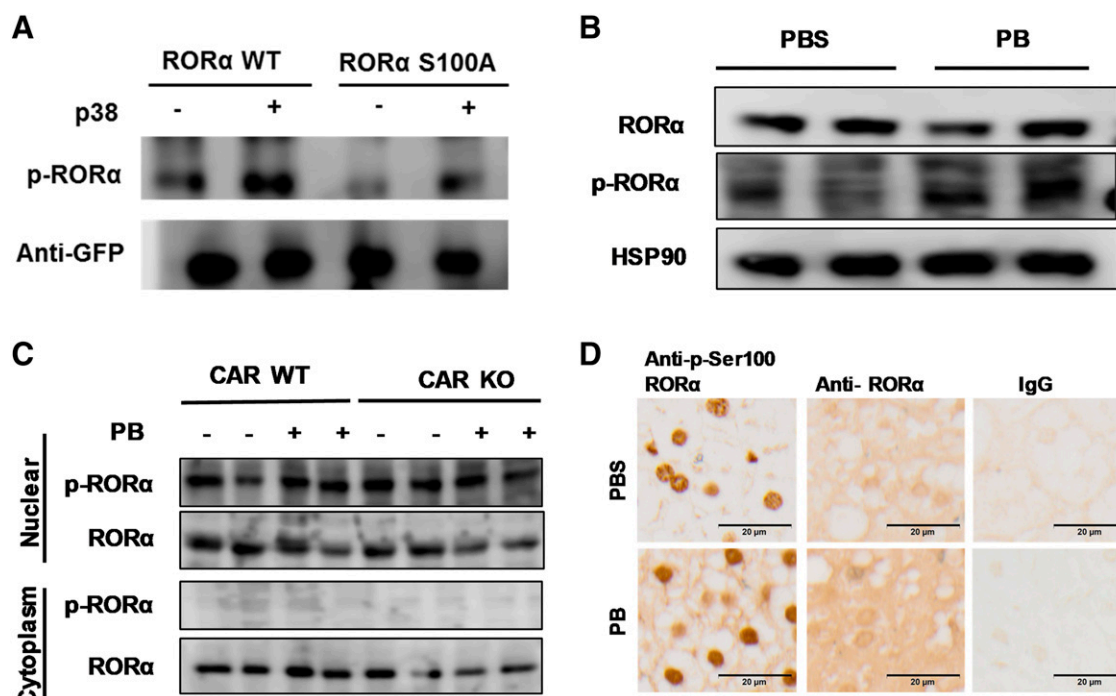
In human primary hepatocyte whole-cell lysate, Western blot analysis revealed that ROR $\alpha$  and p-Ser100 ROR $\alpha$  were expressed (Fig. 5B). Among multiple bands recognized with anti-p-Ser100 ROR $\alpha$  antibody, the band with strongest intensity indicated by the arrow showed exactly the same migration with the band recognized by anti-ROR $\alpha$  antibody in the Fig. 5B upper panel. Consistent with the ChIP assay in Fig 3, PB treatment did not change the p-Ser ROR $\alpha$  expression in these cells. Next, we analyzed Ser100-phosphorylated ROR $\alpha$  subcellular localization in mouse liver (Fig. 5, C and D). We performed Western blotting analysis with nuclear and cytoplasmic extracts (Fig. 5C) and immunohistochemistry analysis of liver sections (Fig. 5D). Ser100-phosphorylated ROR $\alpha$  was clearly observed in mouse liver cells, and it was localized within the nucleus per both Western blot and immunohistochemical staining data.

**PB Induced Change in CYP2B6 Chromatin Conformation in Human Primary Hepatocytes.** We studied chromatin conformation changes within  $-1.8$  kb CYP2B6 promoter using 3C assay in human primary hepatocytes treated with PBS or 1 mM PB (Fig. 6). Restriction enzyme AluI has seven different restriction sites between the PBREM and OARE motifs within the proximal  $-1.8$  kb of the CYP2B6 promoter. As described in the *Materials and Methods* section

above, after AluI digestion and subsequent ligation, the DNAs were amplified using PCR primers designed to amplify DNA fragments between  $-1495$  and  $-195$  in such a way that without digestion the size of the amplicon would be 1.3 kb and with AluI digestion without ligation there would be no amplicon. However, we detected an amplicon size of about 210 bp in the PB-treated but not PBS-treated primary hepatocytes, and the identity of the amplicon was confirmed by sequencing as shown in Fig. 6. This suggested that PB induced a substantial change in the CYP2B6 chromatin conformation that enabled two DNA fragments consisting of PBREM and OARE, respectively, to coexist in the protein-DNA complex and efficiently ligate the two fragments.

## Discussion

We found ROR $\alpha$  siRNAs suppressed expression of CYP2B6 mRNA in human primary hepatocyte cultures. Consistent with this observation, ROR $\alpha$  activated a  $-1.8$  kb CYP2B6 promoter in a cell-based reporter assay with COS-1 cells. Next, we identified a ROR $\alpha$ -responsive DNA element located between  $-668$  and  $-645$  bp upstream of the CYP2B6 transcription start site and named it 2B6-RORE. The assay also revealed that though p-Ser 100-ROR $\alpha$  binding to 2B6-RORE is constitutive and no change was observed after PB treatment in these cells, this protein was enriched on the



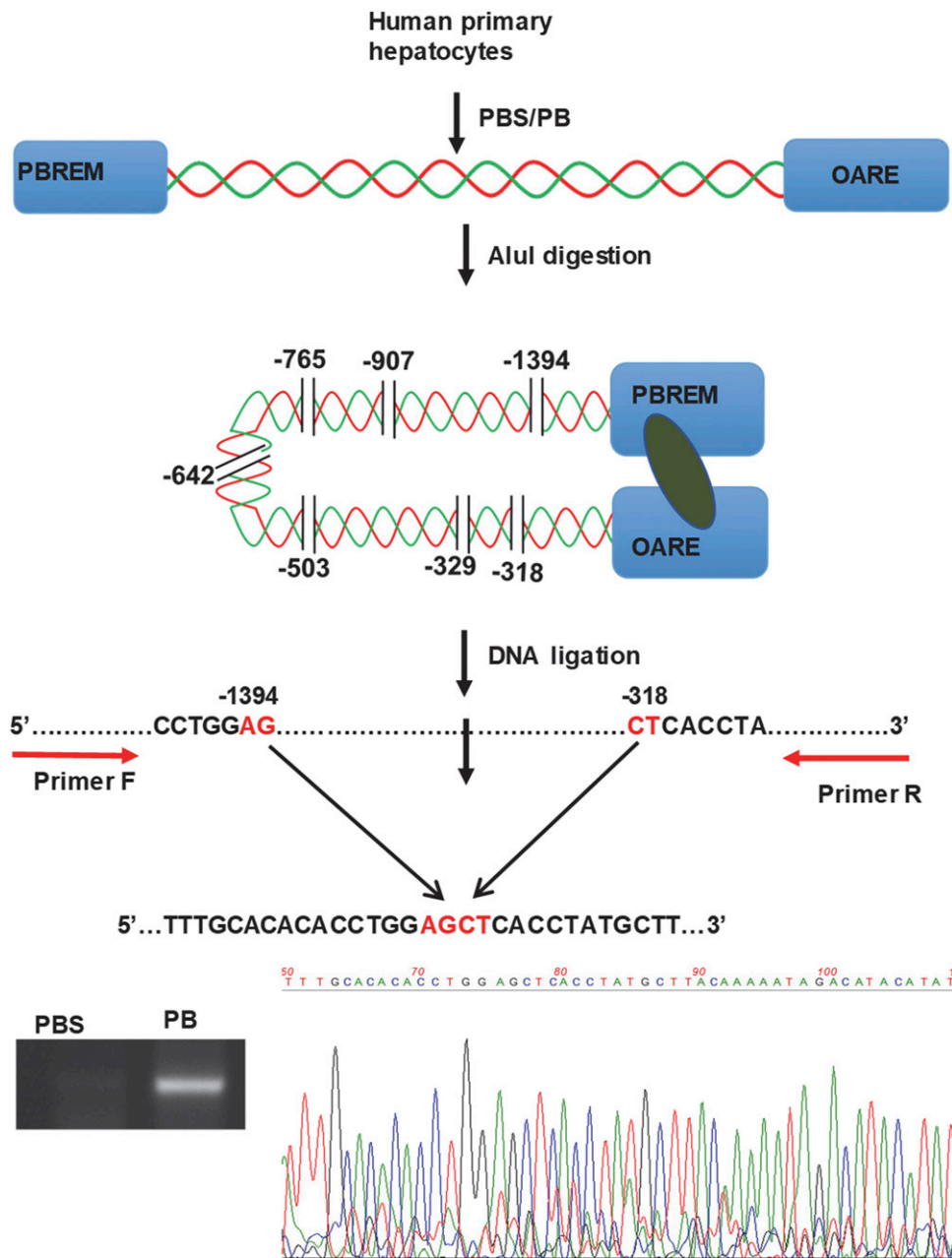
**Fig. 5.** p-Ser100 RORα exists in nucleus in mouse liver. (A) RORα phosphorylation by p38 MAP kinase in HepG2 cell. HepG2 cells were transfected with RORα WT or RORα S100A with p38 MAP kinase expression vectors. Western blot assay was employed to determine phosphorylation of RORα S100 residue. (B) Expression of RORα and p-Ser100 RORα in human primary hepatocytes. The cells were treated with PBS or PB for 24 hours and the Western blot assay was determined in whole-cell lysate by anti-RORα or p-Ser100 RORα antibodies. HSP90, heat shock protein 90. (C and D) CAR WT and CAR KO mice treated with PB for 24 hours, and livers were collected for Western blot (C) or immunohistochemical staining (D) with anti-RORα or anti-p-Ser-RORα antibodies. Both immunohistochemical staining and Western blot assay findings suggested that though RORα was expressed both in the cytoplasm and nuclear fractions of the mouse liver, p-Ser100 RORα expression was limited in the nucleus.

PBREM after PB treatment. Similarly, both HNF4α and CAR were enriched on the PBREM motifs after PB treatment. Although we have no anti-CAR antibody working for ChIP analysis, we consider that RXRα ChIP results in Fig. 3 suggested CAR binding on PBREM, since previous results showed PB treatment of HPH translocate cytoplasmic localized CAR into nucleus (Li et al., 2009), and CAR-RXRα heterodimer strongly binds to the NR1 sequence in PBREM (Sueyoshi et al., 1999). Although it seems unlikely, the possibility of other RXRα partner involved in the PBREM ChIP complex remains. Consistent with the ChIP results, our 3C assay indicated that chromatin containing the CYP2B6 promoter went through dynamic structural modulation by PB treatment, such that both the PBREM and OARE elements existed in the same complex, as evidenced by efficient ligation that occurred between the DNA fragments including these elements. Taking all these observations together, it is inevitable to postulate that through binding their respective response elements, CAR, RORα, and HNF4α mediate the induction of CYP2B6 gene expression in human primary hepatocytes (Fig. 7).

In this hypothetical model (Fig. 7), though RORα and p-Ser100 RORα occupied the 2B6-RORE, HNF4α bound to the OARE in untreated cells. Moreover, since HNF4α was detected on the 2B6-RORE in untreated cells and there is no HNF4α direct binding site in 2B6-RORE, there appears to be an interaction between the RORα on the 2B6-RORE and HNF4α on OARE. This interaction was essential for the modulation of the basal expression of the gene, which was confirmed by the suppression of CYP2B6 mRNAs in RORα-silenced primary hepatocytes. On the other hand, on the basis

of our 3C assay and series of cell-based reporter analyses, it was remarkably evident that PB induced changes in CYP2B6 chromatin conformation. Thus, it is plausible to suggest that PB triggers this chromatin remodeling by inducing activation of nuclear receptor CAR through dephosphorylation of its Thr38 residue (Mutoh et al., 2009, 2013), CAR binding to the PBREM motif (Sueyoshi et al., 1999), and its interactions with RORα and HNF4α. For the interactions, p-Ser100 RORα on the 2B6-RORE was vital, which was indicated by the co-IP assay that CAR preferably interacted with RORα S100D mutant (Fig. 4A). In ChIP analysis shown in Fig. 3, we observed RXRα on the 2B6-RORE in untreated and PB-treated hepatocytes. It is not clear what form of RXRα-containing heterodimer exists on 2B6-RORE at this stage of research, and future analysis on the RXRα complex will be necessary.

In our earlier work using CAR WT/knockout (KO) and RORα WT/RORα<sup>sg/sg</sup> mice models, we reported that though PB induced the mSult1e1 gene in CAR WT or RORα WT mice, it did not induce the gene in either CAR KO or RORα<sup>sg/sg</sup> mice liver (Fashe et al., 2018). Mechanistically, in untreated mouse liver, RORα directly bound to its RORE on mSult1e1 and suppressed gene expression. In PB-treated mouse liver cells, however, RORα was phosphorylated at its S100 residue, which limited its DNA-binding property. Interestingly, this phenomenon facilitated the CAR binding in PB-treated mouse liver cells as a DR4 unit m1E1-PBREM element and RORα binding site through m1E1-RORE shared half-site, and S100 phosphorylated RORα interaction with CAR as coactivator (Fashe et al., 2018). In this study, we observed a statistically significant change in S100-phosphorylated RORα enrichment

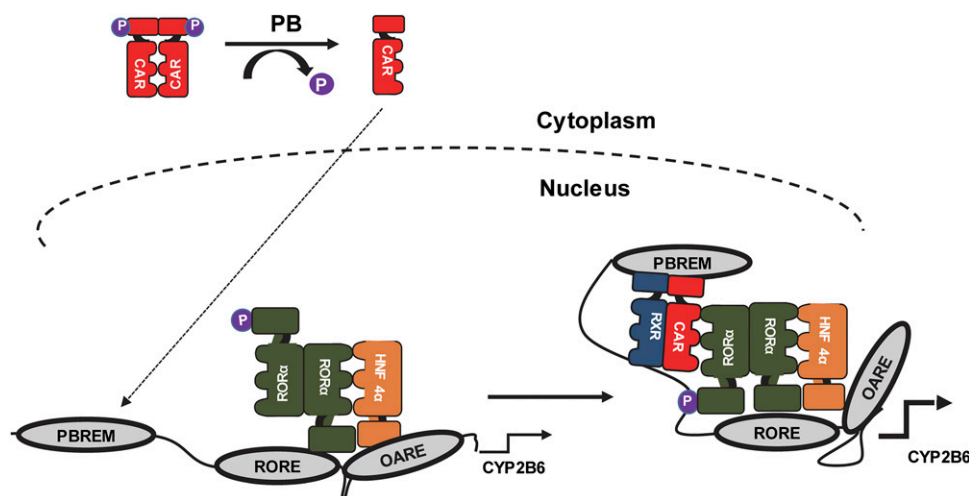


**Fig. 6.** Phenobarbital induced change in CYP2B6 chromatin conformations in human primary hepatocytes. 3C assay was performed using a restriction enzyme AluI and forward and reverse primers starting at  $-1495$  and  $-195$  upstream CYP2B6 transcription site, respectively. After digestion and ligation, the primers amplified a 207-bp DNA fragment only in PB-treated samples, as confirmed by agarose gel electrophoresis and subsequent sequencing.

on PBREM but not on OARE or 2B6-RORE following PB treatment. Thus, it seemed that PB did not affect the S100 ROR $\alpha$  phosphorylation on 2B6-RORE, but it induced change in CYP2B6 chromatin conformation through CAR and p-Ser100 ROR $\alpha$  interaction, which in turn resulted in an increased p-ROR $\alpha$  enrichment on the PBREM in HPH treated with PB. Kinase(s) involved in the phosphorylation of ROR $\alpha$  in CYP2B6 gene regulation is not known at this point. We observed ectopically expressed P38 MAP kinase phosphorylated Ser100 of ROR $\alpha$  in this report (Fig. 5A), and protein kinase C phosphorylated the Ser in previous report (Fashe et al., 2018). A recent publication (Hori et al., 2016) by our group found phosphorylated P38 MAP kinase interacted with CAR in nucleus after CAR was activated and translocated into nucleus. P38 MAP kinase-dependent phosphorylation of CAR subsequently causes inactivation of this receptor. Similarly,

CAR-P38 MAP kinase complex may affect ROR $\alpha$  phosphorylation status after PB activation. However, our ChIP analysis in Fig. 4 and Western blotting in Fig. 5 were not able to detect ROR $\alpha$  phosphorylation increases by PB treatment. Furthermore, we could not find any evidence CAR-P38 MAP kinase interaction affects ROR $\alpha$  phosphorylation observed in Fig. 5A (unpublished data). Thus, physiologic relevance of our finding in Fig. 5A for CYP2B6 gene regulation may not be clear enough at this point, and future analysis will be necessary for establishing P38 MAP kinase roles in ROR $\alpha$  Ser100 phosphorylation.

ROR $\alpha$  is known to be a key element for circadian gene regulation, in which competition between RORs and REV-ERBs for the shared response element RORE in circadian gene brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1 (Bmal1) plays pivotal roles (Guillaumond et al., 2005;



**Fig. 7.** Schematic representation of molecular mechanism of phenobarbital triggered induction of CYP2B6 in human primary hepatocytes. At noninduced condition, ROR $\alpha$  and p-Ser100 ROR $\alpha$  occupied the 2B6-RORE motif while HNF4 $\alpha$  occupied the OARE, and none of the tested factors were detected on the PBREM. In response to PB treatment, the dynamics of the protein-DNA and protein-protein interactions on the CYP2B6 promoter were changed as a result of CAR recruitment onto the PBREM. As previously reported (Mutoh et al., 2009, 2013), dephosphorylated CAR was translocated into the nucleus and commenced the PB induction process. Then p-Ser100 ROR $\alpha$  facilitated the interaction between the factors on the PBREM, RORE, and OARE, thereby enabling the formation of transcriptionally active chromatin complex.

Solt et al., 2011). Although little is known about CYP2B6 circadian activity changes, hepatic clearance of nevirapine, which is largely metabolized by CYP2B6 (Ward et al., 2003), was reportedly higher during daytime in children (Bienczak et al., 2017). Moreover, in rats treated with PB, the magnitude of CYP2B1/2 mRNA induction showed daytime dependence (Kanno et al., 2004), and it has been long known that circadian clock regulates the expression of CYP enzymes (Froy, 2009). Thus, it is conceivable that the ROR $\alpha$  regulation of CYP2B6 can be associated with the differential expression CYP2B6 across the biologic clock and how p-Ser100 ROR $\alpha$  involved in this regulation will be interesting research target.

In the series of recent publication by our group, numerous biologic processes were shown to be regulated by the phosphorylation of highly conserved phosphorylation site in DNA-binding domain of these receptors (Mutoh et al., 2009, 2013; Shindo et al., 2012, 2013; Hashiguchi et al., 2016; Negishi, 2017; Sueyoshi et al., 2019). These phosphorylation sites are located between two zinc finger domain and conserved in 41 out of 48 human nuclear receptors. First, we observed CAR Thr38 dephosphorylation by PB through the chemical's inhibitory effect on epidermal growth factor signaling pathway, and the receptor was translocated into nucleus for activation of target genes (Mutoh et al., 2009, 2013). This phosphorylation was later shown to regulate the intramolecular interaction between DNA-binding domain and ligand binding domain of CAR, causing CAR dimer interphase structure and regulation of active monomer-inactive dimer conversion of the receptor (Shizu et al., 2017, 2018). We also found ER $\alpha$  S212 phosphorylation did not affect its activity as transcription factor, and phospho-mimetic mutant of ER $\alpha$  activated distinct set of genes compared with S212A ER $\alpha$  (Shindo et al., 2012). Ser212-phosphorylated ER $\alpha$  was found in peripheral blood neutrophils, and the cells were found to migrate into the mouse uterus (Shindo et al., 2013). Furthermore, FXR Ser154 phosphorylation made this receptor inactive, and this phosphorylated FXR in the nucleus of centrilobular hepatocytes in FXR ligand treated mice (Hashiguchi et al., 2016). Most recently, RXR $\alpha$  Thr167 phosphorylation was found in fasting mouse white adipose tissue, and genes for key enzymes of energy metabolism in the tissue were differentially regulated in the mutant mice with RXR $\alpha$  Thr167 mutated to Ala (Sueyoshi et al., 2019).

Here, we found ROR Ser100 phosphorylation plays key role in CYP2B6 gene induction, and this represents another example of the roles of conserved phosphorylation sites in regulation of nuclear receptor function. Situated on the newly identified RORE, termed here as 2B6-RORE, positioned between the PBREM and OARE motifs of the CYP2B6 promoter, p-Ser ROR $\alpha$  facilitated the looping CAR-RXR $\alpha$  onto the proximal CYP2B6 promoter to initiate the assembly of the transcription machinery on the CYP2B6 promoter prior to the induction of gene transcription. Given the diverse functions and near-ubiquitous expression of ROR $\alpha$ , numerous biologic processes may be regulated by this ROR $\alpha$  phosphorylation. This report will be a prologue for the future findings of such biologic functions regulated by the ROR $\alpha$  Ser 100 phosphorylation.

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#### Authorship Contributions

*Participated in research design:* Fashe, Hashiguchi, Negishi, Sueyoshi.

*Conducted experiments:* Fashe, Hashiguchi, Sueyoshi.

*Performed data analysis:* Fashe, Negishi, Sueyoshi.

*Wrote or contributed to the writing of the manuscript:* Fashe, Sueyoshi.

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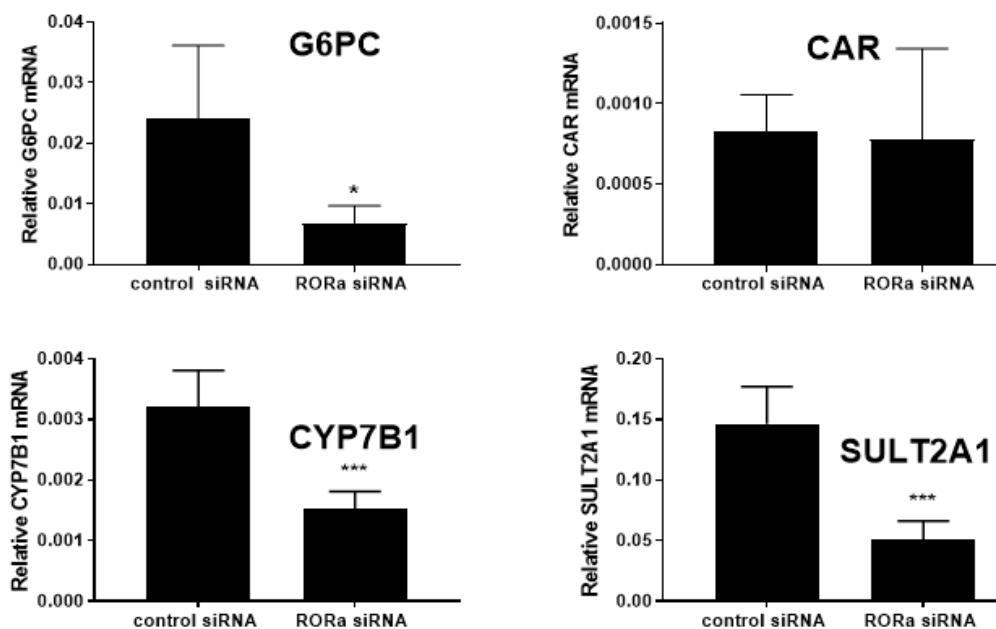
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## Supplemental Data

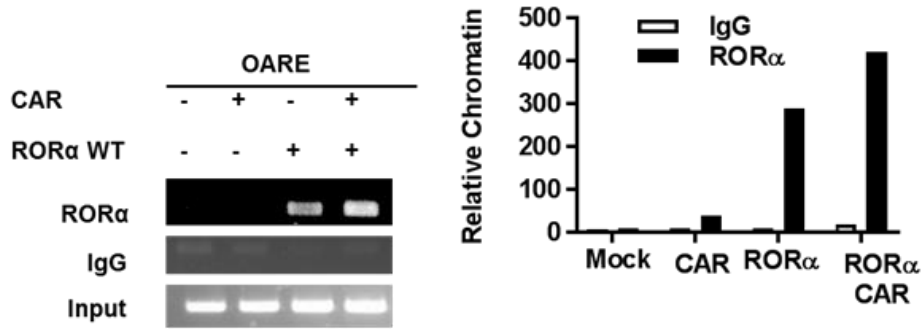
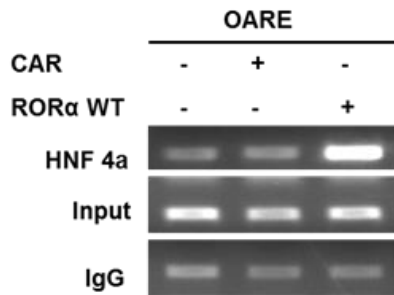
Title: Phosphorylated serine 100 of ROR $\alpha$  orchestrates CAR and HNF4 $\alpha$  to form an active chromatin complex in response to phenobarbital to regulate induction of CYP2B6

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**Supplemental Figure 1.** Expression of ROR $\alpha$  target (G6PC, CYP7B1, and SULT2A1) and non-target gene (CAR) in human primary hepatocytes before and after ROR $\alpha$  knockdown by siRNAs. The cells were treated with control or ROR $\alpha$  siRNAs for 72 hrs. and total RNAs were extracted using Trizol® reagent. cDNAs were prepared by using reverse transcriptase before mRNA estimation using probes RT-PCR.

**A****B**

**Supplemental Figure 2.** (A) CAR enhanced ROR $\alpha$  binding onto the OARE motif. ChIP PCR and quantitative ChIP PCR assay was performed in Huh7 cells transiently expressing CAR, ROR $\alpha$  or both using anti-ROR $\alpha$  antibody. While ROR $\alpha$  could be detected on the OARE sequence, cotransfection of CAR and ROR $\alpha$  into the cells enhanced the ROR $\alpha$  binding at this motif. The left panel shows ChIP-PCR data while the right panel quantitative ChIP PCR results depicts relative chromatin enrichment on the OARE sequence. **B.** ChIP assay depicting the enhancement of HNF 4 $\alpha$  binding at the OARE sequence in Huh7 cells transiently expressing ROR $\alpha$ . Huh7 was transfected with CAR or ROR $\alpha$  expression vectors. Endogenously expressed HNF4 $\alpha$  binding onto the OARE motif was monitored by ChIP PCR.