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Time-dependent interaction between DEC2 and C/EBP α underlies the circadian expression of *CYP2D6* in serum-shocked HepG2 cells

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Running title

DEC2 and C/EBP α regulate circadian expression of *CYP2D6*

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Abbreviations:

DEC2, differentiated embryo chondrocyte-2; HNF4 α , hepatic nuclear factor-4 α ; C/EBP α , CAAT enhancer binding protein- α ; CYPs, cytochrome P450; HDAC1, histone deacetylase-1; TSA, trichostatin A; MAMC, 7-Methoxy-4-(aminomethyl) coumarin; HAMC, 7-hydroxy-4-aminomethylcoumarin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide; bHLH, basic helix-loop-helix

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Abstract

Differentiated embryo chondrocyte-2 (DEC2), also known as bHLHE41 or Sharp1, is a pleiotropic transcription repressor that controls the expression of genes involved in cellular differentiation, hypoxia responses, apoptosis, and circadian rhythm regulation. Although a previous study demonstrates that DEC2 participates in the circadian control of hepatic metabolism by regulating the expression of cytochrome P450 (CYPs), the molecular mechanism is not fully understood. We reported previously that brief exposure of HepG2 cells to 50% serum resulted in 24-h oscillation in the expression of CYP3A4 as well as circadian clock genes. In this study, we found that the expression of CYP2D6, a major drug-metabolizing enzyme in humans, also exhibited a significant oscillation in serum-shocked HepG2 cells. DEC2 interacted with C/EBP α accompanied by forming a complex with histone deacetylase-1, which suppressed the transcriptional activity of C/EBP α to induce the expression of CYP2D6. The oscillation in the protein levels of DEC2 in serum-shocked HepG2 cells was nearly anti-phase to that in the mRNA levels of CYP2D6. Transfection of cells with siRNA against DEC2 decreased the amplitude of CYP2D6 mRNA oscillation in serum-shocked cells. These results suggest that DEC2 periodically represses the promoter activity of CYP2D6, resulting in its circadian expression in serum-shocked cells. DEC2 appears to constitute a molecular link through which output components from the circadian clock are associated with the time-dependent expression of hepatic drug-metabolizing enzyme.

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Introduction

Most living organisms exhibit behavioral and physiological rhythms with a period length of about 24 h. Some of these rhythms are controlled by a self-sustained oscillation mechanism called the circadian clock. Molecular studies of the circadian clock system have revealed that oscillation in the transcription of specific clock genes plays a central role in the generation of 24-h rhythms (Gekakis et al., 1998; Kume et al., 1999). In mammals, the core molecular mechanism of the oscillator consists of two transcriptional activators, CLOCK and BMAL1, and their transcriptional targets, PERIOD (PER) and CRYPTOCHROME (CRY). PER and CRY proteins act as negative regulators of CLOCK/BMAL1 activity, thus forming the major circadian autoregulatory feedback loop (Reppert and Weaver, 2002). The expression of clock genes is also modulated by a second oscillation loop composed of two orphan nuclear receptors, REV-ERB α and retinoid-related orphan receptor- α , which drive the circadian oscillation in *Bmal1* transcription (Preitner et al., 2002; Akashi and Takumi, 2005).

The circadian oscillators in hepatic cells drive rhythmic physiology through these transcriptional factors, which in turn regulate the transcription of downstream genes (Gachon et al., 2006). D-site-binding protein (DBP) and E4 promoter-binding protein 4 (E4BP4) are examples of such output mediators because they are transcriptionally regulated by core oscillator components (Ripperger et al., 2000; Ueda et al., 2005). Clock genes and clock-controlled output genes are expressed rhythmically not only in the suprachiasmatic nucleus (SCN), the center of mammalian circadian clock, but also in other brain regions and peripheral tissues (Sakamoto et al., 1998; Yamamoto et al., 2004). The master clock located in the SCN follows a daily light/dark cycle and, in turn, synchronizes subsidiary oscillators in other brain regions and many peripheral tissues through neural and/or humoral signals (Balsalobre et al., 2000a; Terazono et al., 2003).

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These subsidiary oscillators coordinate a variety of biological processes, producing 24-h rhythms in physiology and behavior. Such rhythmic expression of clock genes is also observed in cultured cells after brief treatment with various compounds (high concentration serum, forskolin, phorbol-12-myristate-13-acetate, calcimycin, or dexamethasone) (Balsalobre et al., 1998; Balsalobre et al., 2000a; Balsalobre et al., 2000b); therefore, the peripheral oscillator in cultured human cells could constitute an in-vitro model for the molecular oscillator in human tissues.

The *CYP* gene superfamily encodes a group of heme-containing monooxygenases, many of which metabolize compounds employed as therapeutic drugs. CYP3A4 is the most abundant CYP expressed in the human liver and small intestine, contributing to the metabolism of approximately half of the drugs in clinical use today (Evans and Relling, 1999). The expression of CYP3A4 and its metabolizing activity exhibit significant circadian oscillation in serum-shocked HepG2 cells (Takiguchi et al., 2007). DBP and E4BP4 regulate the circadian expression of the *CYP3A4* gene, suggesting a molecular link between the circadian clock and xenobiotic metabolism. Differentiated embryo chondrocyte-2 (DEC2), also known as bHLHE41 or Sharp1, is a basic helix-loop-helix (bHLH) transcriptional repressor and acts as an output component of the circadian oscillator. DEC2 regulates the expression of genes involved in cellular differentiation, hypoxia responses, apoptosis, and circadian rhythms (Honma et al., 2002; Miyazaki et al., 2002). A previous study demonstrated that DEC2 also participates in the circadian control of hepatic metabolism by regulating the expression of CYPs (Noshiro et al., 2004); however, the regulation mechanism remains to be fully understood.

Families CYP1, CYP2, and CYP3 encompass the most relevant xenobiotic-metabolizing CYPs in humans (Evans and Relling, 1999). Despite the low content of CYP2D6 isoform in

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human hepatic cells, a variety of drugs are metabolized by this enzyme (Daly et al., 1993; Wolf and Smith, 1999). However, circadian regulation of *cyp2d* genes has been little explored even in experimental animals. In this study, we found that brief exposure of HepG2 cells to 50% serum also induced a significant 24-hr oscillation in the expression of *CYP2D6*. Although hepatic expression of *CYP2D6* is dependent on both hepatic nuclear factor-4 α (HNF4 α) and CAAT enhancer binding protein- α (C/EBP α), DEC2 repressed C/EBP α -induced transactivation of the *CYP2D6* gene. Therefore, we investigated the underlying mechanism of the circadian expression of CYP2D6 in serum-shocked cells by focusing on transcriptional interaction between DEC2 and C/EBP α .

Materials and methods

Cell culture and animals. HepG2 cells were supplied by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (SAFC Bioscience, Kansas City, MI) at 37°C in a humidified 5% CO₂ atmosphere. Male ICR mice were housed under a 12-h light/dark cycle (lights on at Zeitgeber time 0) with food and water *ad libitum*. They were cared for in accordance with the guidelines established by the Animal Care and Use Committee of Kyushu University. Primary culture of hepatocytes were prepared by standard techniques, and cells were maintained in hepatocyte maintenance medium supplemented with 5% FBS, 0.1 μ M insulin, 0.1 μ M dexamethasone, 50 μ g/ml gentamicin, and 50 ng/ml amphotericin B.

Experimental design. To synchronize the circadian clocks in cultured HepG2 cells, serum

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shock was performed as follows: cells were grown to semi-confluence in DMEM supplemented with 10% FBS and then incubated in serum-starved medium for 12 h. On the day of serum shock, 50% FBS or PBS (control) was added for 2 h and then cells were changed back to starvation medium. Cells were harvested for RNA extraction at 0, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, and 60 h after serum treatment. The mRNA levels of *CYP1A2*, *2C9*, *2C19*, and *2D6* were measured by reverse transcription (RT)-PCR. To quantify the protein levels of CYP2D6 and its enzymatic activity, microsomes were prepared from HepG2 cells at 0, 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60 after serum treatment. The protein abundance of CYP2D6 was also determined by immunoblotting. The enzymatic activity of CYP2D6 in microsomal fraction was investigated using 7-Methoxy-4-(aminomethyl) coumarin (MAMC). To investigate the influence of HNF4 α and clock gene products on the expression of endogenous *CYP2D6*, HepG2 cells were transfected with expression plasmids encoding HNF4 α , C/EBP α , CLOCK, BMAL1, PER2, CRY1, DEC2, DBP, E4BP4, retinoic orphan receptor- α (ROR α), or REV-ERB α . At 24 h after transfection, mRNA levels of *CYP2D6* were determined by RT-PCR. To clarify whether endogenous HNF4 α and clock gene products affect the expression of CYP2D6 protein, HepG2 cells were transfected with siRNA against HNF4 α , C/EBP α , or DEC2. Twenty-four hours after transfection, the protein levels of HNF4 α , C/EBP α , DEC2, or CYP2D6 were assessed by Western blotting. The transcriptional mechanism of *CYP2D6* by HNF4 α , C/EBP α , and DEC2 was analyzed using luciferase reporter vectors containing the 5'-flanking region of the *CYP2D6* gene. The luciferase reporter assay was also performed in the presence or absence of trichostatin A (TSA), a histone deacetylase inhibitor. Interactions between DEC2 and C/EBP α were investigated by the immunoprecipitation assay. The binding of endogenous C/EBP α , DEC2, or Histone deacetylase-1 (HDAC1) on the CYP2D6 promoter in HepG2 cells was analyzed by

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chromatin immunoprecipitation (ChIP). To explore the role of DEC2 in the circadian regulation of CYP2D6 expression, HepG2 cells were transfected with siRNA against DEC2 and thereafter treated with 50% FBS as described above. To explore whether the expression of *Cyp2d* gene in the experimental animals exhibit circadian oscillation, the temporal profiles of *Cyp2d9* and *2d22* mRNA were assessed using serum-shocked primary culture of hepatocytes. We also investigated the temporal expression profiles of *Cyp2d9* and *2d22* mRNA in mouse liver kept under 12-h light/dark cycle.

Quantitative reverse transcription-PCR analysis. Total RNA was extracted using RNAiso (Takara Bio Inc., Shiga, Japan). Complementary DNA (cDNA) was prepared via reverse transcription of total RNA using a ReverTra Ace[®] qPCR RT kit (Toyobo Co. Ltd., Osaka, Japan). Diluted cDNA samples were analyzed by real-time or semi-quantitative RT-PCR. Real-time PCR was performed using THUNDERBIRD[™] SYBR[®] qPCR Mix (Toyobo) and the 7500 Real-time PCR system (Applied Biosystems, Foster City, CA). The sequences of primer pairs are listed in Table 1.

Determination of CYP2D6 activity. The enzymatic activity of CYP2D6 in HepG2 cells was determined by assessing the production rate of 7-hydroxy-4-aminomethylcoumarin (HAMC), O-demethylated metabolite of MAMC (Onderwater et al., 1999). Microsomal fractions prepared from HepG2 cells were incubated in 0.1 M potassium phosphate buffer (pH 7.4) containing 10 μ M NADPH and 0.4 mM EDTA. We added the inhibitory antibodies against CYP1A2 into the reaction mixture, because MAMC is also metabolized by CYP1A2 (Onderwater et al., 1999). In fact, antibodies against CYP1A2 decreased the production rate of HAMC in HepG2 microsomal fraction by about 12%, whereas inhibitory antibodies to CYP2D6 reduced the activity by about 85%. After equilibration at 37 °C, MAMC was added into the

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reaction mixture at final concentration of 25 μ M, and the real-time increase in fluorescence was recorded by spectrofluorometer with the excitation wavelength set at 405 nm and emission set at 480 nm. The production rate of HAMC was quantified from the resulting increase in fluorescence. Protein concentration of reaction mixtures was determined using a BCA Protein Assay Kit (Pierce Biotechnology Inc., Rockford, IL). The CYP2D6 activity was expressed as picomoles of HAMC during the 1-min incubation per milligram of protein (pmol/min/mg protein).

Western blotting. Samples (20 μ g protein) were separated on SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. The membranes were reacted with antibodies against human CYP2D6 (Nihon Nosan, Kyoto, Japan), HNF4 α , C/EBP α , DEC2, HDAC1, or ACTIN (Santa Cruz Biotechnology, Santa Cruz, CA). Specific antigen/antibody complexes were made visible using peroxidase-conjugated secondary antibodies and Chemi-Lumi One (Nacalai Tesque Inc., Kyoto, Japan).

Small interference RNA. We designed siRNA for knockdown experiments using BLOCK-iTTM RNAi Designer (<https://rnaidesigner.invitrogen.com/rnaiexpress/>). The target sequences of *C/EBP α* , *HNF4 α* and *DEC2* gene are listed in Table 2. One day before transfection, HepG2 cells were seeded (5 \times 10⁵ per well) in six-well plates containing serum-free DMEM. siRNA against HNF4 α , C/EBP α , DEC2, or scrambled control oligo (200 ng each) was transfected into the cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA).

Construction of reporter plasmids and expression vectors. To construct the luciferase reporter vectors of the human *CYP2D6* gene [*CYP2D6*-Luc], approximately 1.4 kbp fragment (-1399 to +89; +1 indicates the putative transcription start site) derived from the 5'-flanking region of the human *CYP2D6* gene (GenBank accession no. DQ211353) was amplified by PCR

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from genomic DNA of HepG2 cells. The PCR products were purified and ligated into the pGL3 basic vector (Promega, Madison, WI). The mutant construct of *CYP2D6*-Luc was prepared by changing the sequence from CATTGCACAATG to CAAAGCTTAATG (bases –1231 to –1220) and from AGGGCAAAGGCCA to AGGGTAAGCTTCA (bases –55 to –43), respectively. Expression plasmids of HNF4 α , C/EBP α , CLOCK, BMAL1, PER2, CRY1, DEC2, DBP, E4BP4, ROR α , and REV-ERB α were prepared as follows: the coding regions of the transcriptional regulators were obtained by RT-PCR and used after their sequences had been confirmed. All coding regions were ligated into the pcDNA 3.1 vector (Invitrogen, Carlsbad, CA, CA).

Transcriptional assay. On the day before transfection, the cells were seeded (2×10^5 cells/well) into six-well plates containing DMEM supplemented with 10% FBS. Cells were transfected with 100 ng reporter constructs and 1 μ g (total) expression vectors, using Lipofectamine-LTX reagent (Invitrogen) according to the manufacturer's instructions. To correct for variations in transfection efficiency, 0.5 ng pRL-SV40 (Promega) was cotransfected in all experiments. The total amount of DNA per well was adjusted to 1.0 μ g by adding pcDNA3.1 vector (Invitrogen). The transfected cells were incubated in the presence or absence of trichostatin A (TSA), a histone deacetylase inhibitor. At 48 h after transfection, cell extracts were prepared with 500 μ l passive lysis buffer (Promega), and 50 μ l of the extracts were used for assays of firefly luciferase and Renilla luciferase by luminometry. The ratio of firefly luciferase activity (expressed from reporter plasmids) to Renilla luciferase activity (expressed from pRL-SV40) in each sample served as a measure of normalized luciferase activity.

Immunoprecipitation assay. Nuclear fractions of HepG2 cells were prepared at the indicated time points after 50% serum treatment. The fractions were immunoprecipitated by

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anti-DEC2 or anti-C/EBP α antibodies on protein G-agarose beads. The specific bound proteins were released by resuspending beads in 20 μ l loading buffer, divided into equal amounts, and resolved by sodium dodecyl sulfate–polyacrylamide gels (SDS-PAGE). One gel was subjected to Western blotting with anti-DEC2, anti- C/EBP α , or anti-HDAC1 antibodies.

Chromatin immunoprecipitation assay. Cells were crosslinked with 1% formaldehyde in phosphate-buffered saline at 4°C for 10 min. Each crosslinked sample was sonicated on ice and then incubated with antibodies against C/EBP α , DEC2, or HDAC1. DNA was isolated from the immunoprecipitates and subjected to PCR using the following primer pairs: for the surrounding C/EBP α binding site in the CYP2D6 promoter (from bp -1312 to -1074), 5'-TGGTGAAACCCTATCTCTACTG-3' and 5'-TCACTGCAGTCTCGACATCA-3'; for CYP2D6 promoter that does not contain C/EBP α binding sites (from bp -827 to -570), 5'-CCTGTTGCAAACAAGAAGCCATAG-3' and 5'-GGACACGATTACACATGCAGAA-AAT-3'. As negative controls, chromatin immunoprecipitations were performed in the absence of antibody or in the presence of rabbit IgG. PCR products from these samples were not detectable by ethidium bromide staining.

Statistical analysis. The statistical significance of the differences among groups was analyzed by analysis of variance and Dunnett's test or Bonferroni's multiple comparison test. A 5% level of probability was considered significant.

Results

Rhythmic expression of CYP2D6 gene in HepG2 cells after serum treatment. We demonstrated previously that treatment of HepG2 cells with 50% FBS for 2 h induced the

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rhythmic expression of CYP3A4 and CYP2E1 (Takiguchi et al., 2007; Matsunaga et al., 2008). Treatment of cells with high concentration serum transiently induced the expression of CYP1A2 mRNA, but was unable to affect the mRNA levels of CYP2C9 and 2C19 (**Fig. 1A**). On the other hand, serum-shocked HepG2 cells exhibited a significant oscillation in the expression of CYP2D6 ($P < 0.05$; **Fig. 1A**). The rhythmic phase of mRNA levels for CYP2D6 was similar to those of Dbp as reported previously (Takiguchi et al., 2007); however, the oscillation in the levels of CYP2D6 protein was delayed by approximately 8 h relative to its mRNA rhythm (**Fig. 1B**). The rhythmic pattern of CYP2D6 protein expression resembled the overall decreases and increases in the enzymatic activity of CYP2D6 (**Fig. 1C**). These in-vitro results suggest that oscillation in the expression of the CYP2D6 gene is cell autonomous. The rhythmic change in mRNA levels of CYP2D6 seemed to cause the oscillation of its protein abundance and enzymatic activity.

Effects of clock and clock-controlled gene products on the mRNA levels of CYP2D6.

Clock genes, consisting of core oscillation loops, generate 24-hr variations in output physiology through the periodic activation/repression of clock-controlled output genes (Gachon et al., 2006; Ripperger et al., 2000). To explore whether the products of clock genes and/or clock-controlled output genes affect the expression of CYP2D6, we investigated the effects of the transfection of expression plasmids encoding CLOCK, BMAL1, PER2, CRY1, DEC2, DBP, E4BP4, ROR α , or REV-ERB α on the mRNA level of CYP2D6 in HepG2 cells. Cells were also transfected with expression vectors coding HNF4 α or C/EBP α as a positive control (Cairns et al., 1996; Jover et al., 1998). As shown in **Fig. 2A**, the mRNA level of CYP2D6 was elevated significantly when cells were transfected with HNF4 α or C/EBP α expression plasmids ($P < 0.05$ for both), whereas transfection of cells with DEC2 significantly suppressed the endogenous expression of CYP2D6

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mRNA ($P < 0.05$). Consistent with these findings, transfection of HepG2 cells with siRNA against HNF4 α or C/EBP α resulted in an obvious reduction of CYP2D6 protein levels (**Fig. 2B**), whereas transfection with DEC2 siRNA increased CYP2D6 protein abundance. Among the products of clock genes and/or clock-controlled output genes, DEC2 appears to act as a repressor of CYP2D6. In this experiment, the protein levels of C/EBP α were decreased by transfecting with the siRNA against HNF4 α , but transfection of C/EBP α siRNA had little effect on the protein levels of HNF4 α . These results also suggest that HNF4 α may play a predominant role in hepatic C/EBP α expression.

Transcriptional regulation of the *CYP2D6* gene by HNF4 α and DEC2. To investigate the transrepression mechanism of the *CYP2D6* gene by DEC2, we performed the luciferase reporter assay using native or mutated *CYP2D6* reporter constructs. Cotransfection of *CYP2D6*-Luc with HNF4 α resulted in a 6-fold increase in promoter activity (**Fig. 3B**). A similar increase in promoter activity was also detected when *CYP2D6*-Luc was co-transfected with C/EBP α . These transactivation effects of HNF4 α and C/EBP α were abolished when their binding sites mutated (**Fig. 3B**). DEC2 repressed either HNF4 α - or C/EBP α -mediated transactivation of *CYP2D6*. Although co-transfection with 1.0 μ g DEC2 plasmid partially inhibited \square HNF α -mediated transcription, transfection with the same amount of DEC2 plasmid suppressed the C/EBP α -induced promoter activity of *CYP2D6* at the basal level (**Fig. 3C**). These results suggest that DEC2 represses the transcription of *CYP2D6* by mainly interacting with C/EBP α .

HDAC is often involved in transcriptional repression by bHLH transcription factors, and it has been reported that some actions of DEC2 are suppressed by HDAC inhibitors (Sun and Taneja, 2000; Garriga-Canut et al., 2001). We therefore examined the effects of the HDAC

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inhibitor TSA on DEC2-mediated repression. TSA dose-dependently restored the DEC2-mediated repression of C/EBP α -induced CYP2D6 transactivation (**Fig. 4A**). The results of the immunoprecipitation assay revealed that DEC2 precipitated together with C/EBP α (**Fig. 4B**). Endogenously expressed HDAC1 proteins in HepG2 cells were also co-immunoprecipitated together with DEC2.

Although transfection of HepG2 cells with DEC2 expression vectors had little effect on the binding amounts of C/EBP α to its binding site in the *CYP2D6* promoter, the treatment enhanced formation of HDAC1:chromatin complex (**Fig. 4C**). The DEC2-enhanced HDAC1:chromatin formation seemed to be dependent on the C/EBP α protein, since no DNA bands were detected in HDAC1-immunoprecipitated chromatin by using primer pairs for amplifying the *CYP2D6* promoter region that does not contain C/EBP α binding site (**Fig. 4C**). These results suggest that DEC2 promotes HDAC1 recruitment on the *CYP2D6* promoter through the protein-protein interaction with C/EBP α . The correlation between the interaction of these proteins and the transcriptional regulation of CYP2D6 suggests that suppressive action of DEC2 on C/EBP α -mediated transactivation of CYP2D6 is attributable to the sustained recruitment of HDAC1.

Role of DEC2 in the circadian regulation of *CYP2D6* in serum-shocked HepG2 cells.

The levels of DEC2 mRNA also showed obvious 24-hr oscillation in serum-shocked HepG2 cells (**Fig. 5A**). The rhythmic phase of Dec2 mRNA was similar to Per2 mRNA oscillation (Takiguchi et al., 2007). The oscillation in the expression of DEC2 protein was nearly antiphase to that in the mRNA levels of CYP2D6 (**Fig. 5B**), but the protein levels of C/EBP α and HDAC1 failed to show obvious circadian oscillation in serum-shocked cells. Immunoprecipitation experiments revealed that in serum-shocked HepG2 cells, the amount of DEC2 associated with

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C/EBP α increased at the time corresponding to the trough of the CYP2D6 mRNA expression (**Fig. 5C**), whereas a decrease in the amount of DEC2-C/EBP α complex almost matched the peak of CYP2D6 expression. As shown in **Fig. 2B**, transfection of cells with siRNA against DEC2 caused an elevation of CYP2D6 protein levels. Furthermore, treatment of cells with DEC2 siRNA also prevented serum shock-induced oscillation in the expression of CYP2D6 mRNA (**Fig. 5D**). These results indicate that DEC2 protein interacts with C/EBP α in a time-dependent manner. Time-dependent interactions may underlie the circadian expression of CYP2D6 in serum-shocked HepG2 cells.

Rhythmic expression of *Cyp2d9* gene in mouse hepatocytes. In the final set of experiment, we explored whether the expression of *Cyp2d* gene in the experimental animals also exhibited circadian oscillation. Computer-aided analysis identified putative C/EBP α -binding sites in the promoter region of the mouse *Cyp2d9* gene, the murine homolog to human CYP2D6; however, putative binding sites were not found in the promoter region of the mouse *Cyp2d22* gene. The mRNA levels of *Cyp2d9*, but not of *Cyp2d22*, showed significant 24-h oscillation not only in the primary cultured mouse hepatocytes (**p<0.05, Fig. 6A**) but also in the liver of mice (**p<0.05, Fig. 6B**). These findings suggest that putative C/EBP α -binding sites in the promoter region of *Cyp2d9* gene are also functionally important for rhythmic expression of its mRNA.

Discussion

Members of the basic helix-loop-helix (bHLH) family of transcription factors have been shown to play critical roles in cellular differentiation, growth, apoptosis, hypoxia response, and circadian rhythm regulation (Takiguchi et al., 2007; Honma et al., 2002; Miyazaki et al., 2002;

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Azmi, et al., 2004). DEC2 was originally found in rat brain (Rossner et al., 1997) and was subsequently identified in humans and mice (Garriga-Canut et al., 2001; Rossner et al., 1997). DEC2 is expressed in a variety of tissues (Miyazaki et al., 2002; Lu et al., 1999; Fujimoto et al., 2001); however, its role in the circadian regulation of hepatic metabolism has not been fully evaluated. In this study, we showed that DEC2 acts as a potent repressor of C/EBP α . The repressive action of DEC2 on C/EBP α appeared to be the underlying cause of circadian expression of *CYP2D6* in serum-shocked HepG2 cells (**Fig. 7**). The expression levels of several types of CYPs in HepG2 cells have been reported to be lower than those in primary human hepatocytes (Jover et al., 1998; Hara and Adachi, 2002; Westerink and Schoonen, 2007). However, considerable metabolic activity of CYP2D6 was detected in HepG2 cells, and the drug-metabolizing activity also varied in a circadian fashion.

A recent report also demonstrated that DEC2 interacts with C/EBP α to suppress the expression of C/EBP α -target genes (Gulbagci et al., 2009). Protein-protein interaction is dependent on both the bHLH domain and carboxy-terminal region of DEC2 protein. Furthermore, the suppressive actions of DEC2 on C/EBP α -mediated transactivation are suggested to be caused by sustained recruitment of HDAC1. The results of the immunoprecipitation assay revealed that DEC2 interacted with C/EBP α , accompanied by the association with HDAC1. The formation complex with HDAC1 seemed to underlie the suppressive actions of DEC2 on C/EBP α -mediated transactivation. DEC2 also functions as a co-repressor of retinoid X-receptors (RXRs) (Cho et al., 2009). RXRs heterodimerized with pregnane X receptors (PXR) or constitutive androstane receptors (CARs) to regulate the expression of CYP3A4 (Pascussi et al., 2000a; Pascussi et al., 2000b; Chen et al., 2010). Therefore, DEC2 may also contribute to the circadian regulation of CYP3A4 gene in the

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serum-shocked HepG2 cells (Takiguchi et al., 2007).

CYP2D6, a member of the CYP superfamily, is responsible for the metabolism of about 25% of commonly prescribed drugs (Bertilsson and Dahl, 1996). The gene that encodes CYP2D6 has more than 90 variants (Ingelman-Sundberg, 2005; Beverage et al., 2007). Such polymorphism leads to a variety of enzymatic activities and different phenotypes. In fact, the activity of CYP2D6 ranges from complete deficiency to excessive activity, potentially causing medication toxicity or therapeutic failure even at the recommended drug dosage (Ingelman-Sundberg, 2005; Beverage et al., 2007; Zanger et al., 2004). CYP2D6 polymorphism is therefore regarded as the reason for inter-individual differences in the pharmacokinetics and pharmacodynamics of drugs. In addition to inter-individual variation, the present findings using an *in vitro* model of the hepatic circadian clock suggested that there was also intra-individual variation in CYP2D6 activity. In serum-shocked HepG2 cells, significant 24-h oscillation was detected not only in the mRNA levels of CYP3A4 but also in its metabolic activity (Takiguchi et al., 2007). Daily variation in CYP3A4 activity in humans has been suggested by the fact that the pharmacokinetics of several drugs, which are mainly eliminated by CYP3A4 metabolism, vary according to their dosing times (Smith et al., 1986; Min et al., 1997). Although the CYP2D6-mediated drug metabolism in human liver may also vary depending on its dosing time, it has not been clarified whether drug-metabolic activity of CYP2D6 in human liver exhibits circadian oscillation. Further studies are required to investigate this point. The mRNA levels of *Cyp2d9*, the murine homolog to human CYP2D6, exhibited significant circadian oscillation in mouse liver. The oscillation of *Cyp2d9* mRNA levels was nearly anti-phase to that of DEC2 expression (Noshiro et al., 2004). As Computer-aided analysis identified putative C/EBP α -binding sites in the promoter region of the mouse *Cyp2d9* gene, DEC2 may periodically

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repress the transcriptional activity of C/EBP α , thereby inducing the circadian expression of Cyp2d9 mRNA. Taken together these findings suggest the possibility that the expression of CYP2D6 oscillates in human liver.

The individualization of pharmacotherapy has been achieved mainly by monitoring drug concentrations. Consequently, dosage adjustment is based on inter-individual differences in drug pharmacokinetics; however, intra-individual as well as inter-individual variability should be considered to aim at further improving rational pharmacotherapy, because the pharmacokinetics of many drugs also vary depending on rhythmicity in absorption, distribution, metabolism, and elimination (Ohdo et al., 2010). Although the contribution of 24-h variation in CYP2D6 expression to drug metabolism should be clarified, our results suggest a mechanism underlying the dosing time-dependent differences in the pharmacokinetics of drugs and provide a molecular link between the circadian clock and xenobiotic metabolism.

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Authorship contribution

Participated in research design: Matsunaga, Koyanagi, and Ohdo.

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Contributed new reagents or analytic tools: Hanada, Toi, Sato, Fujimoto, and Koyanagi.

Performed data analysis: Matsunaga, Inoue, Yoshiyama, and Koyanagi.

Wrote or contributed to the writing of the manuscript: Matsunaga, Inoue, Koyanagi, and Ohdo.

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Footnotes

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Figure legends

Figure 1 Temporal expression profiles for CYP genes in HepG2 cells after serum treatment.

(A) Quantification of temporal changes in CYP1A2, 2C9, 2C19, and 2D6 mRNA in HepG2 cells after treatment with 50% serum or PBS. Basal level of mRNA (at time point 0) was set at 1.0. Each value is the mean with S.E.M. (N = 3-4). There is a significant time-dependent variation in the mRNA levels of CYP2D6 in serum-treated cells ($P < 0.05$, ANOVA). (B) Temporal expression profile of CYP2D6 protein in HepG2 cells after treatment with 50% serum. Upper panel shows representative electrophoretic image of Western blotting of CYP2D6 protein in the serum shocked HepG2 cells. Protein levels of CYP2D6 were normalized by those of ACTIN, and basal abundance (at time point 0) was set at 1.0. Each value is the mean with S.E.M. (N = 3-4). There is a significant time-dependent variation in the protein abundance of CYP2D6 ($P < 0.05$, ANOVA). (C) Temporal changes in enzymatic activity of CYP2D6 in HepG2 cells after serum treatment. The production rate of HAMC O-demethylated metabolite of MAMC, was assessed as an index of CYP2D6 enzymatic activity. Values are the mean \pm S.E.M (N=6). There is a significant time-dependent variation in the metabolizing activity of CYP2D6 ($P < 0.05$, ANOVA).

Figure 2 Influence of clock gene products on the expression of CYP2D6 in HepG2 cells.

(A) The cells were transfected with 1.0 μ g expression plasmids, encoding HNF4 α , C/EBP α , DBP, E4BP4, ROR α , REV-ERB α , CLOCK, BMAL1, PER2, CRY1, or DEC2. Total RNA was extracted from the cells 24hr after transfection and subjected to RT-PCR of CYP2D6 mRNA. For plots of intensity, the mean value of the control (pcDNA3.1) was set at 1.0. Each value is the mean with S.E.M. (N = 3). * $P < 0.05$ compared with the control (pcDNA3.1) group using Dunnett's test. (B) Influence of the down-regulation of HNF4 α , C/EBP α , or DEC2 on the expression of CYP2D6 protein in HepG2 cells. Cells were transfected with siRNA against

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HNF4 α , C/EBP α , or DEC2 (20 nM each). Protein levels of C/EBP α , HNF4 α , DEC2, and CYP2D6 were assessed by Western blotting.

Figure 3 Transcriptional regulation of CYP2D6 by C/EBP α and DEC2. (A) Schematic representation of human CYP2D6 promoter. Numbers below boxes are nucleotide residues in which C/EBP α and HNF4 α binding sites are positioned relative to the transcription start site (+1). Underlined nucleotide residues indicate mutated sequences of C/EBP α and HNF4 α binding sites. (B) Mutation of sequence of response elements abrogates C/EBP α - or HNF4 α -induced CYP2D6 promoter activity. (C) Repressive action of DEC2 on C/EBP α -induced CYP2D6 promoter activity. Values are the mean \pm S.E.M (N=4).

Figure 4 DEC2 inhibits C/EBP α -mediated transactivation of CYP2D6 by HDAC1 recruitment. (A) Effects of HDAC inhibitor trichostatin A (TSA) on DEC2-mediated transcriptional repression on C/EBP α and HNF4 α . Values are the mean \pm S.E.M (N=4). * P<0.05 for comparison between two groups. (B) DEC2 interacts with C/EBP α , accompanied by forming a complex with HDAC1. Nuclear fractions of HepG2 cells were subjected to immunoprecipitation (IP) with anti-DEC2 or immunoglobulin G (IgG) antibodies, and Western blotted with biotinylated antibodies against DEC2, C/EBP α , or HDAC1. The light chain of immunoglobulin G (IgG-LC) was the positive control. (C) Chromatin immunoprecipitation analysis of CYP2D6 promoter in HepG2 cells. The left panel shows a schematic of the CYP2D6 5'-flanking region. Solid line arrows represent amplification area by PCR. Numbers represent distances in bases from the putative transcriptional start site marked as +1. HepG2 cells were transfected with DEC2 expression vectors. Cross-linked chromatin collected from cells were immunoprecipitated with antibodies against C/EBP α , DEC2, or HDAC1. Representative electrophoretic image of PCR products are shown in right panel.

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Figure 5 DEC2 regulates the circadian expression of CYP2D6 in serum-shocked HepG2 cells. (A) Temporal expression profiles of DEC2 in HepG2 cells after serum treatment. (B) Temporal profiles of protein abundance of C/EBP α , DEC2, and HDAC1 in the nuclear fractions of HepG2 cells after serum treatment. Cells were exposed to 50% serum for 2 h and thereafter nuclear fractions were prepared at the indicated times. (C) Time-dependent interaction between C/EBP α and DEC2 in serum-shocked HepG2 cells. Nuclear fractions were prepared at the indicated time points. Nuclear fractions were subjected to immunoprecipitation (IP) with anti-C/EBP α antibodies, and Western blotted with antibodies against DEC2, C/EBP α , or HDAC1. Band intensities of DEC2 and HDAC1 were normalized by C/EBP α and shown as the mean \pm S.E.M (N = 4-5). * P<0.05 for comparison between two groups. (D) Influence of down-regulation of DEC2 on the circadian oscillation of *CYP2D6* mRNA in serum-shocked HepG2 cells. Cells were transfected with control or Dec2 siRNA on 2 days prior to 50% serum treatment. For all panels, value are the mean \pm S.E.M (N = 4-5). * P<0.05 for comparison between two groups.

Figure 6 Temporal expression profiles for the mRNA levels of *Cyp2d9* and *Cyp2d22* in serum shocked mouse primary cultured hepatocyte (A) and in the liver of mice (B). Values are shown as mean with S.E.M. (n = 3-5). For both panels, levels of *Cyp2d9* mRNA significantly varied in a circadian time-dependent manner (P<0.05; ANOVA).

Figure 7 Schematic representation of molecular mechanism regulating circadian expression of human *CYP2D6* gene in serum-shocked hepatic cells. Hepatic nuclear factors, HNF4 α and C/EBP α , bind to their response elements in the *CYP2D6* promoter to activate its transcription.

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DEC2 periodically interacts with HNF4 α and C/EBP α , thereby repressing their transactivation of the *CYP2D6* gene.

Table 1 Primer sequence used for quantitative RT-PCR analysis

Human <i>CYP1A2</i>	Forward primer	5'-CATCCC CCACAGCACAACAA-3'
	Reverse Primer	5'-TCCCACTTGGCCAGGACTTC-3'
Human <i>CYP2C9</i>	Forward primer	5'-AGCTTGGAAAACACTGCAGT- 3'
	Reverse Primer	5'-CCTGCTGAGAAAGGCATGAAG-3'
Human <i>CYP2C19</i>	Forward primer	5'-CCTGCTGAGAAAGGCATGAAG-3'
	Reverse Primer	5'-CCTGCTGAGAAAGGCATGAAG-3'
Human <i>CYP2D6</i>	Forward primer	5'-CCCATATGACATCCCGTGACATC-3'
	Reverse Primer	5'-TTGGTGATGAGTGTTCGTTCCCTTA -3'
Human <i>β-ACTIN</i>	Forward primer	5'-GACAGGATGCAGAAGGAGATTACT-3'
	Reverse Primer	5'-TGATCCACATCTGCTGGAAGGT-3'
Mouse <i>Cyp2d9</i>	Forward primer	5'-TGGCACAGATAGAGAAGGCCA-3'
	Reverse Primer	5'-TCACGCACCACCATGAGC-3'
Mouse <i>Cyp2d22</i>	Forward primer	5'-GACACCCTTTCAGCCCTAACA-3'
	Reverse Primer	5'-GAAGCGTGGGTCATCGTACT-3'
Mouse <i>β-Actin</i>	Forward primer	5'-CCAGGGTGTGATGGTGGGAA-3'
	Reverse Primer	5'-TTCACGGTTGGCCTTAGGGT-3'

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Table 2 siRNA sequences for target gene

Negative control	5'- UUCUCCGAACGUGUCACGU-3' 5'- ACGUGACACGUUCGGAGAA-3'
anti C/EBP α siRNA	5'-GAUCGCACUAAGGAUGACGACACUAU-3' 5'-AUAGUGUCGUCAUCCUUAGUGCGAUC-3'
anti HNF4 α siRNA	5'-ACCCGGUACUCGUUGCUGUUCUUGU-3' 5'-ACAAGAACAGCAACGAGUACCGGGU-3'
Anti DEC2 siRNA	5'-GCCUAACCAUUAGUACUUA-3' 5'-UAAGUACUAAUGGUUAGGC-3'

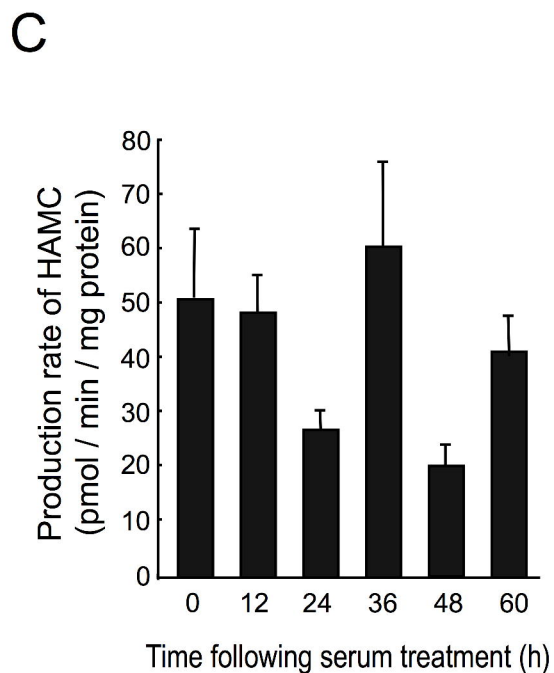
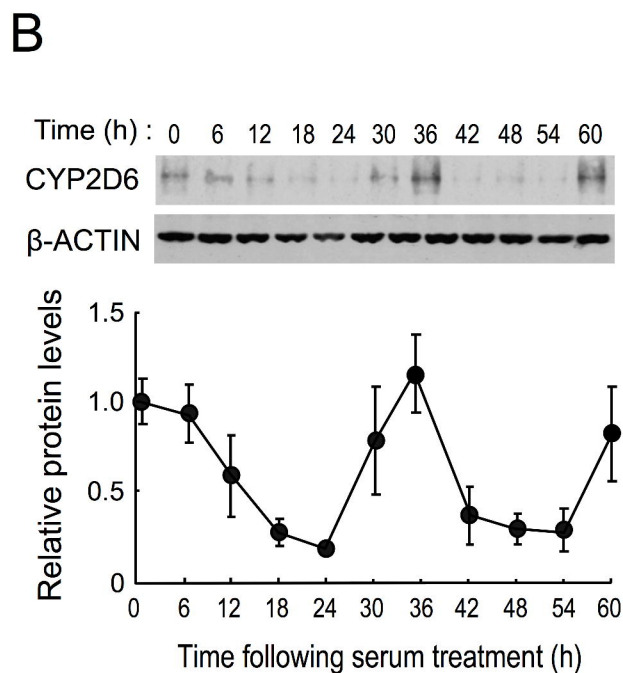
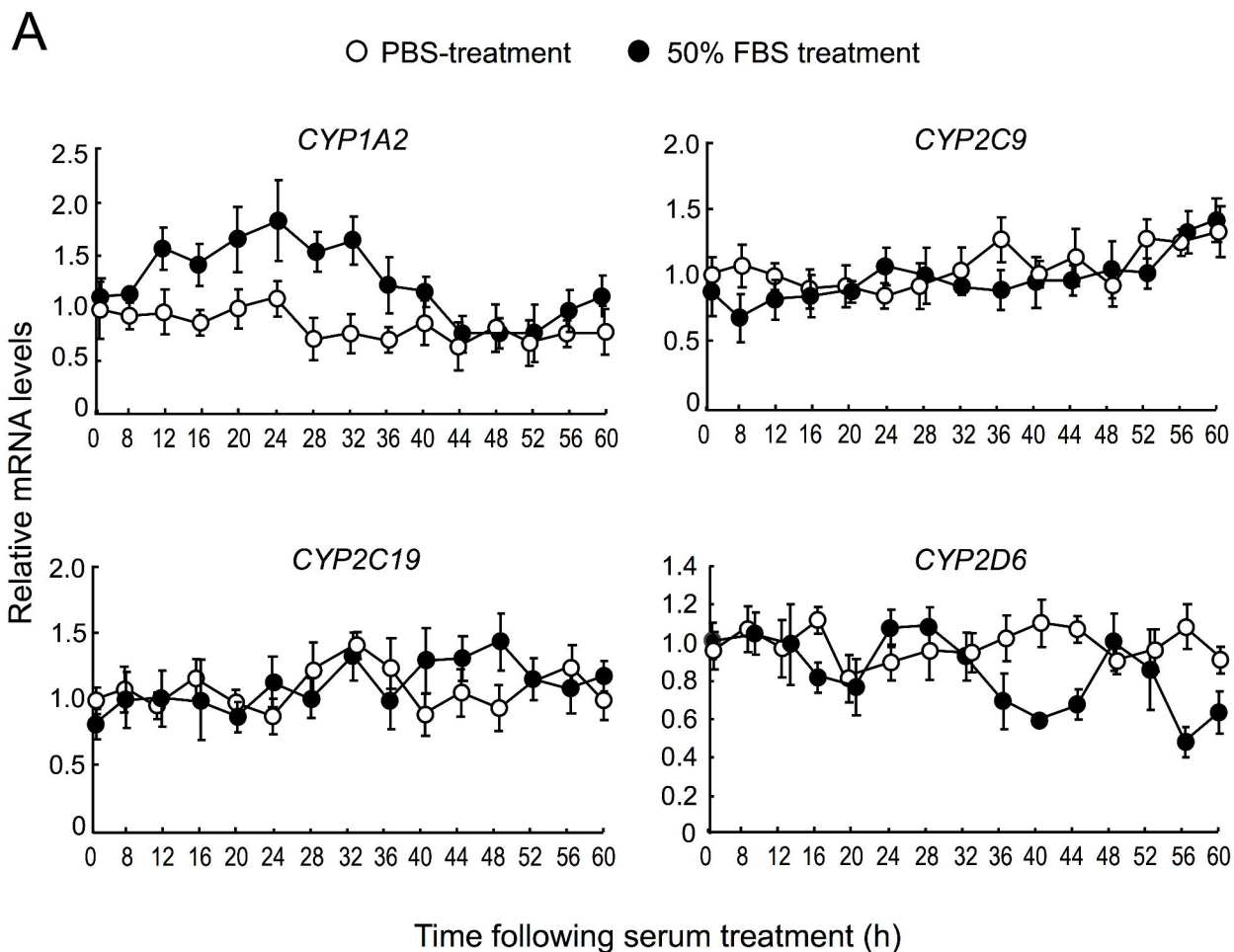
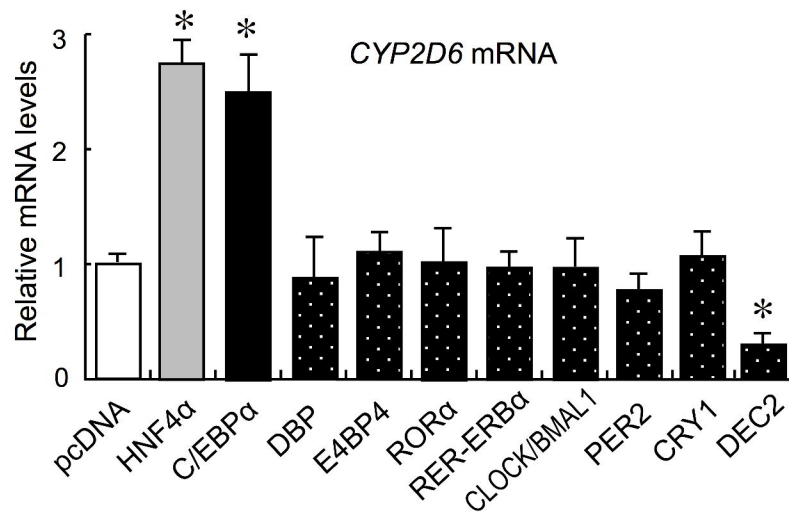


Figure 1

A



B

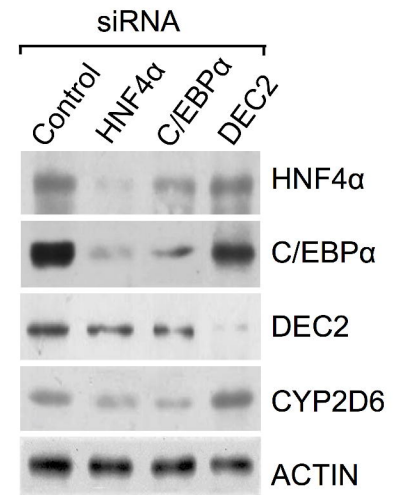


Figure 2

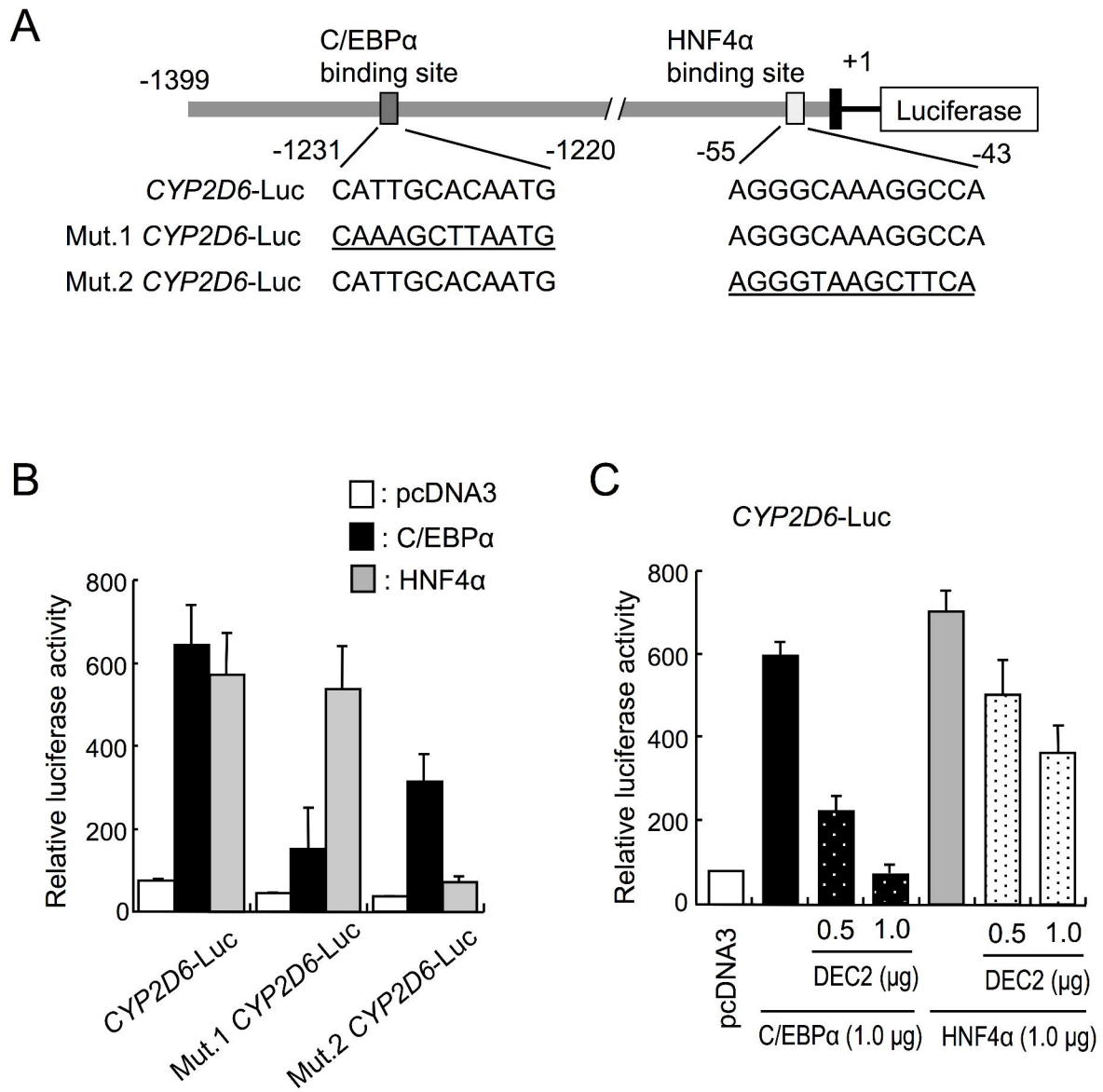


Figure 3

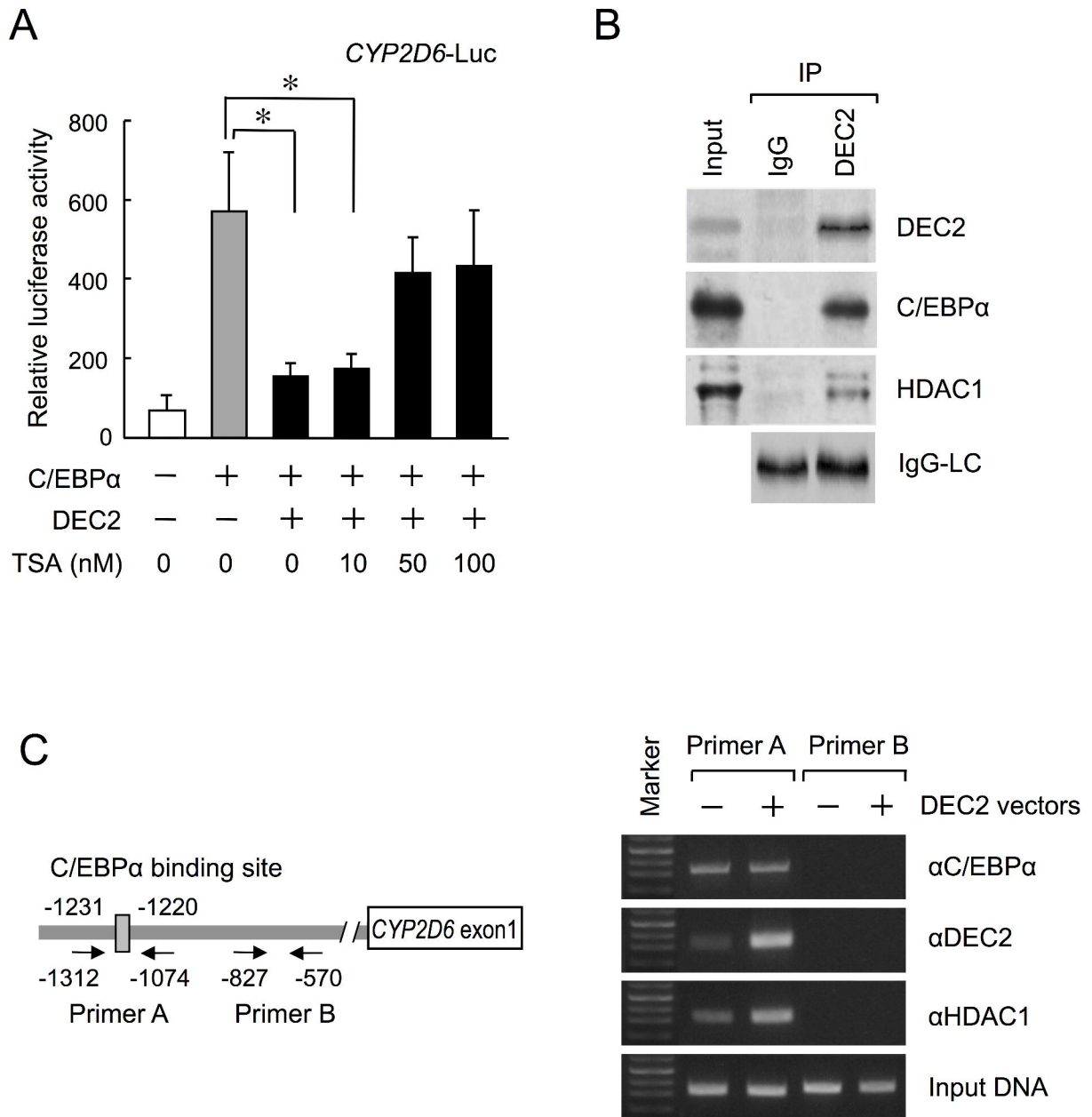


Figure 4

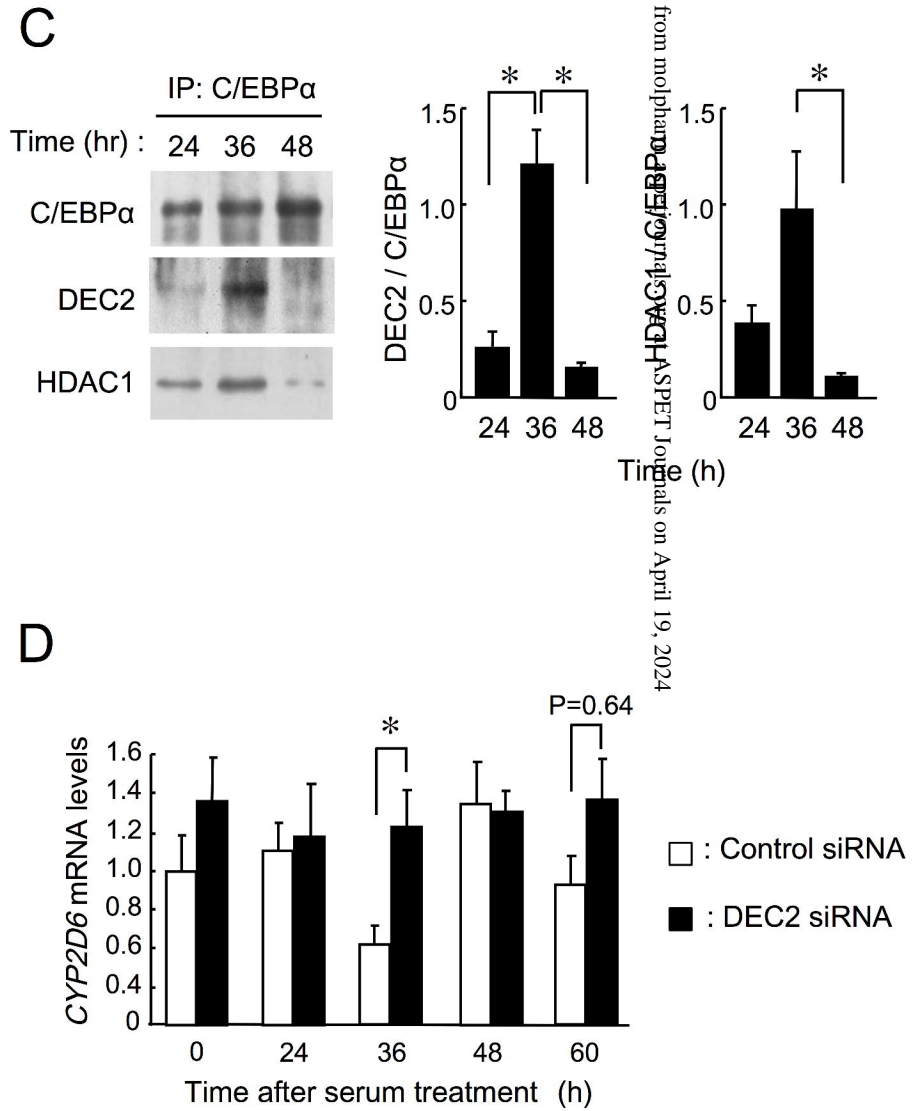
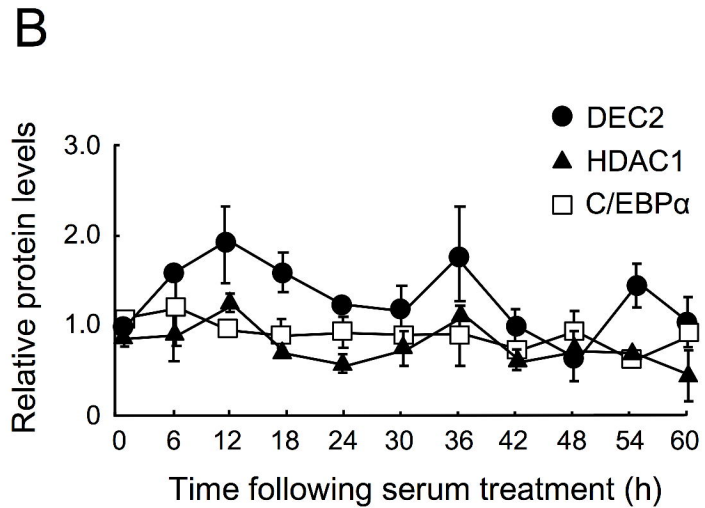
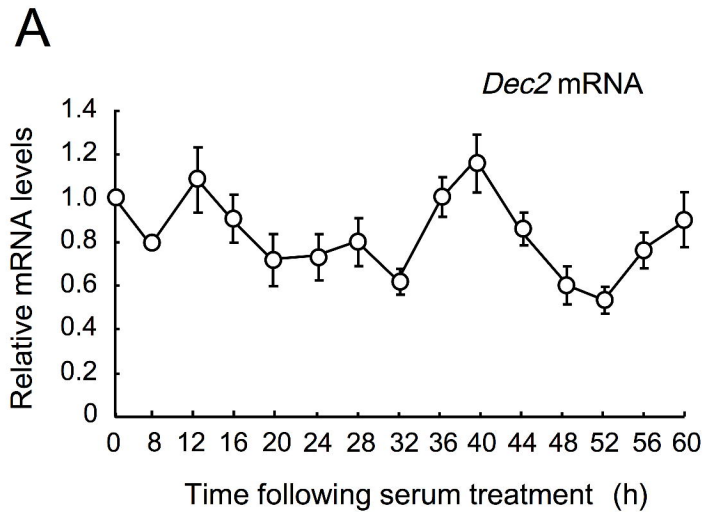


Figure 5

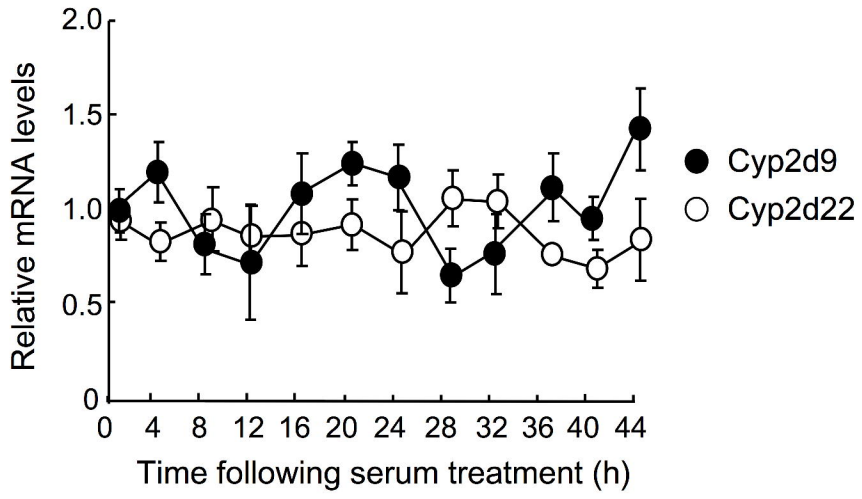
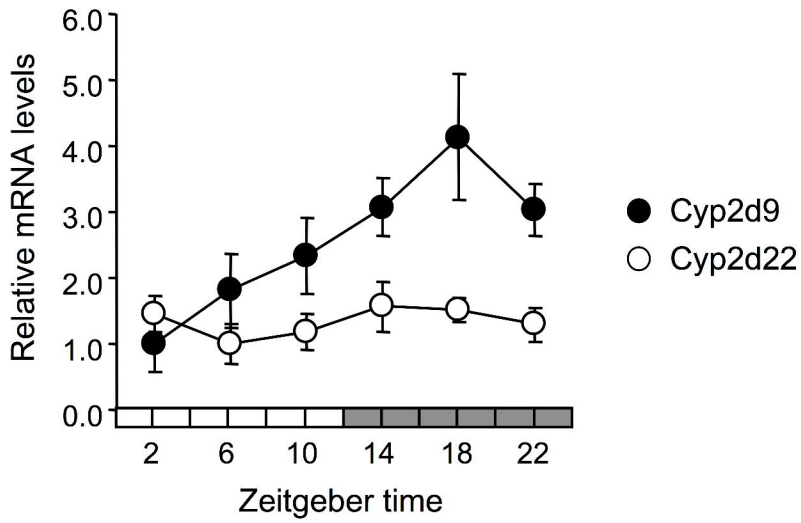
A**B**

Figure 6

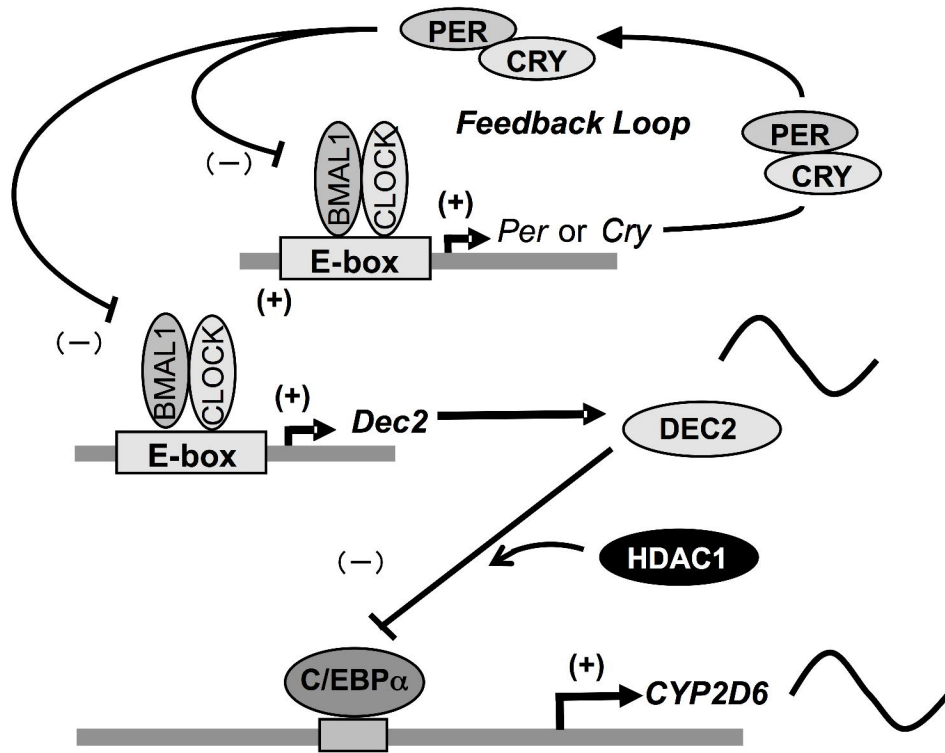


Figure 7