TIMING MATTERS: THE INVOLVEMENT OF CIRCADIAN CLOCK GENES IN DEVELOPMENT AND TOXIN RESPONSES

A Dissertation

by

XIAOYU QU

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2008

Major Subject: Biology

TIMING MATTERS: THE INVOLVEMENT OF CIRCADIAN CLOCK GENES IN DEVELOPMENT AND TOXIN RESPONSES

A Dissertation

by

XIAOYU QU

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee,	David Earnest
Committee Members,	Weston Porter
	Vincent Cassone
	Mark Zoran
Head of Department,	Vincent Cassone

August 2008

Major Subject: Biology

ABSTRACT

Timing Matters: The Involvement of Circadian Clock Genes in Development and Toxin Responses. (August 2008)

Xiaoyu Qu, B.S., Huazhong Agricultural University, China

Chair of Advisory Committee: Dr. David Earnest

Most members of the PAS (PER-ARNT-SIM) protein family are transcription factors, mediating development and adaptive responses to the environment, such as circadian rhythms and toxin responses. Because the PAS domain mediates protein-protein interactions and functional cross-talk between distinct biological processes, we hypothesized that PAS genes in the circadian clockworks, namely *Per1* and *Per2*, may be involved in development and toxin responses, which are modulated by other PAS members. To explore the possible role of clock genes in development, we examined mammary epithelial cells *in vitro* and the mouse mammary gland *in vivo* for evidences of changes in clock gene expression during different stages of development and differentiation. Our results showed that *Per1* and *Bmal1* expression were up-regulated in differentiated HC-11 cells, whereas *Per2* mRNA levels were higher in undifferentiated cells. A similar differentiation-dependent profile of clock gene expression was observed in mouse mammary glands; *Per1* and *Bmal1* mRNA levels were elevated in late pregnant and lactating mammary tissues, whereas *Per2* expression was higher in proliferating virgin and early pregnant glands. These data suggest that circadian clock

genes may play a role in mouse mammary gland development. To examine clock gene function in toxin responses, we evaluated whether disruption or inhibition of *Per1* and/or *Per2* alters toxin-induced activity of the AhR signaling pathway in the mouse mammary gland and liver. We assessed the activation of the AhR signaling pathway in response to 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a prototypical AhR agonist, by analyzing the mRNA abundance of its two target genes, cytochrome P450, subfamily I, polypeptide 1 (Cyp1A1) and *Cyp1B1*. Our results showed that the targeted disruption of *Per1*, but not *Per2*, significantly increases the TCDD-induced *p450* expression in the mammary gland and liver *in vivo*. Similar changes in TCDD-mediated *p450* expression were observed *in vitro* using mammary primary cultures of mammary cells derived from from $Per1^{ldc}$, $Per2^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ mutant mice and Hepa1c1c7 cells subjected to siRNA-mediated inhibition of *Per1* or *Per2*. These discoveries suggest that the clock gene *Per1* may modulate toxin responses perhaps by functioning as a negative regulator for TCDD-mediated activation of the AhR signaling pathway.

DEDICATION

This dissertation is dedicated to my loving parents in China, Qianfang Yuan and Zuxun Qu, and my dear husband, Yang Wu.

ACKNOWLEDGEMENTS

I need to acknowledge many people and groups who have greatly contributed to the course of this study and my training in life science research. I first would like to give great thanks and appreciation to my principle investigator and committee chair, Dr. Earnest, for his continuing intelligent guidance, encouragement and patience. I would also like to thank all members of my graduate committee. Dr. Porter and Dr. Cassone, who are our collaborators as well, have generously provided me with experimental materials and precious expertise in the experimental design. I thank Dr. Zoran for the tedious hours he spent demonstrating the fluorescent microscopy to me and his great contribution in shaping my mind for biological research.

Many thanks also go to the Center of Research on Biological Clocks and the Department of Biology for providing me with advanced facilities to improve my skills, rigorous training to sculpt my brain, as well as a friendly atmosphere to cultivate a positive spirit that is crucial for the success in any career. I would like to thank Ms. Lily Bartoszek for the many hours she spent proofreading the first chapter of this manuscript.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

CIRCADIAN RHYTHMS

The rotating feature of planet Earth has endowed living organisms with endogenous timekeeping systems that allow them to predict and adapt to rhythmic changes in the environment. From cyanobacteria, fungi, algae, plants, flies and birds to man, biological processes within many cells and tissues oscillate with a wide variety of periodicities. Many of these rhythmic processes oscillate with periods of approximately 24 hours and thus are known as circadian rhythms.

Fundamental Properties

Knowledge about the circadian timekeeping system, or "the circadian clock," is largely gained from observing the overt rhythms, which are not the clock itself, but observable molecular, physiological, or behavioral phenomena of the organisms. Such phenomena include the rhythmic development of asexual conidiosporesin in the *bd* strain of *Neurospora crassa*, the day/night fluctuation in the photosynthetic enzymes in plants, the rhythmic secretion of melatonin from the avian pineal, and the rhythmic wheel-running activities of rodents, etc.

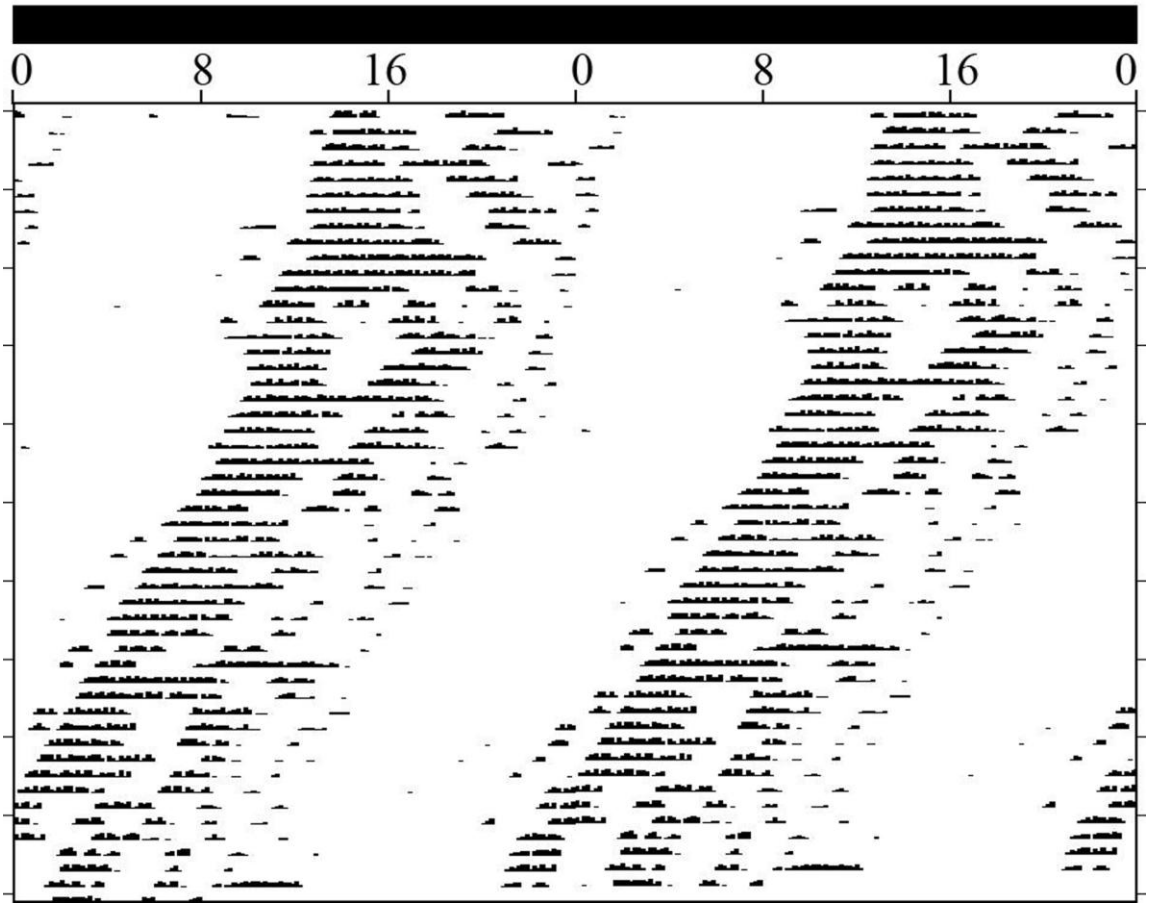
This dissertation follows the style of *Molecular Pharmacology*.

Through characterization of these overt rhythms, several defining properties of circadian rhythms are common among all organisms:

(1) Circadian rhythms have a periodicity of about 24 hours, even in the absence of an environmental cycle (Bell-Pedersen et al., 2005). When an organism is isolated from environmental stimuli and kept in constant conditions, these rhythms persist or "free-run," demonstrating that circadian oscillations are not driven by external signals but instead endogenously generated by an internal biological clock. In the absence of environmental time cues, circadian rhythms and the underlying clock mechanism are maintained with a free-running period, τ (tau), close to, but not exactly, 24 hours (Fig.1).

(2) Circadian rhythms are temperature compensated, such that the period of the underlying clock mechanism remains approximately the same, even when an organism is placed in varying temperatures within its physiological range (Bell-Pedersen et al., 2005).

(3) Circadian rhythms can be entrained or synchronized by environmental stimuli with periods of about 24 hours (Dunlap et al., 2004). Circadian entrainment enables organisms to correctly perceive the time of day, and therefore prepare in advance for the periodic changes in the natural environment. Environmental entraining agents are often referred as zeitgebers, meaning "time givers" in German. Among multiple environmental stimuli that oscillate daily, such as light, temperature, humidity, food availability, and social cues, the most important zeitgeber in mammals is the 24-hour solar cycle.



τ =23.57

Fig. 1. Circadian wheel-running behavior in rodents. Representative activity records of a C57B16 mouse that was maintained under free-running conditions in constant darkness (DD). Actograms are double-plotted over a 48-hour period, and the determination of circadian period by 2 periodogram and fast Fourier transform is indicated at the bottom of the record.

Functional Organization of the Circadian System

The circadian timekeeping systems in various types of organisms are characterized by a common configuration: input/entrainment pathways, a core clockwork, and output pathways (Fig. 2).

The input pathways are the pathways through which the circadian clock receives external stimuli that provide for its entrainment to environmental cycles. The core clockwork is a timekeeping mechanism responsible for the generation of 24-hour oscillations in its own biological processes and in other cells or tissues throughout the body. The output pathways are the pathways through which the clockworks regulate overt rhythms so that behavioral, physiological, and cellular activities of the organism occur at the appropriate times of day.

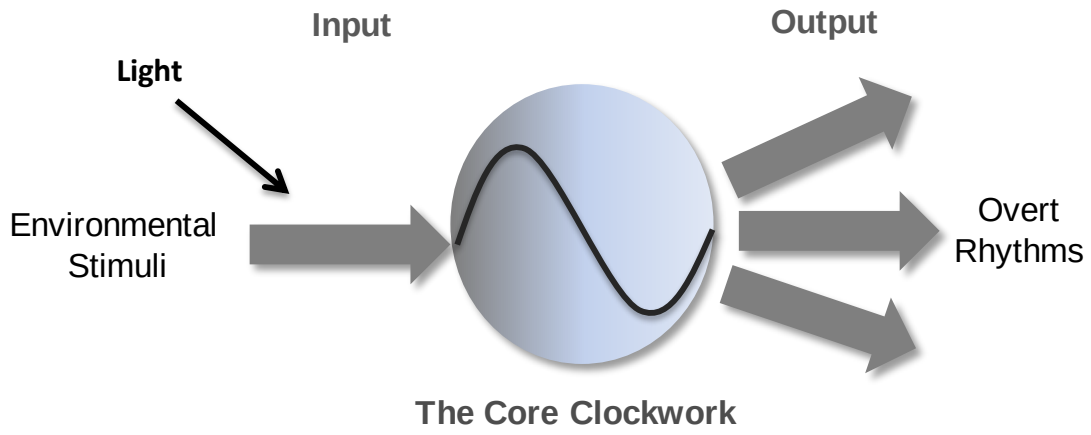


Fig. 2. A simplified model of the circadian timekeeping system. Environmental signals, such as light, synchronize the clockwork to the daily environment through input pathways. The core clockwork then sends temporal cues through the output pathways to maintain the behavioral, physiological, and cellular activity rhythms of the organism.

Circadian Rhythms in Mammals

SCN is the master circadian clock. In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus is the master clock that links environmental time cues to local processes. The role of the SCN as the master clock has been established through functional studies, in which different neuroendocrine structures and nervous tissues were destroyed and the effects on overt circadian rhythms, such as the wheel-running activity, were measured. SCN lesions abolish overt circadian rhythms in many behavioral, endocrine, and biochemical processes (Moore and Eichler, 1972; Schwartz and Zimmerman, 1991; Stephan and Zucker, 1972). Transplantation experiments have provided an important complement to lesion studies and thus a critical test of SCN pacemaker function. In these experiments, transplantation of the SCN tissue from donor animals restores circadian oscillations in arrhythmic SCN-lesioned hosts (Ralph et al., 1990). Interestingly, the restored rhythmicity in the host retains specific circadian properties, namely the period, of the donor. This finding suggests that it is the transplant rather than any remaining SCN cells in the host that is responsible for the restoration of circadian rhythmicity (Ralph et al., 1990). Therefore, the SCN functions as an autonomous timekeeper and as the central pacemaker, which sets the pace of the circadian timing system.

The rhythmic features of the SCN. One fundamental feature of the SCN is that many of its many of its intrinsic molecular and cellular activities are marked by self-sustained oscillations, including cellular metabolism, gene expression, and neuropeptide and neurotransmitter secretion (Earnest and Sladek, 1987; Gillette and Reppert, 1987;

Newman et al., 1992; Reppert and Weaver, 2002; Weaver, 1998). The property of the SCN as a self-sustained circadian oscillator has been supported by the study showing that cultured SCN tissue can maintain robust circadian rhythmicity for up to 32 days *in vitro* (Yamazaki et al., 2000). Furthermore, SCN 2.2 cells, an immortalized cell line derived from rat SCN, can endogenously generate rhythmic gene expression and 2-deoxyglucose uptake for multiple cycles *in vitro* (Allen et al., 2001).

In the SCN, circadian oscillations are not only an ensemble property of the entire nucleus, but are also expressed by individual SCN cells. These cells are therefore often referred to as single-cell oscillators or clock cells. In the intact *in vivo* environment, individual SCN cells communicate phase information to each other and generate synchronized circadian outputs of the central clock, which, through neuronal and humoral signals, regulates the overt rhythms of the organism (Reppert and Weaver, 2001). When removed from the *in vivo* environment and maintained in dissociated cultures fixed on microelectrode arrays, individual SCN neurons continue to demonstrate prominent circadian rhythms in firing rate (Welsh et al., 1995). However, without cell-cell communication through synapses formed *in vivo*, the phase and circadian period of the oscillation in firing rate vary largely among individual SCN neurons that are in dissociated cultures (Welsh et al., 1995; Honma et al., 2004).

Clock Genes and the Circadian Feedback Loops in the SCN

Over the past decade, researchers carried out studies to identify the biochemical and molecular mechanisms responsible for the generation of circadian rhythmicity within the SCN. Experiments using gene mutation or knockout analyses have unveiled

genes that constitute essential components of the clock mechanism. These core clock components include circadian locomotor output cycles kaput (Clock) and brain and muscle ARNT-like protein 1 (*Bmal1*), *Period1* (*Per1*), *Per*2, *Cryptochrome1* (*Cry1*), *Cry*2, and *Rev-erbα*, etc. At the molecular level, these clock genes form interlocked transcriptional/translational feedback loops that are essential for the generation of circadian rhythms in SCN cells (Fig. 3) (Fu and Lee, 2003). Two basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) family members, CLOCK and BMAL1, form dimers, which bind with E-box sequences to stimulate rhythmic transcription of three period genes (*Per1–3*) and two cryptochrome genes (*Cry1* and *Cry2*) at the beginning of each day. By mid-day, CRY and PER proteins begin to accumulate and enter the nucleus and then interact with CLOCK and/or BMAL1 to inhibit their own transcription and enhance Bmal1 transactivation. Increased CLOCK:BMAL heterodimers activate *Per* and *Cry* gene expression and the clock is reset at the beginning of the next day. All clock genes, except *Clock*, demonstrate circadian oscillation in the expression level within the SCN (Reppert and Weaver, 2001).

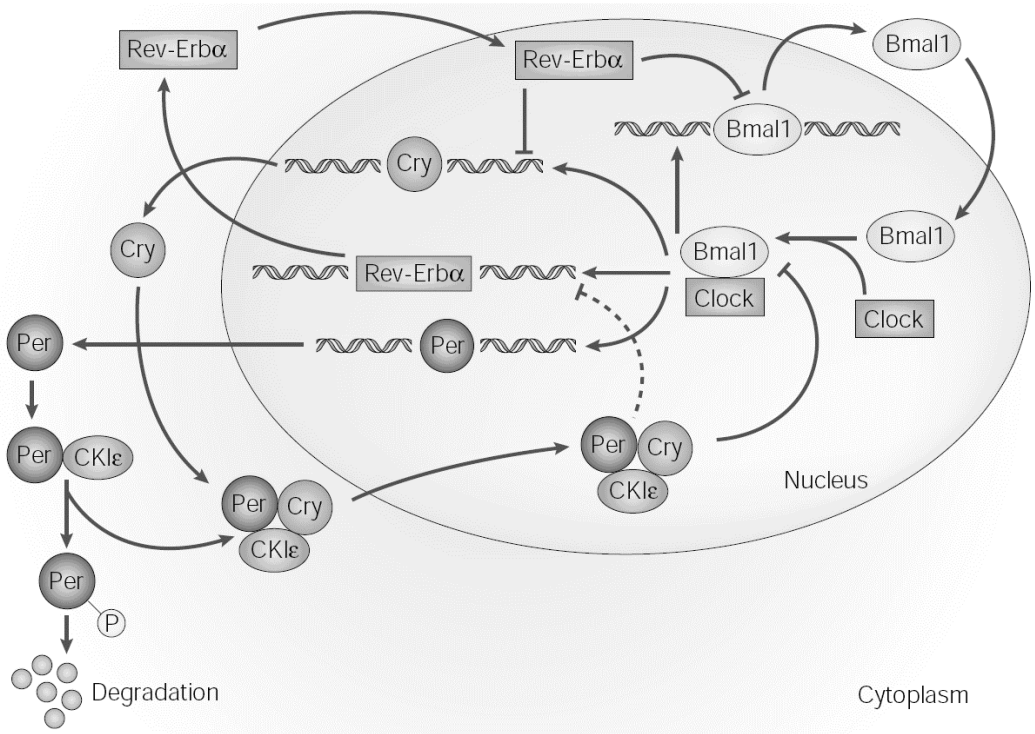


Fig. 3. The model of mammalian circadian feedback loops in a neuron of the suprachiasmatic nucleus (Fu and Lee, 2003).

The essential roles of *Clock*, *Bmal1*, *Per1*, *Per*2, *Cry1*, *Cry*2, and *Rev-erbα* in the circadian clock mechanism are supported observations indicating that mutation or knockout of these genes in mice alters or abolishes the circadian rhythm of activity (Table 1).

TABLE 1
Mouse circadian clock genes (Ko and Takahashi, 2006)

Gene and Allele	Mutant Phenotype under DD
Bmal1 (Arntl)	
Bmal1$^{-/-}$	Arrhythmic
Clock	
Clock$^{\Delta 19/\Delta 19}$	4-h longer period /arrhythmic
Clock$^{-/-}$	0.5-h shorter period
Per1	
Per1^{brd}	1-h shorter period
Per1^{ldc}	0.5-h shorter period /arrhythmic
Per1$^{-/-}$	0.5-h shorter period
Per2	
Per2^{brd}	1.5-h shorter period /arrhythmic
Per2^{ldc}	Arrhythmic
Per3	
Per3$^{-/-}$	0–0.5-h shorter period
Cry1	
Cry1$^{-/-}$	1-h shorter period
Cry2	
Cry2$^{-/-}$	1-h longer period

Clock. Most information about *Clock* has been obtained through studies using homozygous *Clock* mutant mice ($Clock^{\Delta 19/\Delta 19}$), in which exon 19 is missing in the RNA transcripts (King et al., 1997). $Clock^{\Delta 19/\Delta 19}$ mice are characterized by activity rhythms in which the circadian period is increased to 28 hours and by molecular rhythms of low or decreased amplitude. Further analysis revealed that the mutant $Clock^{\Delta 19}$ protein dimerizes with BMAL1 and binds to the *Per1* E-box, but is defective in initiating gene transcription (Gekakis et al., 1998).

Although *Clock* is thought to represent a core element of the circadian timekeeping mechanism based on the disruption of molecular and behavioral rhythms observed above in $Clock^{\Delta 19/\Delta 19}$ mice, the recent analysis of mice with a complete deletion of the *Clock* gene ($Clock^{\Delta 5\text{-}6}$) has challenged our understanding about the function of this gene (Debruyne et al., 2006). $Clock^{\Delta 5\text{-}6}$ animals continue to display robust circadian rhythms of wheel-running activity, with a period similar to that found in wild type animals. At the molecular level, mRNA abundance and protein levels for the core clock genes continue to oscillate with similar amplitudes in the SCN and peripheral tissues of these mice. Based on comparative analysis of data from the $Clock^{\Delta 19/\Delta 19}$ and the $Clock^{\Delta 5\text{-}6}$ mice, it has been proposed that in wild type mice, CLOCK:BMAL1 dimers translocate into the nucleus and positively drive the expression of clock genes such as *Per1*, *Per2*, *Cry1*, *Cry2,* and *Rev-erbα*. When *clock* gene is mutated in the case of the $Clock^{\Delta 19/\Delta 19}$ mutant, BMAL1 binds with the mutant $Clock^{\Delta 19}$ and forms dimers that fail to initiate clock gene expression. In the complete absence of CLOCK, BMAL1 forms a complex with an unknown factor and this complex can still translocate into the nucleus and

initiate gene transcription. However, the complex between BMAL1 and this unknown factor is less efficient than the CLOCK:BMAL1 dimer at activating the transcription of other clock genes.

Bmal1. The essential role of *Bmal1* in the mammalian circadian systems is supported by observations from studies using *Mop3*$^{-/-}$(*Bma1l*$^{-/-}$) mice, which carry a null allele at the mouse *Mop3* locus (Bunger et al., 2000). At the behavioral level, the activity rhythms of *Bmal1*$^{-/-}$ mice entrain to 12:12 light dark (LD) cycles but fail to persist in constant darkness (DD). At the molecular level, *Bmal1*$^{-/-}$ mice exhibit abolished rhythms of *Per1* and *Per2* expression that are marked by extremely low levels of mRNA abundance. Collectively, the observed changes in the behavioral and molecular rhythms in *Bmal1*$^{-/-}$ mutant mice support *Bmal1* function as a positive regulatory element in the molecular feedback loops that form the circadian clockworks in mammals.

***Per1, Per2, and Per3*.** Three homologs of the *Drosophila* clock gene, period, have been discovered in mammals, *Per1*, *Per2*, and *Per3*. Among these genes, *Per1* and *Per2* have been shown to function as essential components of the circadian clock mechanism (Bae et al., 2001; Cermakian et al., 2001; Zheng et al., 2001). *Per1* and *Per2* function in the regulation of circadian behavior is supported by behavioral studies using transgenic mice, in which exon 2-12 of the mouse *Per1* gene (*Per1*^{ldc}) or Exon 6 of the *Per2* gene (*Per2*^{ldc}) were disrupted (Bae et al., 2001). The main findings were that *Per1*^{ldc}, *Per2*^{ldc}, and *Per1*^{ldc}/ *Per2*^{ldc} - mice show normal entrainment to 12:12 LD cycles, but become arrhythmic during exposure to DD, *Per1*^{ldc} and *Per2*^{ldc} mice required 10-21

days in DD before the activity pattern became arrhythmic whereas *Per1*^{ldc}/*Per2*^{ldc} double-mutant mice exhibited arrhythmicity immediately after exposure to DD. The disrupted activity rhythms observed in both *Per1*^{ldc} and *Per2*^{ldc} mice suggest that both *Per* genes are essential for the generation of circadian rhythmicity at the behavioral level.

At the molecular level, mutations of *Per1* or *Per2* have differential effects on clock gene expression in the SCN. In *Per1*^{ldc} mice, the rhythmic expression of clock gene mRNAs is unchanged, although the number of PER2- and CRY1-immunoreactive cells in the SCN is reduced. In contrast, the rhythms of *Bmal1* and *Cry1*expression in the SCN are abolished in *Per2*^{ldc} mice. These findings suggest that *Per1* and *Per2* may serve different roles in the molecular feedback loops. The reduced levels and abolished rhythms of clock gene expression in the SCN of *Per2*^{ldc} mice are compatible with the proposed role of *Per2* as a positive regulator of other core elements of the clockworks. The unaltered clock gene expression profiles and reduced immunostaining for PER2 and CRY1 in the SCN of *Per1*^{ldc} mice suggest that *Per1* does not directly repress or activate other clock genes but instead modulates their interactions within the feedback loops, perhaps by influencing the stability and/or nuclear entry of protein products (Bae et al., 2001).

Cry1 and Cry2. Mammals have two cryptochrome genes, *Cry1* and *Cry2*. Information about the role of the cryptochrome genes in the circadian clock mechanism is mainly derived from studies using mice that carry null alleles for *Cry1*, *Cry2*, and *Cry1*/*Cry2*. Similar to other mutant strains in which clock genes are deleted or disrupted

(van der Horst et al., 1999; Vitaterna et al., 1999), the activity rhythms of *Cry1*$^{-/-}$, *Cry2*$^{-/-}$, and *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice entrain to 12:12 LD cycles. In *Cry1*$^{-/-}$ and *Cry2*$^{-/-}$ mice, behavioral rhythmicity persists in DD, the free-running period is shorter in the former and longer in the latter than that observed in wild-type mice. *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice are distinguished by an instantaneous and complete loss of circadian rhythmicity following exposure to DD. Because *Cry1* and *Cry2* mutations produce opposite changes in the free-running period of the activity rhythm and double knockout mice are completely arrhythmic in DD, these data suggest that *Cry1* and *Cry2* may have antagonistic functions in the circadian clock mechanism and are both necessary for behavioral rhythmicity.

At the molecular level, *Cry1*$^{-/-}$ and *Cry2*$^{-/-}$ mice show robust circadian oscillations of *Per1* and *Per2* mRNA expression in SCN, but the periods of these rhythms are shorter in the former and longer in latter relative to wild-type animals. However, *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice fail to show SCN rhythms of *Per1* or *Per2* expression (Okamura et al., 1999).

CLOCK GENES AND THE PERIPHERAL TISSUES

The molecular dissection of the circadian clock mechanism also brought about the discovery that the capacity to generate endogenous oscillations is not only a functional property of the SCN but also of other tissues and fibroblast cell lines. In addition to SCN, clock genes are rhythmically expressed in peripheral tissues *in vivo*, such as liver, skeleton muscles, testis, heart, kidney, liver, and mammary gland (Kita et al., 2002; Metz et al., 2006 ; Storch et al., 2002; Zylka et al., 1998). Explanted cultures of these peripheral tissues similarly show circadian rhythms of luciferase-reported clock

gene expression *in vitro* (Yamazaki et al., 2000; Yoo et al., 2004). Clock gene oscillations in peripheral tissues are comparable to those found in the SCN but phase delayed by 4-8 hours. Similar to peripheral tissues, Rat-1 and NIH/3T3 fibroblasts and hepatoma cell lines exhibit induced oscillations of clock gene expression for several cycles in response to serum shock, forskolin, or corticosteroid treatment (Balsalobre et al., 1998). These discoveries indicate that the mammalian circadian system consists of multiple, cell-autonomous oscillators arranged in a hierarchy, in which the SCN functions as a central pacemaker and peripheral oscillators provide for the local organization of tissue- or cell-specific processes in time.

Potential Non-Clock Functions of Clock Genes in Peripheral Tissues

Because clock genes oscillate rhythmically in most peripheral tissues, it is not surprising that the disruption of clock genes leads to alterations in physiological processes that have not been previously linked with the circadian clock. Such processes include development, metabolism, reproduction, and tumorigenesis.

Clock genes, development and aging. Clock genes have been shown to serve important roles in the control of cell differentiation and aging. For example, the clock gene, *Bmal1,* has been linked with fundamental processes that mediate aging and adipogenesis. The role of *Bmal1* in the control of aging is supported by studies demonstrating that *Bmal1*-deficient ($Bmal1^{-/-}$) mice show progressive weight loss, higher mortality and decreased activity with age relative to that observed in wild type animals. The decreased weight in these mutant mice is associated with progressive loss of muscle and bone mass and the intra-abdominal and subcutaneous adipose tissue (Bunger et al.,

2005). The decreased activity in *Bmal1*$^{-/-}$ mice is caused by a progressive noninflammatory arthropathy (Bunger et al., 2005), characterized by progressive tendon calcification in the joints. *Bmal1* has also been implicated in the regulation of adipogenesis based on the observations that *Bmal1* expression increases during adipose differentiation and that embryonic fibroblasts derived from *Bmal1*$^{-/-}$ mice do not differentiate into adipocytes until *Bmal1* expression is restored using a retroviral vector (Shimba et al., 2005). The clock genes *Per1* and *Per2* have also been linked to the regulation of development and aging. *Per1*^{brd1}and *Per2*^{brd}mice display phenotypes characterized by premature aging, in which there is a general loss of soft tissues and development of kyphosis at about 12–14 months of age (Lee, 2005).

Clock genes and metabolism. Core elements of the clock mechanism also appear to be involved in the regulation of metabolism. The role of *Clock* in the control of metabolism is supported by the observation that *Clock*$^{\Delta 19/\Delta 19}$ mice are hyperphagic and obese, and develop metabolic syndromes characterized by hyperleptinemia, hyperlipidemia, hepatic steatosis, hyperglycemia, and hypoinsulinemia (Turek et al., 2005). *Clock*$^{\Delta 19/\Delta 19}$ mice also exhibit disrupted diurnal rhythms in glucose and triglyceride levels (Rudic et al., 2004). The involvement of the clock gene *Bmal1* in the regulation of metabolism is suggested by evidence indicating that gluconeogenesis is abolished in *Bmal1*$^{-/-}$ mice (Rudic et al., 2004).

The link between the *Period* genes and metabolism has been revealed by a study examining glucocorticoid rhythms, food intake, and body mass of *Per1*^{Brd} and *Per2*^{Brd} mice (Dallmann et al., 2006). The primary finding of this study is that targeted

disruption of *Per1*, but not *Per2,* severely altered these indices of metabolism. In *Per1*^{Brd} mice, the daily rhythm in glucocorticoid levels which normally peak during the night is abolished, body mass is decreased, but food and water intake per gram body mass and glucose metabolism are increased.

Clock genes and reproduction. Several clock genes have been implicated in the regulation of mammalian reproduction. Evidence for the role of *Clock* in regulating reproduction is derived from the observation that *Clock*$^{\Delta 19/\Delta 19}$ mice are either sterile or distinguished by a premature decline in fertility. As a result of these problems with reproductive competence, colonies of *Clock*$^{\Delta 19/\Delta 19}$ mice cannot be maintained by breeding homozygous pairs. In addition, *Clock*$^{\Delta 19/\Delta 19}$ females exhibit disrupted estrous cycles and inability to maintain full-term pregnancies (Miller et al., 2004).

The importance of *Bmal1* in mammalian reproduction is supported by the finding that both male and female *Bmal1*$^{-/-}$ mice are sterile, albeit for unknown reasons (Kondratov et al., 2007). *Bmal1*$^{-/-}$ males are usually distinguished by smaller seminal vesicles and coagulation glands. It is noteworthy that *Bmal1*$^{-/-}$ females do not produce progeny even when mated with wild-type males.

The possible involvement of *Per* genes in reproduction is suggested by the finding that *Per1*^{brd} and *Per2* ^{brd} mice display a more rapid decline in fertility and subsequent litter sizes with each successive breeding in comparison to wild-type breeders (Lee, 2005). Similar phenomena were observed in the *Per1*^{ldc}/*Per2*^{ldc} mice maintained in our animal facility (Earnest and Qu, unpublished data).

Clock genes and tumorigenesis. Clock genes have important roles in cell cycle control, DNA damage response, and tumor suppression *in vivo* and *in vitro*. Evidence for *Clock* involvement in cell cycle control is based on studies indicating that mutation of this gene affects the expression of multiple genes mediating the cell cycle and proliferation in liver and skeletal muscle (Miller et al., 2007). Further support for the role of *Clock* in regulating the cell cycle is provided by the finding that ~~the~~ proliferation rates are decreased in fibroblasts derived from *Clock* mutant embryos relative to those obtained from wild-type animals (Miller et al., 2007).

The role of *Per2* in regulating cell cycle and tumorigenesis is supported by several lines of evidence. *In vivo* studies demonstrated that the targeted disruption of *Per2* in mice results in significant increases in neoplastic and hyperplastic growth of the salivary glands and in their susceptibility to genotoxic stress. When challenged with gamma-radiation, $Per2^{Brd}$ mice are distinguished by rapid hair graying, a loss in p53-mediated apoptosis in thymocytes, and robust tumor development (Fu et al., 2002). This increased sensitivity to genotoxic stress in $Per2^{Brd}$ mice is consistent with the altered expression profiles for cell cycle genes in these animals. In this regard, the rhythms of *Cyclin D1*, *Cyclin A*, *Mdm-2*, and *Gadd45α* were significantly disrupted in the liver of $Per2^{Brd}$ mice. *In vitro* studies using mammalian cancer cell lines have provided further support for the role of *Per2* as a tumor repressor. The induction of *Per2* expression in cancer cell lines has been shown to produce growth inhibition, cell cycle arrest, apoptosis, and complete inhibition of colony formation in soft agar cultures (Gery et al., 2005). The molecular mechanism by which *Per2* influences the cell cycle and

tumorigenesis is currently unknown. Recent evidence indicates that *Per2* expression is up-regulated in human leukemia cell lines by CCAAT/enhancer-binding proteins (C/EBPs), which are a family of transcription factors that regulate cell growth and differentiation (Gery et al., 2005). Based on these findings, *Per2* is thought to represent a downstream target of the C/EBPα gene involved in acute myeloid leukemia (AML), and its disruption may be associated with the initiation and/or progression of AML.

Per1 has also been implicated as a tumor suppressor because the level of *Per1* expression is inversely related to tumorigenesis. Clinical studies comparing normal and cancerous tissues showed that *Per1* mRNA levels were significantly lower in human lung cancer and breast cancer tissues relative to matching normal tissues from the same patients (Gery et al., 2006). *In vitro* studies examining the converse aspect of this relationship showed that the ectopic expression of *Per1* significantly inhibits the growth of cancer cell lines derived from colon, lung, breast, and endometrial tissues (Gery et al., 2006). Further evidence for the inverse relationship between *Per1* expression and tumorigenesis is provided by *in vitro* observations showing that the rate of apoptosis under genotoxic stress is directly correlated with *Per1* mRNA levels. Over-expression of *Per1* increases the apoptotic rate of colon cancer cells exposed to ionizing radiation, whereas siRNA knockdown of *Per1* decreases the apoptotic rate of similarly treated cells (Gery et al., 2006). Collectively, these observations suggest that *Per1* may play a role in tumor suppression by sensitizing cancer cells to DNA-damage induced apoptosis. The molecular mechanism through which *Per1* induces apoptosis is unknown. However, Gery and colleagues have suggested that PER1 may regulate the cell cycle by directly

interacting with cell cycle checkpoint proteins and repressing the expression of cell cycle genes such as *Wee1*, *Cyclin B1*, and *Cdc2* (Gery et al., 2006).

Clock genes and responses to drugs of abuse. Animals carrying clock gene mutations are also characterized by altered responses to drugs of abuse.

The mutation of the *Clock* gene has been associated with altered responses to cocaine. *Clock* mutant mice show increased sensitization to cocaine, especially its rewarding effects (McClung et al., 2005). Similar to other animals with elevated cocaine sensitization and reward, *Clock* mutant mice were characterized by increased excitability of their dopamine neurons in the midbrain ventral tegmental area (VTA) in response to dopamine. This phenotype is presumably related to altered expression of genes that may directly regulate dopaminergic transmission, such as tyrosine hydroxylase (TH). CLOCK is therefore thought to regulate the VTA dopamine system by modulating the expression of genes that control dopaminergic neurotransmission (McClung et al., 2005).

The *Period* genes have similarly been implicated in responses to drugs of abuse, such as cocaine and alcohol. $Per1^{brd}$ and $Per2^{brd}$ mice exhibit altered sensitization to cocaine. Compared to the behavioral responses of wild type mice, sensitization after repeated exposure to cocaine is abolished in $Per1^{brd}$ and enhanced in $Per2^{brd}$ mice (Abarca et al., 2002). Conditioned place preference experiments have yielded similar findings that the knockout of *Per1* gene results in a complete loss of cocaine reward behavior, whereas the mutation of *Per2* gene leads to enhanced cocaine reward responses relative to those observed in wild-type animals (Abarca et al., 2002).

Period gene mutations not only alter responses to cocaine, but also to alcohol. *Per2*^{brd} mice voluntarily consume more alcohol than wild-type animals (Spanagel et al., 2005). The behavioral phenotype of increased alcohol consumption was associated with the reduced expression of the glutamate transporter Eaat1 in these animals. As a result, it is likely that *Per2*^{brd} mice exhibit reduced glutamate uptake by astrocytes and thus hyperactive glutamatergic neurotransmission in the brain (Spanagel et al., 2005). On the other hand, the targeted disruption of *Per1* had no significant effects on ethanol reinforcement, seeking, or relapse behavior in mutant mice (Zghoul et al., 2007).

GO PAS THE CLOCK

The impact of clock gene mutations on other biological processes is not surprising, because the central elements of the circadian clockworks, such as CLOCK, BMAL1, and the period proteins, are transcriptional regulators belonging to the Per-Arnt-Sim (PAS) family. PAS family members commonly express DNA-binding motifs and regulate gene expression in many important biological processes, including responses to environmental toxins, development, and the circadian rhythm (Crews and Fan, 1999; Kewlay et al., 2004).

Overview

The majority of PAS proteins share a common organization: a basic helix-loop-helix (bHLH) motif located within the N-terminal end followed by two PAS domains and a C-terminus that is poorly conserved (Fig. 4). However, there are exceptions to this arrangement. For example, PERIOD proteins contain PAS motifs but lack the bHLH domain. The bHLH was ubiquitously discovered in a wide range of transcription factors,

in which the basic region directs interactions with DNA and the HLH portion functions as the interface for dimerization. The PAS domain, on the other hand, functions as a secondary dimerization domain that dictates partner specificity and increases the strength of dimerization (Furness et al., 2007). Members of the PAS family can be categorized into two classes based on their dimerization potential: α-class proteins or class-I factors, which often act as sensors responsible for the communication of environmental signals to the nucleus, and β-class proteins or class-II factors, which are constitutively expressed in the nucleus and typically serve as broad-spectrum partners involved in specifying genomic targets of heterodimeric complexes (Furness et al., 2007; Gu et al., 2000). Formation of dimers is necessary for the DNA-binding activity of bHLH/PAS proteins. These protein dimers tend to recognize variant forms of classic E-box enhancer sequences and thus initiate transcriptional responses in a wide spectrum of biological pathways mediating the detection of and adaptation to environmental changes (Kewley et al., 2004).

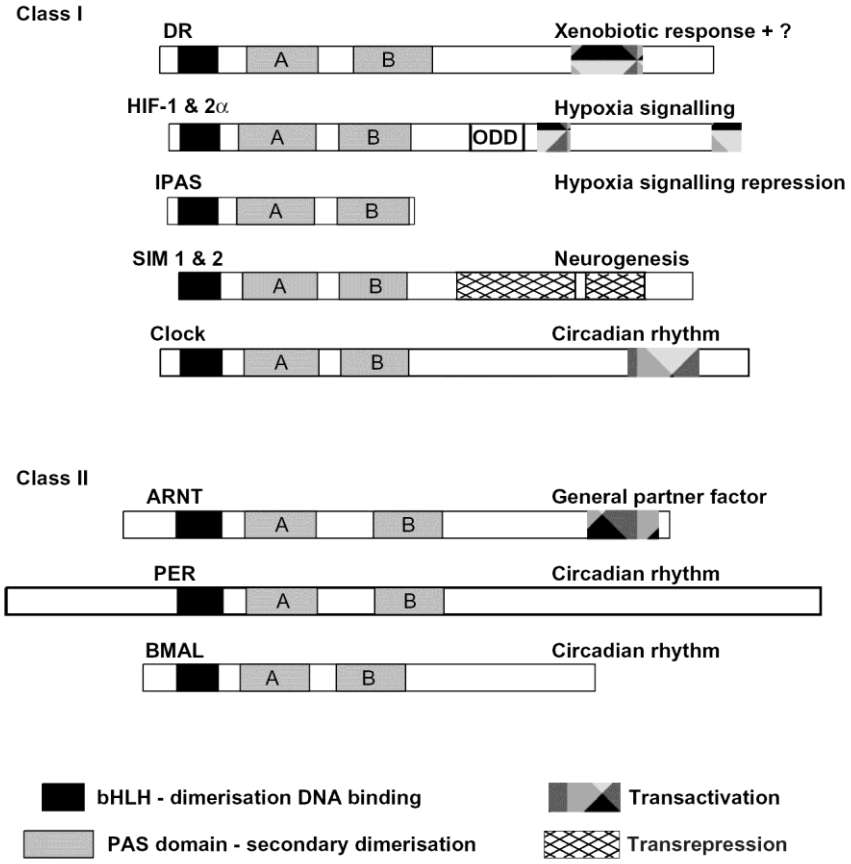


Fig. 4. Schematic representation of the bHLH/PAS family of transcriptional regulators. PAS family members have in common an N-terminal bHLH DNA binding/dimerisation domain (with the exception of PER proteins, which lack the basic DNA binding domain). The PAS domain functions as a secondary dimerization domain that enhances interactions between family members in addition to providing specificity between family members, such that members in the Class I sensing/responsive unit are obligated to heterodimerize with a Class II family member. The PAS domain consists of two hydrophobic repeat regions, A and B, and in the case of the aryl hydrocarbon receptor (AhR), PAS-B functions as a ligand-binding domain (Furness et al., 2007).

The Function of PAS Proteins in Regulating Circadian Rhythms

Clock, *Bmal1*, and the *Per* genes (*Per1-3*) are members of the PAS family that as described earlier form molecular feedback loops underlying the circadian clockworks in mammals. CLOCK protein is categorized as a class I factor, which is a sensing/responsive element, while BMAL1 is a class II factor, which is a general binding partner (Furness et al., 2007; Gu et al., 2000). PER proteins lack a DNA-binding domain and are thus thought to function as repressive components in circadian rhythm function.

Like most class I factors of the PAS family, CLOCK protein has been shown to translocate from the cytoplasm into the nucleus, bind to its partner, and initiate the expression of their downstream responsive genes. Unlike the constitutive expression of other class II factors in the nucleus, BMAL1 is readily translocated between the cytoplasm and nucleus. In fact, the subcellular localization of CLOCK is BMAL1-dependent because CLOCK immunoreactivity is absent in the nuclear fraction of embryonic fibroblasts derived from *Bmal1*$^{-/-}$ mice (Kondratov et al., 2003). Further investigation revealed that CLOCK fails to show nuclear accumulation after mutation of a functional nuclear localization signal (NLS) in the N-terminal or the nuclear export signal (NES) in the PAS domain of BMAL1 (Kwon et al., 2006).

The Function of PAS Proteins in Development

Although the molecular pathways for PAS protein function in development are not well documented, various findings suggest that many of these proteins are indispensable elements for normal development. For example, single-minded (SIM), one of the founding members of the bHLH–PAS protein family, is a key regulator of central

nervous system development for midline cells in *Drosophila*. The importance of the Sim genes in mammalian development is supported by the results of mutagenic studies. Homozygous *Sim1* null mice die shortly after birth. Histological evidence for developmental defects in these mutant mice is based on the absence of secretory neurons containing oxytocin, vasopressin, thyrotropin-releasing hormone, corticotropin-releasing hormone, or somatostatin, in the paraventricular nucleus (PVN), the anterior periventricular nucleus (aPV), and the supraoptic nucleus (SON) (Michaud et al., 1998). This observation suggests that *Sim1* may be essential for normal development of specific neuronal populations in mammals.

Another PAS gene, the neuronal PAS domain protein 3 (NPAS3) also has an important role in neuronal development. NPAS3 is a brain-enriched transcription factor, and mutation of this gene in mice affects neural development of the hippocampus. *Npas3*$^{-/-}$ mice show decreased expression of hippocampal fibroblast growth factor (FGF) receptor subtype 1 mRNA and an associated reduction (84%) in basal neural precursor cell proliferation within the dentate gyrus relative to wild-type littermates (Pieper et al., 2005).

PAS proteins have also been associated with the development of reproductive organs. Although the aryl hydrocarbon receptor (AhR), is best known for its role as a transcriptional factor in the signaling pathway in response to xenobiotics, this PAS family member is also involved in the development of reproductive organs, the liver, and the immune system (Barouki et al., 2007). Recent evidence suggests that the AhR may influence follicular growth in the ovary via alteration in its estradiol regulation and

responsiveness. *AhR*-null mice exhibit lower serum and follicle-produced estradiol levels and decreased estrogen receptor 1 and 2 mRNA expression when compared to wild type mice (Benedict et al., 2000; Barnett et al., 2007). The import role of AhR in the development of liver and the immune system is supported by the observation that AhR null mice demonstrated decreased accumulation of lymphocytes in the spleen and lymph nodes, reduced liver size, and bile duct fibrosis (Fernandez-Salguero et al., 1995).

The Function of PAS Proteins in Toxin Responses

The importance of the PAS genes in responses to environmental toxins such as polycyclic aromatic hydrocarbons (PAHs) is well documented. Toxin responses are mediated by the AhR signaling pathway, which includes a class I factor in the PAS family, AhR, and a class II factor, AhR nuclear translocator (ARNT). In vertebrates, AhR is the only bHLH/PAS protein known to bind and be activated by small chemical ligands. Many synthetic and naturally occurring polyaromatic xenobiotics, such as PAHs, can act as ligands for the AhR. The PAH, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), is a prototypical Ahr agonist (Furness et al., 2007). Under normal conditions, AhR exists in the cytoplasm in a dormant state, forming a complex with chaperone proteins such as the Heat shock protein 90 (HSP90) (Fig. 5), the aryl hydrocarbon receptor interacting protein (*Aip*), and p23. Upon entry into the cell, PAHs bind with AhR and cause its dissociation from the complex. The PAH-bound AhR then translocates into the nucleus and dimerizes with ARNT. AhR/ARNT heterodimers then bind with the *cis*-acting DNA element, known as the xenobiotic response element (XRE), to regulate the expression of downstream target genes. The majority of their

target genes encode cytochrome *p450* enzymes of the A and B subfamily, including *Cyp1A1*, *Cyp1A2*, and *Cyp1B1* (Fujii-Kuriyama and Mimura, 2005). Cytochrome p450s catalyze oxidation of PAHs to reactive metabolites suitable for conjugation by phase II detoxifying enzymes, including glutathione S-transferases and UDP-glucuronosyl transferases. The resulting conjugates are generally less reactive, more hydrophilic molecules that are easier to excrete. If not rendered less reactive or excreted, oxidative PAH metabolites can form DNA adducts leading to mutations and increased cancer risk.

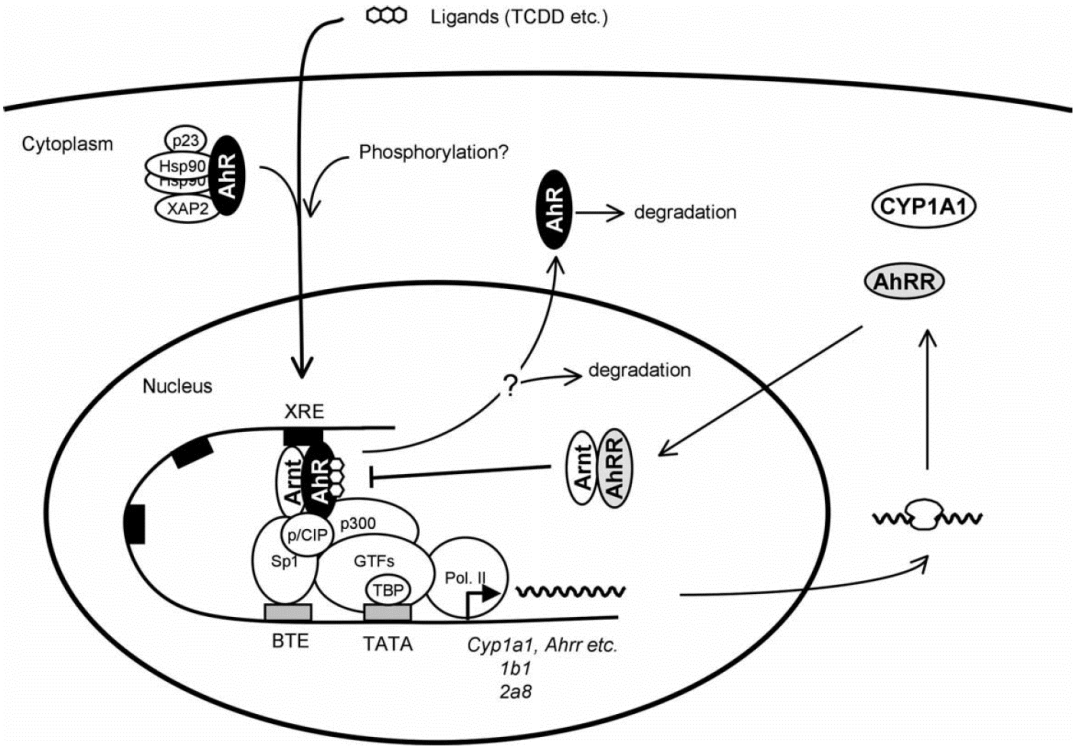


Fig. 5. A model of the AhR signaling pathway **(**Fujii-Kuriyama and Mimura, 2005**).**

Many studies indicate that the majority, if not all, of the toxic effects of dioxin are mediated through the AhR signaling pathway. For example, benzo[a]pyrene, another ligand of the AhR, is carcinogenic in wild type animals, but its carcinogenicity is not manifested in AhR null mice (Shimizu et al., 2000).

Interactions between PAS Proteins Regulating Different Biological Processes

Because PAS proteins typically function as heterodimers, in which a sensor protein is associated with a general binding partner, it is possible that bHLH-PAS proteins may function in several different cellular pathways and thus the activation of one pathway may modulate signaling in another (Gu et al., 2000). In fact, interconnections between PAS protein-regulated pathways occur through a variety of mechanisms, including competition for binding partners (Woods and Whitelaw, 2002), functional interference (Moffett et al., 1997), direct interaction (Hogenesch et al., 1998), and transcriptional regulation (Chilov et al., 2001).

The proteins encoded by the *Sim* genes, SIM1 and SIM2, provide good examples of PAS proteins that interact with other family members mediating responses to hypoxia and environmental toxins such as dioxin. The interaction between the SIM proteins and other PAS members regulating hypoxia responses has been observed in studies demonstrating that SIM1 and SIM2, when stably expressed in human embryonic transformed kidney cells, compete with hypoxia inducible factor α (HIFα) for binding to ARNT and thereby repress HIFα-mediated gene transcription during hypoxia. The *Sim* genes also appear to interact with PAS members mediating toxin responses because the ectopic expression of SIM1 or SIM2 *in vitro* has been shown to block the AhR-activated

XRE reporter activities. Such repression is likely due to the competition of SIMs with AhR for ARNT, because it is relieved by the over-expression of ARNT. The SIM1/ARNT and SIM2/ARNT heterodimers do not interact with the XRE. Therefore, the sequestering of ARNT by the SIM proteins leads to the repression of the gene transcription activated by AhR (Woods and Whitelaw, 2002). PAS proteins identified as components of the circadian timekeeping mechanism also play a role in the regulation of non-clock processes through interactions with other PAS proteins. For example, *in vitro* translation experiments demonstrate that the clock protein, BMAL1, forms dimers with AhR (Hogenesch et al., 1997).

SUMMARY AND SPECIFIC AIMS

Clock genes form the molecular core of the timekeeping mechanism responsible for the generation of circadian rhythms. The targeted disruption of one or multiple clock genes often results in changes in fundamental properties of overt circadian rhythms, such as period, responses to light, and rhythm amplitude. However, emerging evidence suggests that clock gene function may extend beyond the mere regulation of circadian rhythms. Because clock genes are rhythmically expressed in the central nervous system (CNS) as well as in peripheral tissues, it is likely that clock genes may have significant roles in regulating other biological processes throughout the body.

What other processes are likely to be regulated by clock genes? We propose that clock genes may be involved in biological pathways by which PAS genes regulate development and responses to toxins. This hypothesis is founded on the observation that central elements of the circadian clock mechanism belong to the PAS gene family, and

interactions between PAS genes may provide for functional links between different pathways. This dissertation describes experiments designed to investigate clock gene involvement specifically in development and toxin response pathways.

Specific Aim 1: Are Clock Genes Differentially Expressed During Mammary Gland Development

The mammary gland provides a unique model for developmental research, because its development mainly occurs post-embryonically during puberty and undergoes repeated cycles in the female's life through pregnancy, lactation, and involution. Many identified mediators of mammary gland development exhibit changes in gene expression during different stages of the gland's normal development and during tumorigenesis. Therefore, experiments were conducted to determine whether expression of the clock genes *Per1*, *Per2*, *Bmal1*, and *Cry1* changes during different stages of mouse mammary gland development.

Specific Aim 2: Does Disruption of the Clock Genes Per1 and/or Per2 Affect the Activation of the AhR signaling Pathway in the Mammary Gland by TCDD

Activation of the AhR signaling pathway has been shown to affect tumorigenesis in the mammary gland, the effect of which vary largely depending on the type of ligand (Schlezinger et al., 2006). Evidence for interactions between the clock genes, *Per1* and *Per2*, and components of the AhR signaling pathway is derived from studies indicating that *Drosophila* PER forms dimers with AhR and ARNT via the PAS domain, and this process interferes with the DNA binding activity of AhR/ARNT heterodimers (Lindebro et al., 1995). The function of clock genes in the AhR signaling pathway is also suggested

by studies demonstrating that BMAL1 interacts with AhR (Hogenesch et al., 1997). Consequently, experiments were conducted to determine whether targeted disruption of the clock genes, *Per1* and/or *Per2*, affects the activation of cytochrome p450s and other components of the AhR signaling pathway in the mammary gland by the protypical AhR ligand, TCDD.

Specific Aim 3: Does Disruption of the Clock Genes Per1 and/or Per2 Alter TCCD-Mediated Responses of the AhR Signaling Pathways in the Liver

The primary function of the liver is to detoxify xenobiotics such as drugs and environmental contaminants. Because cells in the liver function as peripheral oscillators that rhythmically express all elements of the canonical clockworks (Yamazaki et al., 2000) and many drug-metabolizing enzymes in the liver exhibit circadian profiles (Furukawa, 1999), we propose that the clock genes, *Per1* and *Per2*, may also play a role in regulating xenobiotic responses of the liver. To address this hypothesis, experiments were conducted to determine if the targeted disruption of *Per1* and/or *Per2* alters the TCDD-mediated regulation of the AhR signaling pathway and induction of cytochrome p450 genes in the liver.

Specific Aim 4: Do PAS Gene Components of the Circadian Clockworks Directly Interact with Central Regulators of the AhR Signaling Pathway

The mechanism by which the *Per* genes modulate responses of the AhR signaling pathway to TCDD is unknown. One possibility is that the *Per* genes may influence TCDD-mediated regulation of the AhR signaling pathway by changing the binding efficiency of the AhR protein to the XRE of the *Cyp1A1* gene. This speculation

is supported by the finding that ectopic expression of the *Drosophila* PERIOD protein inhibits the recruitment of AhR protein onto the XRE of the *Cyp1A1* gene (Lindebro et al., 1995). Therefore, experiments in this aim were conducted to determine whether siRNA inhibition of *Per1* or *Per2* alters TCDD-induced AhR binding to the XRE and RNA Polymerase II association with the promoter of *Cyp1A1* in Hepa1c1c7 cells *in vitro*.

CHAPTER II

THE EXPRESSION OF CIRCADIAN GENE IN MOUSE MAMMARY EPITHELIAL CELLS AND IN THE DEVELOPING MOUSE MAMMARY GLAND*

INTRODUCTION

Biological clocks play a key role in adaptation to daily and seasonal environmental changes. The circadian clock regulates daily oscillations in metabolism, hormone, and neurotransmitter levels and a variety of behaviors, including activity, drinking, and feeding (Reppert and Weaver, 2001). In mammals, these 24-hr rhythms are controlled centrally by the suprachiasmatic nucleus (SCN), a small region in the anterior hypothalamus that functions as the master circadian clock and receives light input from the retina.

The basic molecular clockworks underlying the generation of circadian rhythms consist of interacting positive and negative transcriptional/translational feedback loops. The dimerization of two basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) family members, circadian locomotor output cycles kaput (Clock) and brain and muscle ARNT-like protein (Bmal1), drives the rhythmic transcription of three Period genes (*Per1*, *Per2*, and *Per3*) and two Cryptochrome genes (*Cry1* and *Cry2*) at the beginning of each day.

*Reprinted with permission from "Circadian Clock and Cell Cycle Gene Expression in Mouse Mammary Epithelial Cells and in the Developing Mouse Mammary Gland" by Metz RP, Qu X, Laffin B, Earnest D and Porter WW (2006) *Dev Dyn* **235:**263-271, Copyright [2005] by Wiley-Liss, Inc.

By mid-day, CRY and PER proteins begin to accumulate and enter the nucleus and then interact with CLOCK and/or BMAL1 to inhibit their own transcription and enhance Bma1l transactivation. Thus, increased CLOCK:BMAL1 heterodimers activate *Per* and *Cry* gene expression, and the clock is reset at the beginning of the next day (Reppert and Weaver, 2001).

The expression of these clock genes and their rhythmic regulation are not unique to the SCN but, instead, are widely distributed in many cells and tissues. For example, the period genes are expressed and rhythmically regulated in a variety of peripheral tissues, including liver, lung, and skeletal muscle (Zylka et al., 1998; Yamazaki et al., 2000). These peripheral oscillations in the expression of core clock elements are thought to provide for local coordination of cell populations in specific tissues and the circadian control of downstream genes, including *Cyclin D1* and *c-Myc*, which regulate cell cycle progression (Nagoshi et al., 2004). It is interesting that the cyclic expression of clock genes does not occur within some tissues such as testis and thymus (Alvarez et al., 2003; Morse et al., 2003). Thus, the local coordination of time between individual cells may be suspended in differentiating tissues or perhaps clock genes may also have noncircadian functions in developing cells.

The mammary gland is a unique tissue because its development mainly occurs postembryonically during puberty. In addition, the mammary gland undergoes repeated cycles of development that sequentially consist of proliferation, differentiation, and regression, coinciding, respectively, with pregnancy, lactation, and involution. Numerous hormones and growth factors exert positive or negative effects to regulate

these developmental transitions. These regulatory pathways include the hormones estrogen, progesterone, and prolactin as well as locally produced signaling molecules, including members of the transforming growth factor beta (TGF-), Wnt, epidermal growth factor (EGF), fibroblast growth factor (FGF), and matrix metalloprotease (MMP) families (Muller and Neville, 2001). Studies have shown that many of these signals governing mammary gland development and differentiation are regulated in a circadian manner. Ultimately, these orchestrated signals activate critical pathways, which control cell growth, migration, differentiation, and apoptosis, in a time-dependent manner to ensure proper branching morphogenesis and regulation of milk production.

The expression and functional implications of clock genes in mammary gland development have not been explored. Recent discoveries have revealed that the circadian clock and disruption of its core molecular components affect fundamental aspects of cellular function, including cell cycle progression, apoptosis, and differentiation (Matsuo et al., 2003; Xiao et al., 2003; Granda et al., 2005). Many of the genes identified in these studies as targets for circadian clock regulation are known mediators of normal mammary gland development. In addition, animal studies indicate that DNA synthesis and mitosis in the terminal end buds of developing adolescent mouse mammary glands occurs in a rhythmic manner with a peak in DNA synthesis occurring in the middle of the dark phase (Berger and Daniel, 1982). Therefore, the purpose of these studies was to determine whether developmental changes in clock gene expression occur within mammary epithelial-derived cell lines and mouse mammary tissues. Using quantitative polymerase chain reaction (qt-PCR), the expression of clock genes was compared with

genes with distinctive developmental profiles in undifferentiated and differentiated mouse epithelial cells (HC-11) *in vitro* and in mouse mammary glands at different stages of reproductive development.

MATERIALS AND METHODS

HC-11 Cell Culture

HC-11 cells are prolactin-responsive cells derived from the COMMA-1D mouse mammary epithelial cell line (Ball et al., 1988). Without hormone stimulation, these cells divide and do not express markers of the differentiated mammary gland. When treated with the lactogenic hormones hydrocortisone and prolactin, cell division is arrested and induction of *β-casein* expression, which is the marker of differentiation, is observed.

HC-11 cells were maintained in RPMI 1640 medium (Life Technologies) containing 10% fetal bovine serum, 50 g/ml gentamicin (Life Technologies), 5 g/ml insulin (Sigma) 10 ng/ml, and EGF (JRH Biosciences) in a humidified incubator in 95% air, 5% CO2 at 37°C. For induction of *β-casein* expression, cells were grown to confluence and growth medium was replaced on a daily basis for 3 consecutive days. On the fourth day, cells were washed three times with phosphate buffered saline (PBS) and priming medium (RPMI 1640 medium containing 10% charcoal-stripped serum, 50 g/ml gentamicin, 5 g/ml insulin, and 1 g/ml hydrocortisone [Sigma]) was added. Twenty-four hours later, cells were exposed to induction medium (priming medium containing 1 g/ml ovine prolactin [National Hormone and Pituitary Program, Harbor UCLA Medical Center, Torrance, CA]). Cells were maintained in induction medium for 4 days with

daily replacement. Total RNA was isolated and DNA was removed by on-column digestion using the RNeasy mini extraction kit (Qiagen).

Animals

Female C57Bl/6J mice were obtained from Charles River (Wilmington, MA). All animals were housed three per cage and maintained under a standard 12-hr photoperiod (LD 12:12; lights-on at 6:00 AM or Zeitgeber Time [ZT] 0). Animals were provided with access to food and water ad libitum. The procedures used in this study were approved by the University Laboratory Animal Care Committee at Texas A&M University.

For characterization of gene expression during mammary gland development, female mice were analyzed at seven developmental stages: virgin weeks 4 and 10 (V4 and V10), pregnancy days 6 and 16 (P6 and P16), lactation days 1 and 7 (L1 and L7), and involution day 3 (i3). At each stage of development, animals (n = 3-5) were killed by cervical dislocation around 11 hr after lights-on (ZT11) and mammary gland tissues were collected for later extraction of total RNA. To profile differences in the daily pattern of clock gene expression during development, mammary gland tissues were collected from mice at V10 and L1 (n = 3) killed by cervical dislocation at 6-hr intervals for 24 hr beginning at ZT0. For each animal, approximately 30 mg of mammary tissue was homogenized in 1 ml of Trizol reagent (Invitrogen) and total RNA was extracted from the aqueous phase. The final RNA pellet was suspended in 100 l RNase-free water, and subjected to purification and on-column DNase digestion using the RNeasy mini extraction kit (Qiagen).

Real-Time PCR

Quantification of relative mRNA abundance was performed using TaqMan or SYBR-Green real-time PCR technology (Applied Biosystems, Inc. [ABI], Foster City, CA) as described previously (Allen et al., 2004). To generate single-strand cDNAs, total RNA (1μg) was reverse transcribed using Superscript II (Invitrogen) and random hexamers or oligo-dTs. For each sample, the cDNA equivalent of 50 ng of total RNA was amplified in an ABI PRISM 7700 sequence detection system. Because limited information is available on clock gene expression in mouse mammary gland tissue or cell lines, each sample was analyzed in triplicate to ensure the accuracy of our data. To control for differences in sample RNA content, either Cyclophilin A (CypA) or *β-actin* mRNA was amplified with the cDNA equivalent of 1 ng of total RNA from the same samples. Results generated from the same experiments using different internal controls were consistent with each other.

The comparative CT method described in the ABI Prism 7700 Sequence Detection System User Bulletin #2 (PE-ABI) was used to calculate the relative mRNA abundance for a given target gene. Using this method, the amount of target gene mRNA in each sample was normalized first to corresponding *CypA* or *β-actin* mRNA levels, and then relative to a calibrator consisting of pooled cDNA from multiple samples that was analyzed on each reaction plate. For comparison of the daily patterns of clock gene expression in V10 and L1 mice, relative abundance of target mRNA was represented as a percentage of the maximal value obtained in the V10 group. Primer sequences for PCR amplification are listed in Table 2.

TABLE 2
Real-time PCR primers of the mammary developmental study

Gene	Primer Sequences
Cyclin D1	
Forward	5’-TCCGCAAGCATGCACAGA-3’
Reverse	5’-GGTGGGTTGGAAATGAACTTCA-3’
C-Myc	
Forward	5’-ACAGCAGCTCGCCCAAATC-3’
Reverse	5’-CGAGTCCGAGGAAGGAGAGA-3’
Arnt	
Forward	5’-GCCAGCCTGAGGTCTTTCAA-3’
Reverse	5’-AATTCTTCATTGTTGTAGGTGTTGCT-3’
β-casein	
Forward	5’-TGTGCTCCAGGCTAAAGT TCACT-3’
Reverse	5’-GGTTTGAGCCTGAGCATATGG-3’
Per1	
Forward	5’-AAACCTCTGGCTGTTCCTACCA-3’
Reverse	5’-AATGTTGCAGCTCTCCAAATACC-3’
Per2	
Forward	5’-ATGCTCGCCATCCACAAGA-3’
Reverse	5’-GCGGAATCGAATGGGAGAAT-3’
Bmal1	
Forward	5’-CCAAGAAAGTATGGACACAGACAAA-3’
Reverse	5’-GCATTCTTGATCCTTCCTTGGT-3’
Cry1	
Forward	5’-AGTTCCCCTCCCCTTTCTCTT-3’
Reverse	5’-GGGTTCCCTTCCATTTTGTCA-3’
β-actin	
Forward	5’-GCAACGAGCGGTTCCG-3’
Reverse	5’-CCCAAGAAGGAAGGCTGGA-3’
CypA	
Forward	5’-TGTGCCAGGGTGGTGACTT-3’
Reverse	5’-TCAAATTTCTCTCCGTAGATGGACTT-3’

Statistical Analyses

For *in vitro* observations, statistical analysis was performed using independent sample t-tests to determine whether the mRNA levels for a target gene were significantly different between undifferentiated and differentiated HC-11. For analysis of the mammary gland *in vivo*, statistical analyses were performed using one-way ANOVA to determine whether mRNA levels for a given gene were significantly different across different stages of development (V4, V10, P6, P16, L1, L7, and I3). Fisher's least significant difference (LSD) post hoc analyses were used if significant main effects of development were obtained. The daily patterns of gene expression in mammary gland were assayed using two-way ANOVAs with developmental stage (V10 vs. L1) and time (ZT0, 6, 12, and 18) as two independent variables. To identify rhythmic variation in mammary gland patterns of gene expression, time-point determinations within V10 and L1 mice were evaluated separately using one-way ANOVAs. Paired comparisons between determinations at specific time points were analyzed post hoc for statistical differences using Fisher's LSD tests. Further statistical analyses were performed using t-tests to compare V10 and L1 animals for differences in gene expression at ZT12, because mammary tissues were collected around this time for developmental comparisons. The α value was set at 0.05 for all statistical analyses.

RESULTS

Expression of Circadian Clock Genes and Differentiation Markers in Mouse Mammary Epithelial-Derived Cells

Because tissues composed of differentiating cells show developmental changes in the regulation of molecular components of the circadian clockworks (Alvarez et al., 2003), the present studies were conducted to determine whether development affects clock gene expression in the mammary gland, which is capable of repeated cycles of differentiation. We first examined the influence of development on clock gene expression in the HC-11 mammary epithelial cell line. HC-11 cells are an immortalized, but nontransformed, line isolated from the mid-pregnant mouse mammary gland (Ball et al., 1988). Similar to the mammary gland *in vivo*, HC-11 cells stop dividing and differentiate into epithelial cells that show high expression of the milk protein *β-casein* after stimulation with prolactin and hydrocortisone.

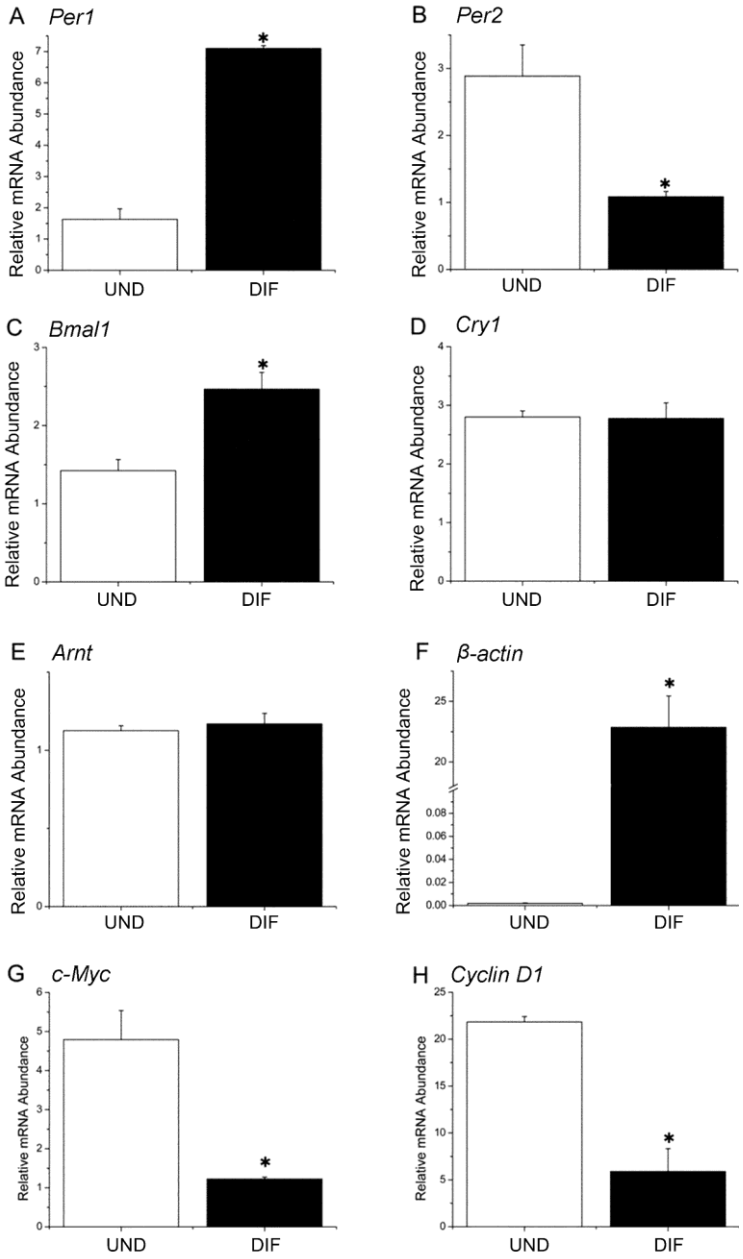


Fig. 6. Expression of circadian clock, cellular proliferation, and mammary differentiation marker genes in undifferentiated and differentiated mouse mammary epithelial-derived HC-11 cells. A-H: *Per1* (A), *Per2* (B), *Bmal1* (C), *Cry1* (D), *Arnt* (E), *β-casein* (F), *c-Myc* (G), and *Cyclin D1* (H) mRNA levels were analyzed in undifferentiated (UND) and differentiated (DIF) HC-11 cells. Total RNA was isolated from three plates per treatment and analyzed by quantitative real-time reverse transcriptase-polymerase chain reaction. Data are expressed as the mean (±SEM) for each differentiation state. Asterisks indicate significant differences in gene expression levels between UND and DIF HC-11 cells.

Thus, HC-11 cells differentiated by exposure to these lactogenic hormones were examined in relation to undifferentiated cells for differences in clock gene expression by qt-PCR. Interestingly, differentiated HC-11 cells were found to express significantly higher levels of *Per1* (Fig. 6A) but lower levels of *Per2* ($P < 0.05$) mRNA than undifferentiated cells (Fig. 6B). Levels of *Bmal1* expression in differentiated HC-11 cells were also significantly elevated ($P < 0.05$) relative to those found in undifferentiated cells (Fig. 6C). These differentiation-induced increases in *Per1* and *Bmal1* mRNA levels were 4.7-fold and 1.7-fold, respectively, whereas *Per2* expression was decreased by 3-fold in differentiated cells. However, undifferentiated and differentiated HC-11 cells exhibited no significant differences in *Cry1* expression. Further analysis of the temporal profiles of clock gene expression revealed no evidence of circadian or regular rhythmic variations in serum-induced HC-11 cells that were either undifferentiated or differentiated by exposure to lactogenic hormones (data not shown). To examine the effect of differentiation on expression of a noncircadian clock member of the bHLH-PAS family, levels of *Arnt* mRNA were also analyzed in undifferentiated and differentiated HC-11 cells. Although abundant *Arnt* expression was observed in HC-11 cells, relative mRNA levels were not altered by differentiation (Fig. 6E).

To provide an index of developmental state, expression of mammary differentiation and proliferation markers was also analyzed in the same HC-11 cultures. Expression of *β-casein*, a major milk protein and widely used marker of mammary epithelial cell differentiation, was significantly elevated ($P < 0.05$) in differentiated HC-11 cells (Fig. 6F). In contrast, expression of two principal markers of mammary epithelial cell proliferation, *c-Myc* and *Cyclin D1*, were significantly higher ($P < 0.05$) in undifferentiated HC-11 cells (Fig. 6G and 6H).

Expression of Circadian Clock Genes in the Developing Mouse Mammary Gland

Because our *in vitro* studies suggest that clock gene expression may be developmentally regulated in mouse mammary epithelial cells, we extended this analysis to determine whether clock gene mRNA levels are similarly altered during mammary gland development *in vivo*. Using qt-PCR, clock gene expression was analyzed in mouse mammary tissues collected between Zeitgeber Time (ZT) 10-12 at seven different stages of development. Our results indicate that clock gene expression in the mammary gland is developmentally regulated (Fig. 7).

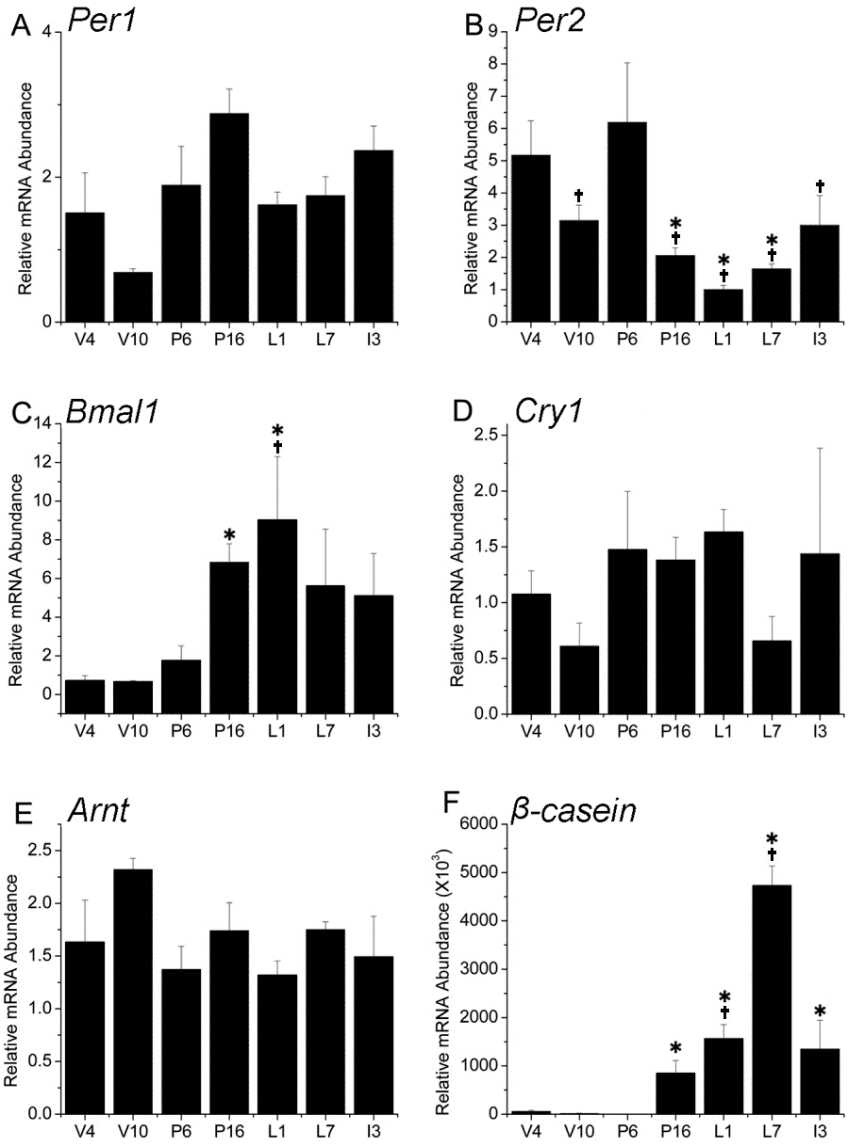


Fig. 7. Developmental regulation of circadian clock gene expression in the mouse mammary gland. A-F: *Per1* (A), *Per2* (B), *Bmal1* (C), *Cry1* (D), *Arnt* (E), and *β-casein* (F) mRNA levels were analyzed in mouse mammary glands during different stages of development. Total RNA was isolated between ZT10 and ZT12 from mammary tissue obtained from at least three individual mice per time point and analyzed by quantitative real-time reverse transcriptase-polymerase chain reaction. Time points were chosen to represent significant developmental stages, including early (4-week-old virgin, V4) and late (10-week-old virgin, V10) adolescence, early (pregnancy day 6, P6) and late (pregnancy day 16, P16) pregnancy, early (lactation day 1, L1) and late (lactation day 7, L7) lactation, and mid-involution (day 3 involution, I3). Mean values that were significantly different ($P < 0.05$) from virgin week 4 and pregnancy day 6 are denoted by an asterisk and a dagger, respectively.

Analysis with one-way analysis of variance (ANOVA) revealed a main effect of developmental stage on the levels of *Per2* and *Bmal1* [$F_{Per2}(6, 16) = 4.551$; $F_{Baml1}(6,16) = 3.721$; $P < 0.05$], but not *Per1* and *Cry1*, mRNA in the mammary gland. Of these developmentally regulated clock genes, *Per2* and *Bmal1* exhibited the most distinctive patterns of expression and the largest differences between stages. *Per2* expression in the mammary gland was relatively high during virgin stages and early pregnancy, decreased during the latter stage of pregnancy, remained low throughout lactation, and increased slightly during involution. The developmental pattern of *Bmal1* expression in the mammary gland was inversely related to that for *Per2*; mammary content of *Bmal1* mRNA was low in virgin and P6 animals, increased during late pregnancy, and remained high throughout lactation and involution. Although morphological changes occur throughout virgin, pregnancy, lactation, and involution stages of mammary gland development, we focused on critical comparisons of clock gene expression at V10 and L1, because these stages serve to distinguish fully mature, but undifferentiated, mammary gland from differentiated tissues that resemble the developmental status of HC-11 cells stimulated with lactogenic hormones. Consistent with our *in vitro* observations, *Per1* expression in the mammary gland at L1 was approximately two fold higher than that at V10. In contrast, *Per2* expression was highest in early adolescent (V4) and pregnant (P6) glands, which are periods associated with rapid expansion of the mammary epithelial compartment through active proliferation (Fig. 7B). Post hoc analysis showed that *Per2* mRNA levels in the mammary gland were significantly higher ($P < 0.05$) at V4 than at P16, L1, and L7. In addition, *Per2* expression was also

significantly greater ($P < 0.05$) in P6 than in V10, L1, L7, and I3 glands. Changes in *Bmal1* expression also occurred during the mammary gland development (Fig. 7C) such that mRNA levels were significantly higher ($P < 0.05$) at P16 and L1 than those observed in V4 mice. Although *Cry1* showed a trend for increased expression during pregnancy and early lactation (P6, P16, and L1), no significant differences in *Cry1* mRNA levels were detected over the course of mammary gland development (Fig. 7D). Similar to *in vitro* observations in HC-11 cells, the nonclock bHLH-PAS gene *Arnt* exhibited no significant differences in expression between developmental stages (Fig. 7E). In contrast, expression of the milk protein and mammary differentiation marker *β-casein* was significantly increased [$F(6, 14) = 22.16$; $P < 0.05$] in late pregnancy and throughout lactation relative to levels found in virgin and early pregnancy glands (Fig. 7F).

Expression of Cell Cycle Regulation Genes in the Developing Mouse Mammary Gland

Similar to the circadian clock, cell division is cyclically regulated involving precise transitions between component processes. Specific regulatory proteins gate transitions between resting state, DNA synthesis, and mitosis. Control checkpoints at these transitions ensure that progression into the cell cycle is timed and tightly regulated. Defects in these checkpoint proteins are responsible for uncontrolled proliferation that can lead to mutations, genomic instability, and ultimately cancer. Several studies have shown that the central circadian clock regulates cell-cycle progression in numerous tissues, including the intestine, oral epithelium, skin, and bone marrow progenitor cells

(Scheving et al., 1983; Buchi et al., 1991; Garcia et al., 2001; Reddy et al., 2005). Because *Per2* has been implicated in the regulation of *Cyclin D1* and *c-Myc* expression in the liver (Fu et al., 2002; Fu and Lee, 2003), we examined mammary gland expression of these two cell cycle genes and compared their developmental profiles with those observed for clock genes. *c-Myc* (Fig. 8A) and *Cyclin D1* (Fig. B) expression was similarly altered during mammary gland development such that mRNA levels for both genes were low in adult virgin mice (V10) and increased during early (P6) and late pregnancy (P16) when the epithelial cells proliferate in preparation for milk production. Decreases in both *c-Myc* and *Cyclin D1* mRNA levels were observed during lactation and early involution. However, analysis with one-way ANOVA revealed a main effect of developmental stage on *c-Myc* expression [$F(6,14) = 9.414$; $P < 0.05$] but not *Cyclin D1*. Mammary gland levels of *c-Myc* mRNA during pregnancy (P6 and P16) were significantly greater ($P < 0.05$) than those observed at V4 and during lactation and early involution. The lack of significant differences in *Cyclin D1* expression among stages is probably related to the high degree of variation of *Cyclin D1* mRNA levels in P6, P16, and L1 glands (Fig. 8B).

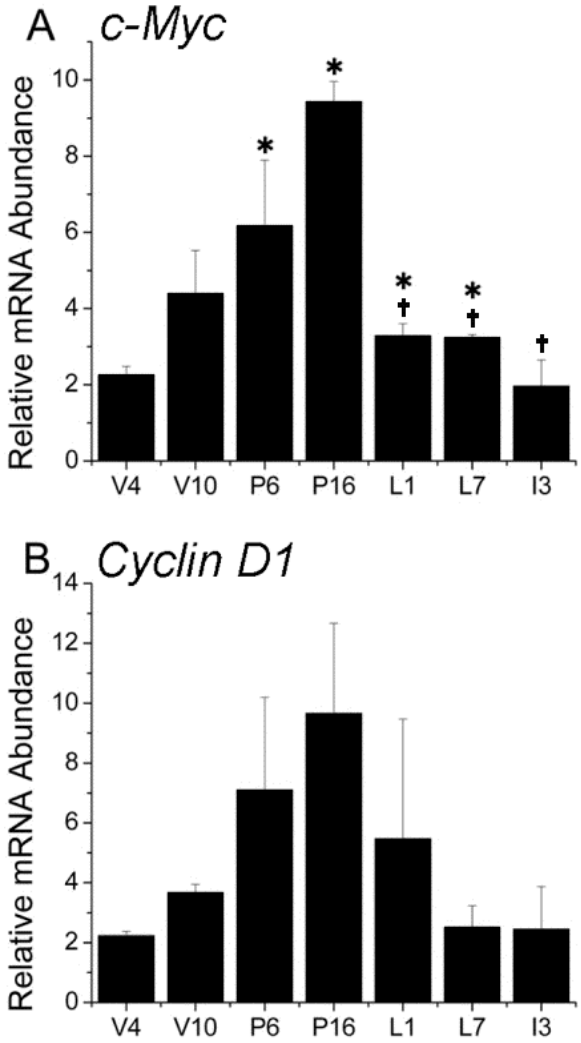


Fig. 8. Expression of *c-Myc* and *Cyclin D1* in the developing mouse mammary gland. Real-time polymerase chain reaction analysis of *c-Myc* (A) and *Cyclin D1* (B) expression in mouse mammary glands (n = 3-5) at virgin week 4 (V4) and 10 (V10), pregnancy day 6 (P6) and 16 (P16), lactation day 1 (L1) and 7 (L7), and involution day 3 (I3). Data are expressed as the mean (±SEM) at each developmental time point. Mean values that were significantly different ($P < 0.05$) from virgin week 4 (V4) and pregnancy day 6 (P6) are denoted by an asterisk and a dagger, respectively.

Circadian Rhythmicity of Mammary Clock Gene Expression Is Altered During Differentiation

Clock gene expression is rhythmically regulated in many peripheral tissues (Reppert and Weaver, 2001; Reddy et al., 2005). Because the mammary tissue used in our developmental analysis was obtained around the same time of day (between ZT10 and ZT12) for all stages, it is possible that the observed changes in clock gene expression during development may reflect alterations in the circadian phase of clock gene oscillations, rather than their relative expression. To explore this possibility, we analyzed the daily patterns of clock gene expression in V10 and L1 mice (Fig. 9), because these two stages showed the greatest differences in our developmental panel.

Mammary tissues were characterized by rhythmic expression of *Per1*, *Per2*, *Bmal1*, and *Cry1* (Fig. 9A, B, C and D). In the mammary gland of V10 animals, the rhythmic peak in *Per1* expression occurred during the day at ZT6 (Fig. 9A). This rhythmic pattern of *Per1* expression is similar to that observed in the kidney (Fig. 9F) and that reported in other peripheral tissues (Tong et al., 2004). Maximal levels of *Per2* mRNA in V10 mammary tissues were observed at ZT12 (Fig. 9B), which coincides with the peak in DNA synthesis and mitosis found in developing mouse mammary glands (Berger and Daniel, 1982). *Bmal1* and *Cry1* mRNA levels in the virgin mammary gland oscillated in an antiphase relationship to the rhythm of *Per2* expression with a nocturnal peak occurring at ZT18 (Fig. 9C, D). During lactation, mammary gland levels of *Per1*,

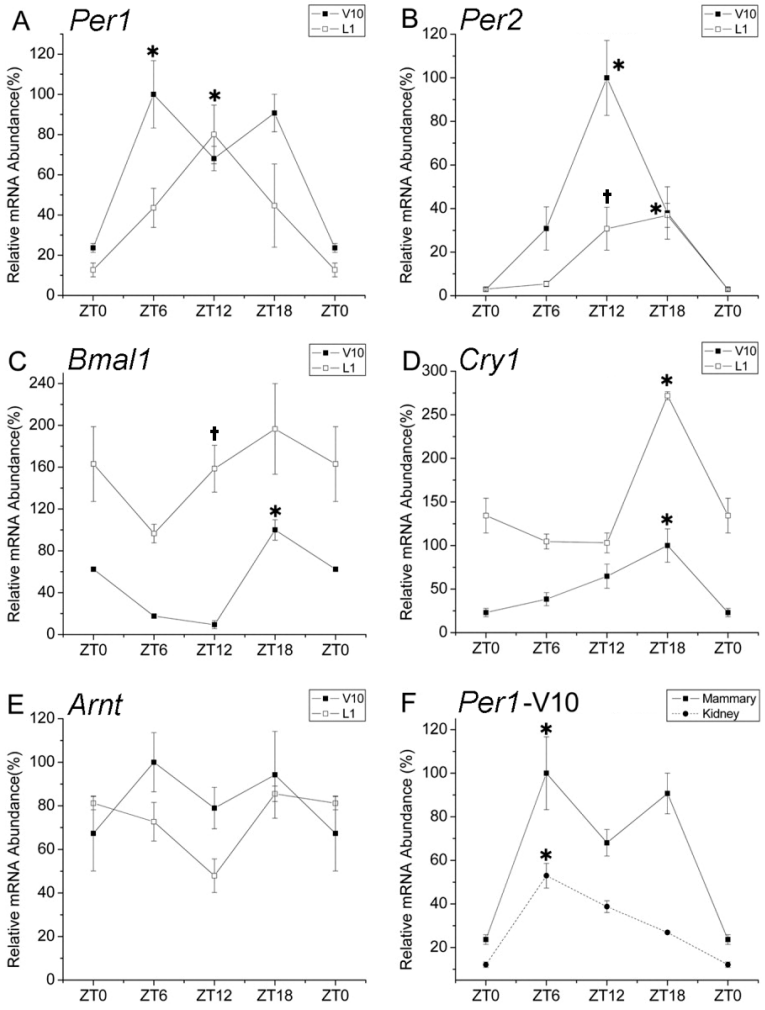


Fig. 9. Daily expression of circadian clock genes in 10 week virgin and day 1 lactating mouse mammary glands. A-E: Temporal patterns of *Per1* (A), *Per2* (B), *Bmal1* (C), *Cry1* (D), and *Arnt* (E) expression in 10-week-old virgin (V10; filled squares, solid line) and day 1 lactating (L1; open squares, solid line) mouse mammary glands. Symbols denote polymerase chain reaction determinations of mRNA levels (mean ± SEM) in V10 and L1 mammary glands (n = 3) collected at 6 hr intervals for 24 hr. The plotted values correspond to the ratios of mouse *Per2*, *Bmal1*, or *Cry1*/*CypA* or *β-actin* mRNA signal and are represented as a percentage of the maximal value obtained in the V10 group. F: Daily pattern of *Per1* expression in kidney tissues from V10 animals is also depicted in comparison with its temporal profile in the mammary gland. Asterisks indicate time points during which peak values for gene expression were significantly greater ($P < 0.05$) than those observed during preceding or succeeding minima of the same developmental stage. Because all tissues in Figure 7 were collected around ZT10-ZT12, V10 and L1 were compared further for differences in clock gene expression at ZT12. Mean values at ZT12 that were significantly different ($P < 0.05$) between V10 and L1 animals are denoted by a dagger.

Per2, and *Cry1*, but not *Bmal1*, mRNA fluctuated rhythmically. Further comparison between developmental stages revealed differences in the phase of these diurnal oscillations such that *Per1* and *Per2* gene expression levels peaked 6 hours later in L1 mice than in V10 animals. However, the implications of these developmental differences in rhythm phase are somewhat restricted by the limited temporal resolution associated with the 6-hr sampling interval used in this experiment. During lactation, the amplitude of the mammary gland rhythm in *Per2* expression was altered such that peak-to-trough differences were greater at V10 (100-fold) than at L1 (40-fold). It is also noteworthy that *Cry1* mRNA levels were consistently higher in L1 than V10 animals at all time points examined (Fig. 9D), although rhythm amplitude remained similar during lactation. In contrast to the clock genes, expression of the bHLH-PAS gene *Arnt* was similar, but not rhythmic, in V10 and L1 mammary glands (Fig. 9E).

DISCUSSION

Using an *in vitro* mouse mammary epithelial cell model, we have found that mammary epithelial cell differentiation is associated with increases in *Per1* and *Bmal1* mRNA levels. Growth and proliferation in undifferentiated HC-11 cells were associated with higher levels of *c-Myc* and *Cyclin D1* expression, which coincided with high levels of *Per2* mRNA. Conversely, differentiation of HC-11 cells, which is delineated by high levels of *β-casein* expression, occurred in conjunction with high levels of *Per1* and *Bmal1* mRNA. These findings indicate that the clock genes *Per1*, *Per2* and *Bmal1* in the HC-11 mammary epithelial cell line are differentially expressed during development in association with expression of specific differentiation markers.

Our *in vitro* results were corroborated *in vivo* through the analysis of a mouse mammary gland developmental panel, which indicates that *Per1* and *Bmal1* expression are associated with periods of increased cellular differentiation. Normally, BMAL1 and CLOCK induce *Per1*, *Per2*, and *Cry1* expression. However, our results show that during times when *Bmal1* expression is up-regulated, *Per1*, but not *Per2* mRNA levels are increased. The variation in gene expression amplitude indicate that circadian clock genes may have certain roles in regulating the mammary gland development, and they may be cooperating with each other differently from the normal feedback mechanisms in the SCN.

Clock genes are known to oscillate not only in the central oscillator, but also in multiple peripheral tissues (Zylka et al., 1998; Yamazaki et al., 2000). Therefore, the changes in clock gene expression we observed in the mammary gland development could be due to a simple shift in phase instead of the alteration of the overall gene expression amplitude. This is unlikely to be the primary reason. Our data showed that clock genes are rhythmically expression in the virgin stage of the gland development, when the mainly cellular event is cell division and growth. Interestingly, as the gland enters its lactating stage, when the cell division is arrested and cells have differentiated, the rhythm amplitude is greatly altered. *Bmal1* was expressed arrhythmically and at higher levels during lactation. Such alteration in the amplitude is also accompanied with changes in phase.

The above observations are consistent with previous developmental studies on testes and thymus. Clock genes *Bmal1* and *Per1* were found to be constantly expressed

through out day and night in testes and thymus. During pregnancy and early lactation stage, the mammary gland is composed primarily of differentiating cells, similar to testes and thymus. However, unlike testes and thymus, the mammary gland goes through repeated cycles and only goes into the differentiating stage if stimulated by pregnancy. This may be the reason why the gland appears to have regular clock gene oscillation similar to other peripheral organs during the virgin stage.

Comparison between the expression pattern of clock genes and cell cycle genes bring forth another layer of information. When mammary gland levels of *C-Myc* mRNA were relatively low on P6, peak expression of *Per2* was observed, whereas elevated *c-Myc* expression on P16 coincided with low *Per2* mRNA abundance. This inverse relationship between *c-Myc* and *Per2* expression during pregnancy may have implications for interactions between these genes in mammary gland development and carcinogenesis.

The analysis of clock gene expression during mammary gland development *in vivo* also provides us with the opportunity to measure changes in clock gene expression during apoptosis. In our study, clock gene *Per1* and *Per2* demonstrate trends of increase in gene expression during the involution stage of the mouse mammary gland development. Because apoptosis is a major event that characterizes the mammary involution stage, our observation indicates the possible role of *Per1* and *Per2* gene in stimulating apoptosis. The correlation between *Per* genes and apoptosis in cancerous cells was demonstrated in previous studies. The direct correlation between the rate of apoptosis and the *Per1* mRNA level has been revealed through *in vitro* studies showing

that the over-expression of *Per1* increases the apoptotic rate of colon cancer cells exposed to ionizing radiation, whereas siRNA inhibition of *Per1* decreases the apoptotic rate of similarly treated cells (Gery et al., 2006). The role of *Per2* in stimulating apoptosis has been presented in the study showing that intratumoral delivery of *Per2* stimulates apoptosis in transplanted lung carcinoma and therefore inhibits tumor growth (Hua et al., 2007). Our study using normal mammary tissues suggests a similar role of *Per1* and *Per2* in the initiation of apoptosis during the involution stage of regular mouse mammary gland development.

In summary, the current study demonstrated that the expression of clock genes, *Per1*, *Per2*, and *Bmal*, are altered during the mammary gland development. It is unknown what the cause of such changes is. Nakamura and colleagues showed that the mRNA level of *Per1* and *Per2* are affected in rats by 17beta-estradiol (E2) (Nakamura et al., 2005). Thus, clock gene expression could be affected by E2 during mammary gland development as well. On the other hand, our group observed that the ductile branching and alveolar growth is disrupted in virgin female mice with mutation in *Per1* and *Per2* (Unpublished data if Porter and Metz). Therefore, circadian clock genes may play indispensible roles in mouse mammary gland development and differentiation.

CHAPTER III

DISRUPTION OF CLOCK GENE EXPRESSION ALTERS RESPONSES OF THE AHR SIGNALING PATHWAY IN THE MOUSE MAMMARY GLAND*

INTRODUCTION

Members of the Per-Arnt-Sim (PAS) family of transcriptional regulators are involved in development and in sensing and adapting to environmental conditions. PAS proteins control diverse biological processes such as morphogenesis, circadian rhythms, and responses to hypoxia and toxins (Crews and Fan, 1999). The PAS domain is a multi-functional protein motif governing ligand and DNA binding as well as interactions between PAS and non-PAS proteins. Most PAS proteins function as heterodimers consisting of a sensor protein associated with a general binding partner. For example, the aryl hydrocarbon receptor (AhR) partners with the AhR nuclear translocator (Arnt) to mediate the transcriptional activation of xenobiotic metabolizing enzymes while circadian locomotor output cycles kaput (Clock) associates with brain, muscle ARNT-like protein 1 (Bmal1) to form core elements of the circadian clock mechanism in mammals. Not surprisingly, PAS protein-regulated pathways are interconnected through

---

* Reprinted with permission from "Disruption of Clock Gene Expression Alters Responses of the Aryl Hydrocarbon Receptor Signaling Pathway in the Mouse Mammary Gland" by Qu X, Metz RP, Porter WW, Cassone VM, Earnest DJ (2007) *Mol Pharmacol*. **72**:1349-58, Copyright [2007] by *Molecular Pharmacology Online* (URL http://www.molpharm.aspetjournals.org) by American Society for Pharmacology and Experimental Therapeutics.

a variety of mechanisms including competition for binding partners (Woods and Whitelaw, 2002), functional interference (Moffett et al., 1997), direct interaction (Hogenesch et al., 1998) and transcriptional regulation (Chilov et al., 2001).

The importance of the PAS genes, *AhR* and *Arnt*, responding to environmental toxins such as polycyclic aromatic hydrocarbons (PAHs) is described in Chapter I. Briefly, upon binding with PAH, AhR translocates from the cytoplasm into the nucleus, where it dimerise with ARNT. AhR:ARNT heterodimers then bind to the xenobiotic response elements (XRE) in target gene promoters affecting their expression. Principal targets of AhR signaling are cytochrome *p450* enzymes of the A and B subfamily including *Cyp1A1*, *Cyp1A2* and *Cyp1B1*, which are linked to the toxic and mutagenic effects of PAHs.

The PAS genes, *Clock*, *Bmal1*, Period 1 (*Per1*) and *Per2*, are important components of the circadian clock mechanism in mammals. These PAS genes form interacting positive- and negative-feedback loops in which the transcription of core components is rhythmically regulated by their protein products. PER1 and PER2 form heterodimeric complexes with the protein products of the Cryptochrome (*Cry*) genes and following a delay, these complexes are translocated to the nucleus (Kume et al., 1999; Yagita et al., 2000). CRY proteins then inhibit the transcription of *Clock* and *Bmal*1 (Griffin et al., 1999). In turn, CLOCK and BMAL1 close the feedback loop by forming heterodimers that positively regulate the rhythmic transcription of the *Per* and *Cry* genes via the activation of E-box elements (Gekakis et al., 1998; Hogenesch et al., 1998; Jin et al., 1999). CLOCK:BMAL1 complexes also mediate the activation of clock-controlled

genes that serve as outputs from the clock and function to regulate downstream rhythmic processes throughout the body.

Recent evidence suggests that molecular components of the circadian clock serve important functions in other PAS gene-regulated processes including development, tumorigenesis and drug metabolism. For example, *Per1* and *Per2* have been implicated in mammary gland development and differentiation based on changes in their expression during different stages of development and of the cell cycle. *Per1* and *Per2* involvement in the regulation of neoplastic growth is supported by the observations that *Per2*-deficient mice are more susceptible to the development of spontaneous and γ-radiation-induced tumors (Fu et al., 2002) and that PER1 and PER2 expression is down regulated in human breast tumors relative to normal surrounding tissue (Chen et al., 2005).

Because PAHs are potent carcinogens, and PAS proteins can interact with one another, we examined whether core elements of the clock mechanism also play some role in PAH responses mediated by the PAS gene, *AhR*. Previous studies indicate that *Drosophila* PER forms dimers with AhR and ARNT via the PAS domain and this process interferes with the DNA binding activity of AhR/ARNT heterodimers (Lindebro et al., 1995). Clock gene function in AhR signaling is also suggested by studies demonstrating that BMAL1 interacts with AhR (Hogenesch et al., 1997). Consequently, experiments were conducted to determine whether targeted disruption of the clock genes, *Per1* and/or *Per2*, affects the activation of cytochrome *p450*s and other components of the AhR signaling pathway in the mammary gland by the protypical AhR ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Our results demonstrate that disruption of the

circadian clock produces hyperinduction of host responsiveness to environmental toxicants.

MATERIALS AND METHODS

Animals

Experimental subjects were female wild type (WT) 129/sv mice (N=38) purchased from Charles River (Wilmington, MA) and *Per1*^{ldc}, *Per2*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mutant mice (@N=18) derived from breeding pairs generously provided by Dr. David Weaver (University of Massachusetts Medical School, Worcester, MA). Establishment and characterization of these transgenic mice has been described previously (Bae et al., 2001). Animals were maintained in the vivarium at Texas A&M University System Health Science Center under a standard 12h light: 12h dark cycle (LD 12:12; lights-on at 0600 hr) with access to food and water *ad libitum*. Procedures used in this study were approved by the University Laboratory Animal Care Committee at Texas A&M University.

Experiment 1: Effects of Targeted Disruption of *Per1, Per2* and *Per1/Per2* on TCDD-Induced Responses of the AhR Signaling Pathway in the Mouse Mammary Gland *in vivo*

Responses of the AhR signaling pathway were examined in 8-week-old female mice treated with TCDD (provided by Dr. Stephen Safe, Texas A&M University School of Veterinary Medicine, College Station, TX) at a dose of 10µg/kg body weight. Previous studies showed a single dose of 5µg/kg TCDD or higher for 24 hours significantly induces hepatic *Cyp1A1* expression in mice (Narasimhan et al., 1994). In

the current study, animals received an intraperitoneal injection of vehicle (corn oil) or TCDD about 6 hours after lights-on in the LD12:12 cycles (1200 hr; Zeitgeber Time [ZT] 6). Twenty-four hours after treatment, animals were sacrificed by cervical dislocation at ZT6) and mammary gland tissues were collected in RNA Stabilization Reagent (RNA *later*, Qiagen, Valencia, CA) for later extraction of total RNA. For each tissue sample, approximately 30 mg of mammary tissue was homogenized and processed for extraction of total cellular RNA using the RNeasy Lipid Tissue Mini Kit (Qiagen). The final RNA pellet was subjected to on-column DNase digestion (Qiagen), suspended in 100µl RNase-free water, and then stored at –80°C.

Experiment 2: Effects of Targeted Disruption of *Per1, Per2* and *Per1/Per2* on TCDD-Induced Responses of the AhR Signaling Pathway in Primary Cultures of the Mouse Mammary Gland

Mammary gland cells were collected from 12- to 14-week old female mice, and primary cultures of these cells were established using methods similar to those described previously (Pullan and Streuli, 1996; Seagroves et al., 1998). For each experiment, mammary gland cultures were obtained from WT mice and compared with those from the mutant mice (*Per1*^{ldc}, *Per2*^{ldc} or *Per1*^{ldc}/*Per2*^{ldc}) at the same age (@N=3). Briefly, cells were extracted from mouse mammary glands and cultured on serum/fetuin-coated 6-well-plates in DMEM/F12 medium (Invitrogen, Carlsbad, California) containing 5µg/ml insulin (Sigma-Aldrich, St. Louis, MO), 1µg/ml hydrocortisone (Sigma-Aldrich), 5ng/ml epithelial growth factor (EGF) (QED Biosciences, San Diego, CA), 50µg/ml gentamicin (Invitrogen), 100U/ml penicillin/streptomycin (Invitrogen) and 5% FBS at

37°C in a humidified incubator with 5% CO_2. Confluent cultures were treated with vehicle (N=3; dimethyl sulfoxide [DMSO], Sigma-Aldrich) or 20nM TCDD (N=3) for 24 hours. After treatment, cultures were collected by trypsinization and total RNA was extracted using RNeasy mini kit (Qiagen). The dose and duration of TCDD treatment in these experiments were based on previous observations indicating that robust increases in *Cyp1A1* and *Cyp1B1* mRNA and protein levels occur within human mammary epithelial cells *in vitro* following exposure to TCDD for 24 hours (Chen et al.,, 2004).

Experiment 3: Time-Dependent Effects of TCDD Treatment On the AhR Signaling Pathway in the Mouse Mammary Gland *in vivo*

To determine whether TCDD-induced effects on the AhR signaling pathway *in vivo* vary as a function of treatment time, WT mice were injected intraperitoneally with vehicle or 10ug/kg body weight TCDD at the midpoint of either the light phase (1200hr; ZT 6; N=22) or dark phase (0000hr; ZT 18; N=12) and mammary gland tissues were collected 24 hours after treatment as described in Experiment 1.

Quantitative RT-PCR Analysis

Quantification of relative mRNA abundance was performed using SYBR-Green real-time PCR technology (Applied Biosystems, Inc. [ABI], Foster City, CA) as described previously (Metz et al., 2006). Total RNA (1ug) was reverse transcribed using Superscript II (Invitrogen) and random hexamers. For each sample, the cDNA equivalent to 1.25ng total RNA per 12.5ul reaction was amplified in an ABI 7500 Fast real-time PCR System using 9600 emulation modes. To control for differences in sample RNA content, cyclophilin A (*CypA*) or *β-actin* was amplified from the same samples. Primer sequences for PCR amplification of target and control genes are listed in Table 1.

The comparative C_T method was used to calculate the relative mRNA abundance for a given target gene. Using this method, the amount of target gene mRNA in each sample was normalized first to corresponding *CypA* or *β-actin* mRNA levels, and then relative to a calibrator consisting of pooled cDNA from multiple samples that was analyzed on each reaction plate.

TABLE 3
Real-time PCR primers of the AhR signaling pathway study

Gene	Primer Sequences
Cyp1A1	
Forward	5’-CCTCTTTGGAGCTGGGTTT-3’
Reverse	5’-AGGCTCCACGAGATAGCAGT-3’
Cyp1B1	
Forward	5’-TCTTTACCAGATACCCGGATG-3’
Reverse	5’-CACAACCTGGTCCAACTCAG-3’
AhR	
Forward	5’-CAAATCAGAGACTGGCAGGA-3’
Reverse	5’-AGAAGACCAAGGCATCTGCT-3’
Arnt	
Forward	5’-GCCAGCCTGAGGTCTTTCAA-3’
Reverse	5’-AATTCTTCATTGTTGTAGGTGTTGCT-3’
β-actin	
Forward	5’-CTTCCTTCTTGGGTATGGAATC-3’
Reverse	5’-ACGGATGTCAACGTCACACT-3’
CypA	
Forward	5’-TGTGCCAGGGTGGTGACTT-3
Reverse	5’-TCAAATTTCTCTCCGTAGATGGACTT-3’

Statistical Analysis

In Experiments 1 and 2, statistical analyses were first performed on the raw data using two-way analyses of variance (ANOVAs) with treatment (vehicle vs. TCDD) and genotype (WT, *Per1*^{ldc}, *Per2*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc}) as two independent variables. If significant main effects of treatment were identified, planned comparisons using independent *t*-tests were applied to compare gene expression between control and TCDD groups of the same genotype. The fold differences in gene expression between these treatment groups were then analyzed using one-way ANOVA and, if required, Fisher's least significant difference (LSD) post hoc analyses to determine whether genotype had a significant effect on TCCD-induced changes in mRNA levels for a given gene. In Experiment 3, the raw data were first analyzed using two-way ANOVAs with treatment (vehicle vs. TCDD) and time (ZT 6 vs. ZT 18) as two independent variables. If significant main effects were identified, planned comparisons using independent *t*-tests were applied to compare gene expression between control and TCDD groups at the same treatment time. For the *p450* genes, the fold differences in TCDD-induced gene expression were also analyzed using independent *t*-tests to determine the significance of treatment time. The α value was set at 0.05 for all statistical analyses.

RESULTS

Effects of Targeted Disruption of *Per1, Per2* and *Per1/Per2* on TCDD-Activated AhR Signaling Pathway in the Mouse Mammary Gland *in vivo*

Expression and TCDD-mediated induction of key genes in the AhR signaling pathway was compared between WT, *Per1*^{ldc}, *Per2*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mutant mice

(Bae et. al., 2001). Consistent with previous findings (Narasimhan et al., 1994), basal levels of *Cyp1A1* mRNA expression were observed in the mammary glands of all vehicle-treated WT and mutant mice (Fig. 10A). Relative to vehicle controls, TCDD had a robust effect in inducing *Cyp1A1* expression within the mammary gland. In both WT and mutant mice, mammary gland levels of *Cyp1A1* mRNA were significantly greater ($p<0.05$) in TCDD-treated animals than vehicle controls. Genotype-related differences were evident in the absolute values of TCDD-induced *Cyp1A1* expression in the mammary gland (Fig. 10A). In the mammary glands of $Per1^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ mutant mice, the TCDD-induced *Cyp1A1* expression was significantly ($p<0.05$) and about 3 times higher than that found in WT animals. Analysis of the fold difference in gene expression between the TCDD- and vehicle-treated groups for each genotype revealed further distinctions in the activation of the AhR signaling pathway among mutant mice with targeted disruptions of the *Per1* and *Per2* genes (Fig. 10B). The fold differences in the TCDD-induced *Cyp1A1* expression within the mammary gland were significantly greater in $Per1^{ldc}$ ($p<0.05$) and $Per1^{ldc}/Per2^{ldc}$ ($p<0.05$) mutant mice than in WT animals. In fact, the inductive effects of TCDD on *Cyp1A1* expression within the mammary glands of $Per1^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ mutant mice were respectively increased by 17.9- and 5.9-fold relative to that found in WT mice.

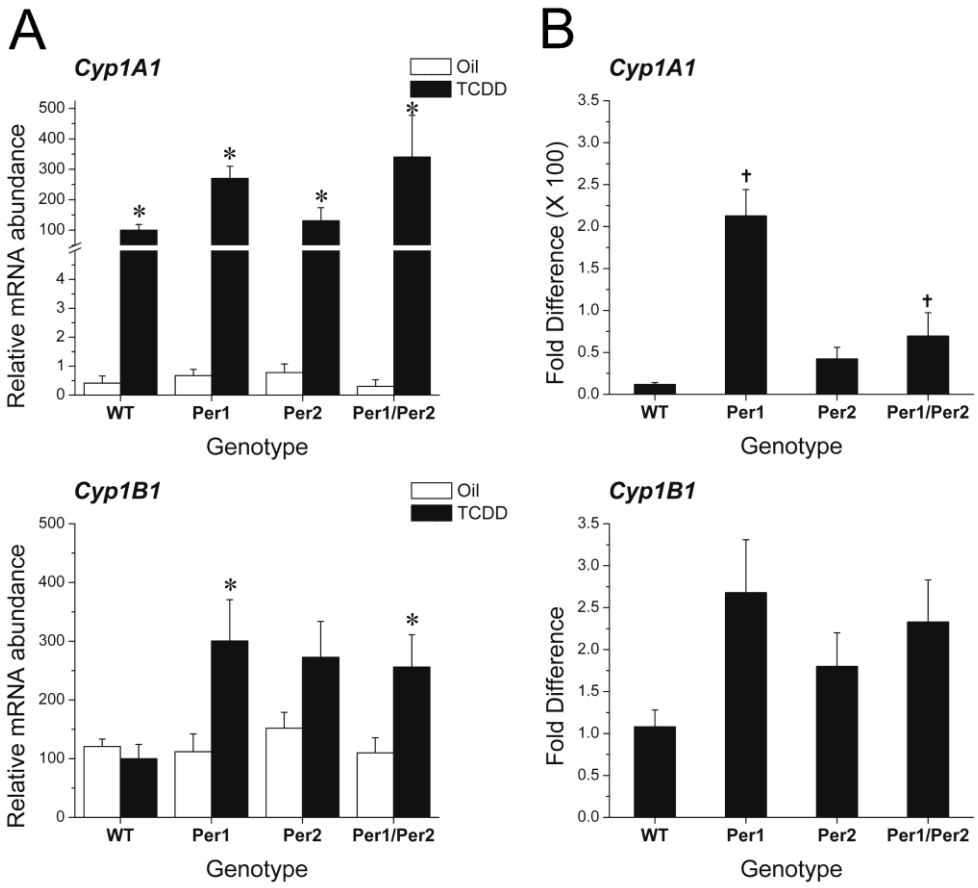


Fig. 10. Effects of targeted mutations of *Per1* (*Per1*^{ldc}), *Per2* (*Per2*^{ldc}), and *Per1*/ *Per2* (*Per1*^{ldc}/*Per2*^{ldc}) on TCDD-induced expression of *p450* genes in the mouse mammary gland. For *Cyp1A1* and *Cyp1B1*, the relative mRNA abundance (A) and fold differences (B) in their expression after TCDD treatment were analyzed in the mammary glands from WT, *Per1*^{ldc}, *Per2*^{ldc}, and *Per1*^{ldc}/*Per2*^{ldc} mice. Data are expressed as the mean (±SEM) for each experimental group. The plotted values for the relative mRNA abundance correspond to the ratios of species-specific *Cyp1A1* or *Cyp1B1*/*CypA* mRNA signal that were adjusted in relation to the average for TCCD-treated WT mice, which was arbitrarily set at 100. The values for fold differences in TCDD-induced *Cyp1A1* expression are represented at x100. Asterisks denote comparisons for each genotype, in which *p450* gene expression in the mammary gland of TCDD-treated mice was significantly greater ($p<0.05$) than that observed in oil-treated controls. For each genotype, fold differences in *p450* gene expression between the TCDD- and oil-treated groups were determined by normalizing all values to the average of oil-treated controls, which was arbitrarily set at 1. The fold differences in the TCDD-induced *Cyp1A1* and *Cyp1B1* expression within the mammary gland were significantly greater in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} (=, $p < 0.05$) mutant mice than in WT animals.

TCDD-mediated effects on mammary gland expression of another *p450* gene in the AhR signaling pathway, *Cyp1B1*, followed a similar trend. *Cyp1B1* mRNA levels in the mammary gland were consistently low and similar among all vehicle-treated WT and mutant mice (Fig. 10A). In comparison with vehicle controls, TCDD treatment produced significant increases ($p<0.05$) in mammary gland levels of *Cyp1B1* mRNA in $Per1^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ mice but not in WT and $Per2^{ldc}$ animals. Despite the lack of significant variation among genotype-based comparisons, the fold differences in TCDD-induced *Cyp1B1* expression within the mammary glands of $Per1^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ mutant mice was respectively increased by 2.5- and 2.2-fold relative to that found in WT animals (Fig. 10B).

Two major regulators of TCDD-induced responses, *AhR* and *Arnt* were also analyzed in our study. Similar levels of *AhR* expression were observed in mammary glands of all vehicle-treated WT and mutant mice (Fig. 11). No significant differences in mammary gland levels of *AhR* mRNA were evident among vehicle control and TCCD-treated mice, regardless of their genotype. Similar to *AhR*, *Arnt* mRNA expression in the mammary gland was comparable in all mice with no major treatment- or genotype-based differences (Fig. 11).

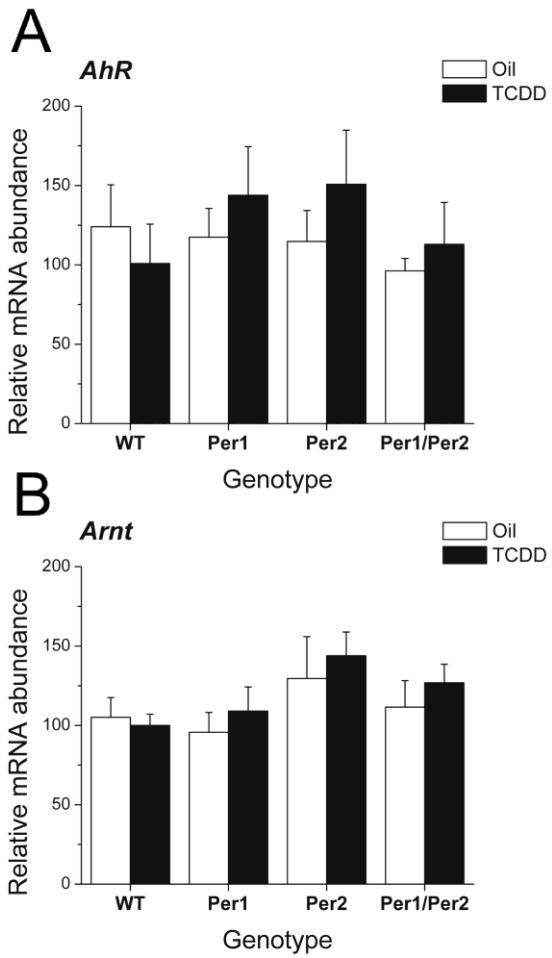


Fig. 11. Relative abundance of *AhR* and *Arnt* mRNA in the mammary glands of oil- and TCDD-treated *Per1*^{ldc}, *Per2*^{ldc}, and *Per1*^{ldc}/*Per2*^{ldc} mice. Data are expressed as the mean (±SEM) for each experimental group. The plotted values for the relative mRNA abundance correspond to the ratios of species-specific *AhR* or *Arnt*/*CypA* mRNA signal that were adjusted in relation to the average for TCCD-treated WT mice, which was arbitrarily set at 100. TCDD treatment or genotype had no significant effects on mammary gland levels of *AhR* and *Arnt* mRNA.

Effects of Targeted Disruption of *Per1, Per2* and *Per1/Per2* on TCDD-Activated AhR Signaling Pathway in Primary Cultures of the Mouse Mammary Gland

Because the AhR signaling pathway is influenced by steroid hormones such as glucocorticoids and 17β-estradiol *in vivo* (Gorski et al., 1988; Christou et al., 1995; Prough et al., 1996) and because serum levels and the rhythmic regulation of steroid hormones are altered in *Per1*-deficient mice (Dallmann et al., 2006), parallel *in vitro* analysis was conducted to indirectly address the role of *Per*-mediated hormonal changes in the potentiation of TCDD-induced AhR signaling in mutant mice. Primary cultures of the mouse mammary gland were used to determine whether the observed amplification of TCDD-induced *p450* expression in *Per* mutant mice persists *in vitro* in the absence of hormonal influences.

Basal levels of *Cyp1A1* expression were observed among all vehicle-treated mammary gland cultures derived from WT and mutant animals (Fig. 12A). Relative to vehicle controls, treatment with 20nM TCDD for 24 hours induced significant increases ($p<0.05$) in *Cyp1A1* expression in all mammary gland cultures. Genotype-based distinctions were evident in the fold differences in *Cyp1A1* expression between TCDD- and vehicle-treated cultures. Consistent with the results of our *in vivo* study, the fold differences in the TCDD-induced *Cyp1A1* expression were significantly greater in mammary gland cultures derived from $Per1^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ ($p<0.05$) mutant mice than in those from WT animals (Fig. 12B). The TCDD-mediated induction of *Cyp1A1* mRNA levels was 5.7- and 4.2-fold higher in $Per1^{ldc}$ and $Per1^{ldc}$ /$Per2^{ldc}$ mammary cells than in WT cultures. In mammary gland cultures from $Per2^{ldc}$ mice, the fold differences in the induction of *Cyp1A1* expression by TCDD were reduced relative to WT cells.

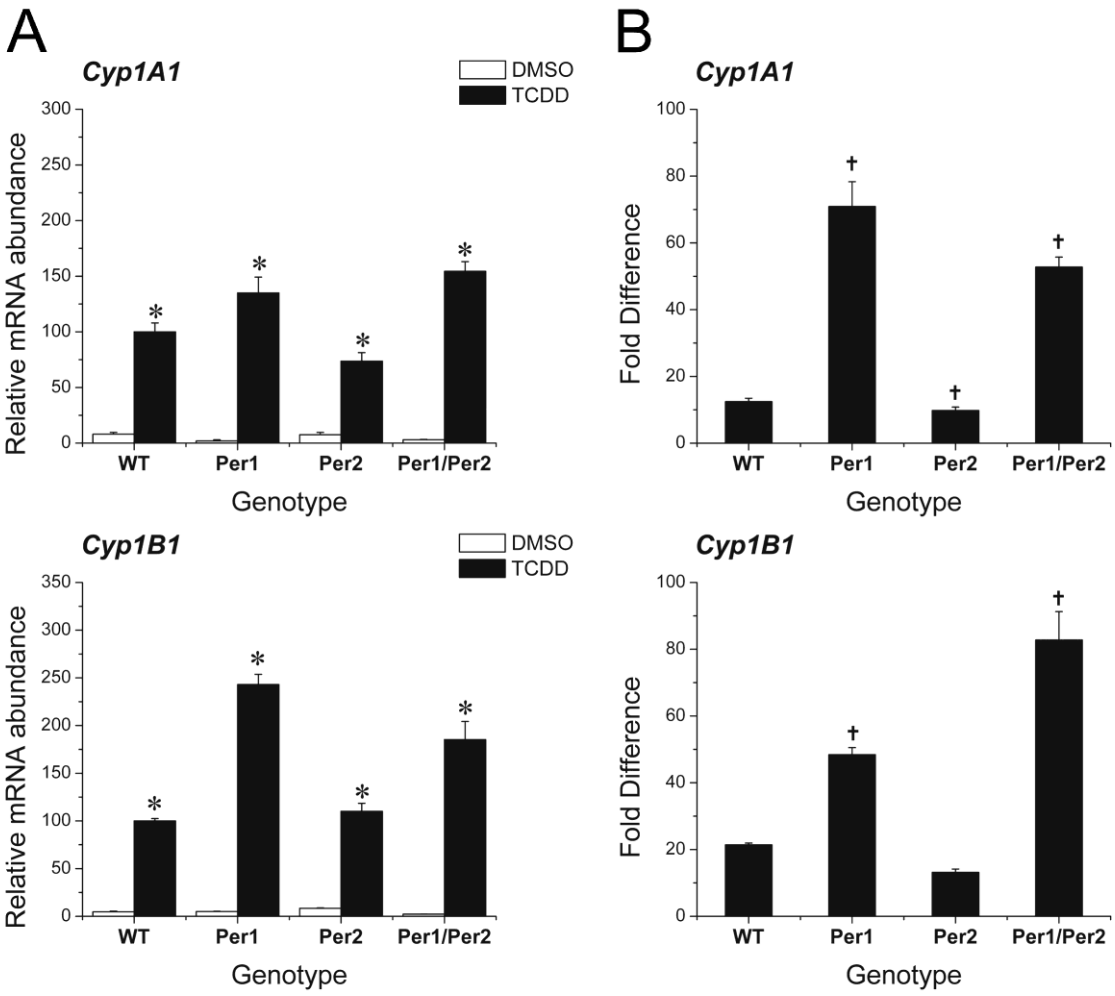


Fig. 12. Effects of targeted mutations of *Per1*, *Per2*, and *Per1*/ *Per2* on TCDD-mediated induction of *p450* genes in mouse mammary cells *in vitro*. For *Cyp1A1* and *Cyp1B1*, the relative mRNA abundance (A) and fold differences (B) in their expression after TCDD treatment were analyzed in primary cultures of mammary tissue derived from WT, $Per1^{ldc}$, $Per2^{ldc}$, or $Per1^{ldc}/Per2^{ldc}$ mice. Data are expressed as the mean (±SEM) for each experimental group. The plotted values for the relative mRNA abundance correspond to the ratios of species-specific *Cyp1A1* or *Cyp1B1*/ *β-actin* mRNA signal that were adjusted in relation to the average for TCCD-treated cells from WT mice which was arbitrarily set at 100. Asterisks denote comparisons for each culture genotype, in which *p450* gene expression in TCDD-treated mammary cells was significantly greater ($p<0.05$) than that observed in DMSO-treated cultures. The fold differences in *p450* gene expression between the TCDD- and DMSO-treated cultures of each genotype were determined by normalizing all values to the average of DMSO-treated controls, which was arbitrarily set at 1. The fold differences in TCDD-induced *Cyp1A1* and *Cyp1B1* expression were significantly greater (=, $p<0.05$) in $Per1^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ mammary gland cultures than in WT cells.

Similar to *Cyp1A1*, *Cyp1B1* expression was consistently low in vehicle-treated mammary cells (Fig. 12A). TCDD had a significant effect in inducing *Cyp1B1* expression in all WT and mutant cultures ($p<0.05$). Interactions between treatment and genotype were comparable to those observed *in vivo*. TCDD treatment produced increases in *Cyp1B1* expression in $Per1^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ mammary cells that were significantly greater ($p<0.05$) than those found in WT cultures (Fig. 12A). Further analysis revealed that the fold differences in TCDD-induced *Cyp1B1* expression were significantly greater ($p<0.05$) in $Per1^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ mammary gland cultures than in WT cells (Fig. 12B). Fold differences in TCDD-mediated *Cyp1B1* induction in $Per1^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ cells were respectively 2.3- and 3.9-times higher than that in WT cultures.

The central regulators of TCDD-induced signaling, *AhR* and *Arnt*, were differentially expressed and affected by this toxin in primary cultures of the mouse mammary gland. Among vehicle-treated mammary cells, it is interesting that *AhR* mRNA expression in all mutant cultures were significantly greater ($p<0.05$) than WT levels. The highest levels of *AhR* expression in vehicle-treated cells were observed in cultures derived from $Per1^{ldc}$ mice. TCDD had a significant effect in reducing *AhR* mRNA levels in both WT and mutant mammary cells ($p<0.05$) (Fig. 13). In response to TCDD exposure, *AhR* expression was reduced to comparable levels among WT and mutant cells with exception of cultures derived from $Per1^{ldc}$ mice. Following treatment, *AhR* mRNA levels in $Per1^{ldc}$ mammary gland cultures were significantly (about 2 times) higher ($p<0.05$) than those found in WT cells exposed to TCDD. In contrast to *AhR*, there was no significant effect of either treatment or genotype on *Arnt* expression in mammary gland cultures (Fig. 13). Similar levels of *Arnt* mRNA were expressed by both WT and mutant cells following treatment with vehicle or TCDD.

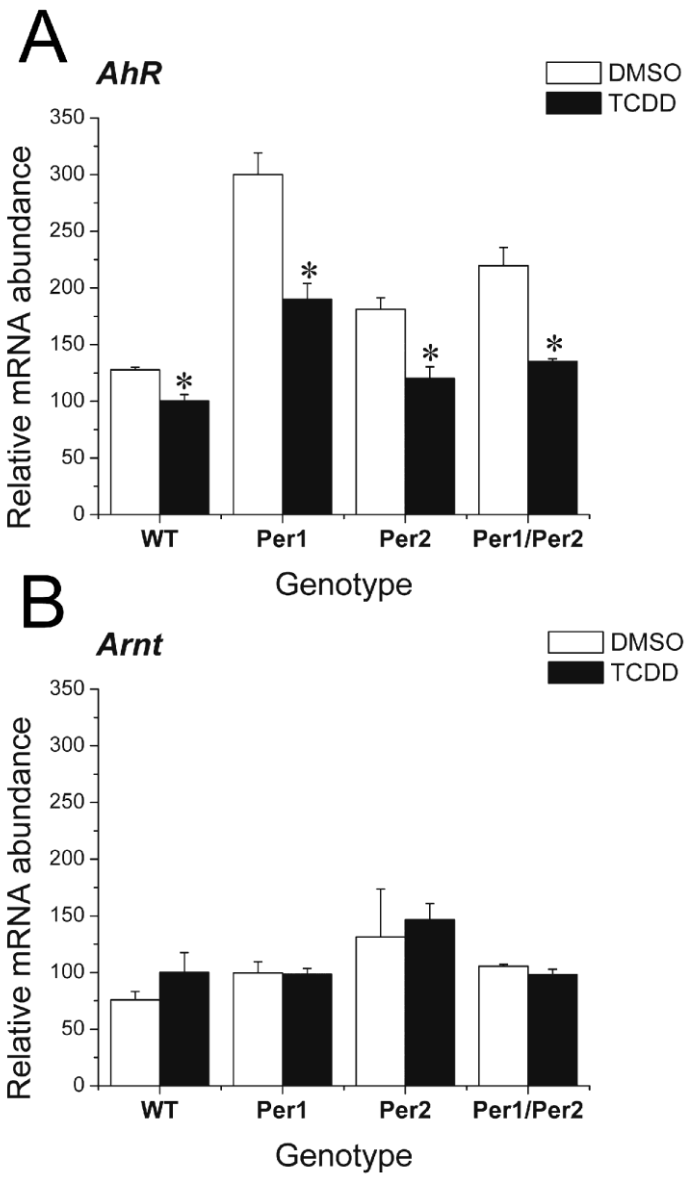


Fig. 13. Relative abundance of *AhR* and *Arnt* mRNA in DMSO- and TCDD-treated mammary cultures derived from WT, *Per1*^{ldc}, *Per2*^{ldc}, or *Per1*^{ldc}/*Per2*^{ldc} mice. Data are expressed as the mean (±SEM) for each experimental group. The plotted values for the relative mRNA abundance correspond to the ratios of species-specific *AhR* or *Arnt*/ *β-actin* mRNA signal that were adjusted in relation to the average for TCCD-treated cells from WT mice, which was arbitrarily set at 100. Asterisks denote comparisons for each culture genotype, in which *AhR* mRNA levels in TCDD-treated mammary cultures was significantly decreased (p<0.05) relative to that observed in DMSO-treated cells.

Time-Dependent Effects of TCDD Treatment on the AhR Signaling Pathway in the Mouse Mammary Gland *in vivo*

Because the results of our *in vivo* and *in vitro* experiments indicate that changes in *Per* gene expression affect TCDD-induced responses of the AhR signaling pathway, we next determined whether the inductive effects of this toxin on *p450* gene expression varies endogenously in accord with the diurnal *Per* rhythms that are known to occur in mouse mammary gland (Metz et al., 2006). In oil-injected WT mice, *Cyp1A1* mRNA levels in the mammary gland were low irrespective of treatment time (Fig. 14A). TCDD administered during the day and at night both triggered significant increases in mammary gland levels of *Cyp1A1* mRNA ($p<0.05$). Interestingly, treatment time had a significant effect ($p<0.05$) on the fold differences in the TCDD-mediated *Cyp1A1* induction in the mammary gland such that the increase in the expression of this *p450* gene triggered by toxin injection at ZT 18 was 8.6-fold higher than that following treatment at ZT 6 (Fig. 14B). Unlike the pattern for *Cyp1A1*, *Cyp1B1* expression in the mammary glands of vehicle-treated animals was marked by significant variation over time ($p<0.05$) with mRNA levels at ZT 6 that were substantially greater than those at ZT 18. Consequently, TCDD had no significant effects on mammary gland levels of *Cyp1B1* mRNA when treatment was administered at ZT 6 but induced significant increases ($p<0.05$) in expression of this *p450* gene following exposure at ZT 18 (Fig. 14A). Moreover, the fold differences of TCDD-induced *Cyp1B1* expression within the mammary gland at ZT 18 were significantly ($p<0.05$) and about 2 times higher than those observed at ZT 6 (Fig. 14B).

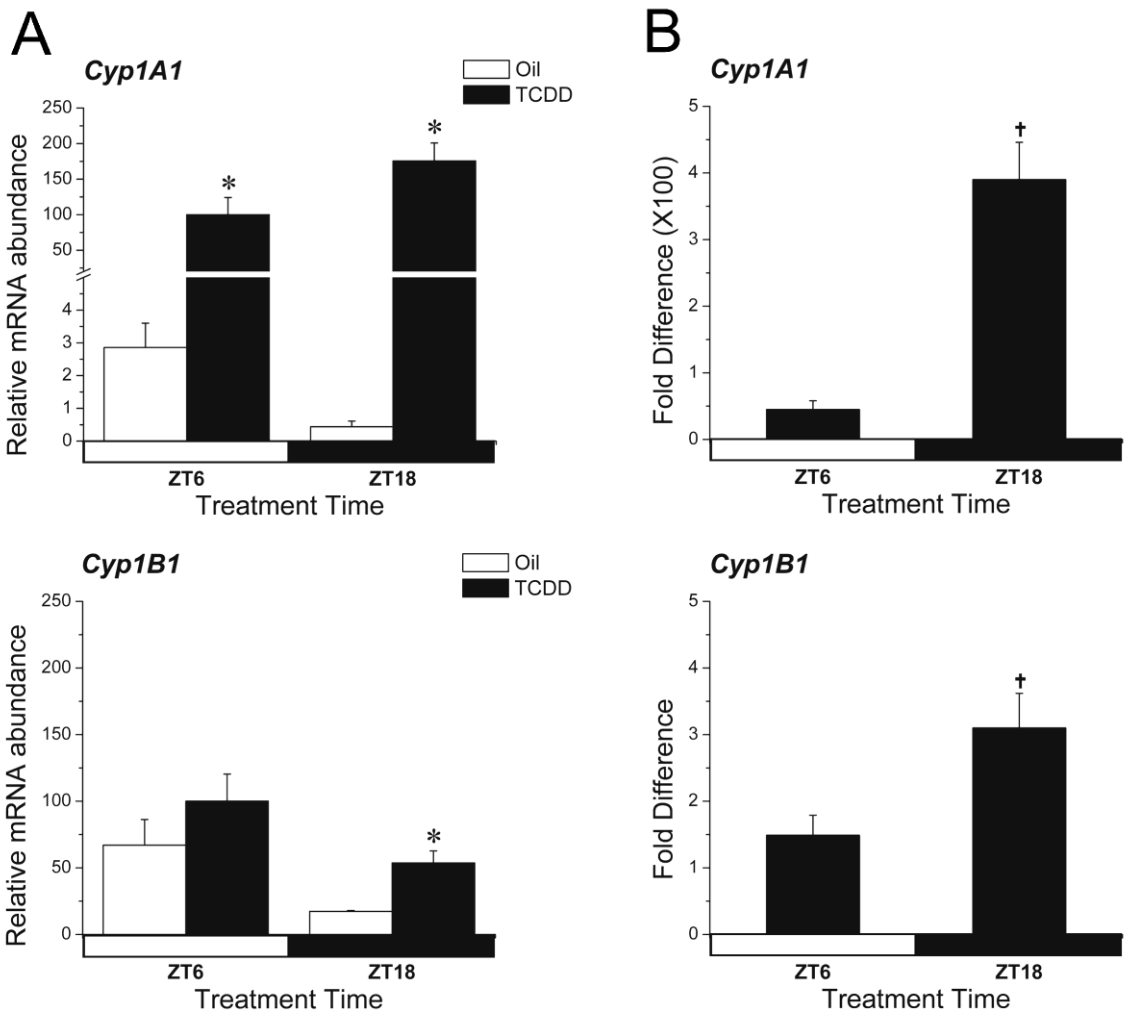


Fig. 14. Effects of treatment time on TCDD-mediated induction of *p450* genes in the wild type mouse mammary gland. For *Cyp1A1* and *Cyp1B1*, the relative mRNA abundance (A) and fold differences (B) in their expression after TCDD treatment during the daytime (ZT 6) and nighttime (ZT 18) were analyzed in the mammary glands of WT mice. Data are expressed as the mean (±SEM) for each experimental group. The plotted values for the relative mRNA abundance correspond to the ratios of *Cyp1A1* or *Cyp1B1*/*CypA* mRNA signal that were adjusted in relation to the average for WT mice exposed to TCDD at ZT6, which was arbitrarily set at 100. The values for fold differences in TCDD-induced *Cyp1A1* expression are represented at x100. Asterisks denote treatment times, in which TCDD induced significant ($p<0.05$) increases in *Cyp1A1* and *Cyp1B1* expression within the mammary gland relative to that observed in oil-treated controls. For each treatment time, fold differences in *p450* gene expression between the TCDD- and oil-treated groups were determined by normalizing all values to the average of oil-treated controls, which was arbitrarily set at 1.

To investigate if the diurnal oscillation of the TCDD-mediated *p450* induction persists in the absence of *Per* rhythms, we compared responses of *Per1*^{ldc}/*Per2*^{ldc} mice to TCDD given at ZT6 and ZT18. In oil-injected *Per1*^{ldc}/*Per2*^{ldc} mice, *Cyp1A1* mRNA levels in the mammary gland were low irrespective of treatment time (Fig. 15A). TCDD administered during the day and at night both triggered significant increases in mammary gland levels of *Cyp1A1* mRNA (p<0.05). Interestingly, the diurnal oscillation in TCDD-induced *Cyp1A1* expression observed in the wild type mammary is completely abolished in the *Per1*^{ldc}/*Per2*^{ldc} animals. Such abolishment of TCDD induced *Cyp1A1* expression is evident from the comparison of the absolute mRNA abundance (Fig. 15A) as well as fold increases in expression levels (Fig. 15B). Similar to *Cyp1A1*, *Cyp1B1* expression in the mammary gland of vehicle-treated animals remains similar between day and night (Fig. 15A), without showing the diurnal variation demonstrated by vehicle treated wild type animals. TCDD significantly increases the *Cyp1B1* mRNA abundance in *Per1*^{ldc}/*Per2*^{ldc} mouse mammary gland (p<0.05), regardless of the treatment time. The induction of *Cyp1B1* by TCDD, as demonstrated by the absolute values of gene expression (Fig. 15A) and fold increases in mRNA levels (Fig. 15B), is higher at ZT18 than at ZT6. In *Per1*^{ldc}/*Per2*^{ldc} mice, the fold differences of TCDD-induced *Cyp1B1* expression within the mammary gland at ZT 18 were significantly (p<0.05) and about 6 times higher than those observed at ZT 6 (Fig. 15B). Therefore, the diurnal oscillation of TCDD triggered *Cyp1B1* induction is further enhanced in *Per1*^{ldc}/*Per2*^{ldc} mice comparing to wild type.

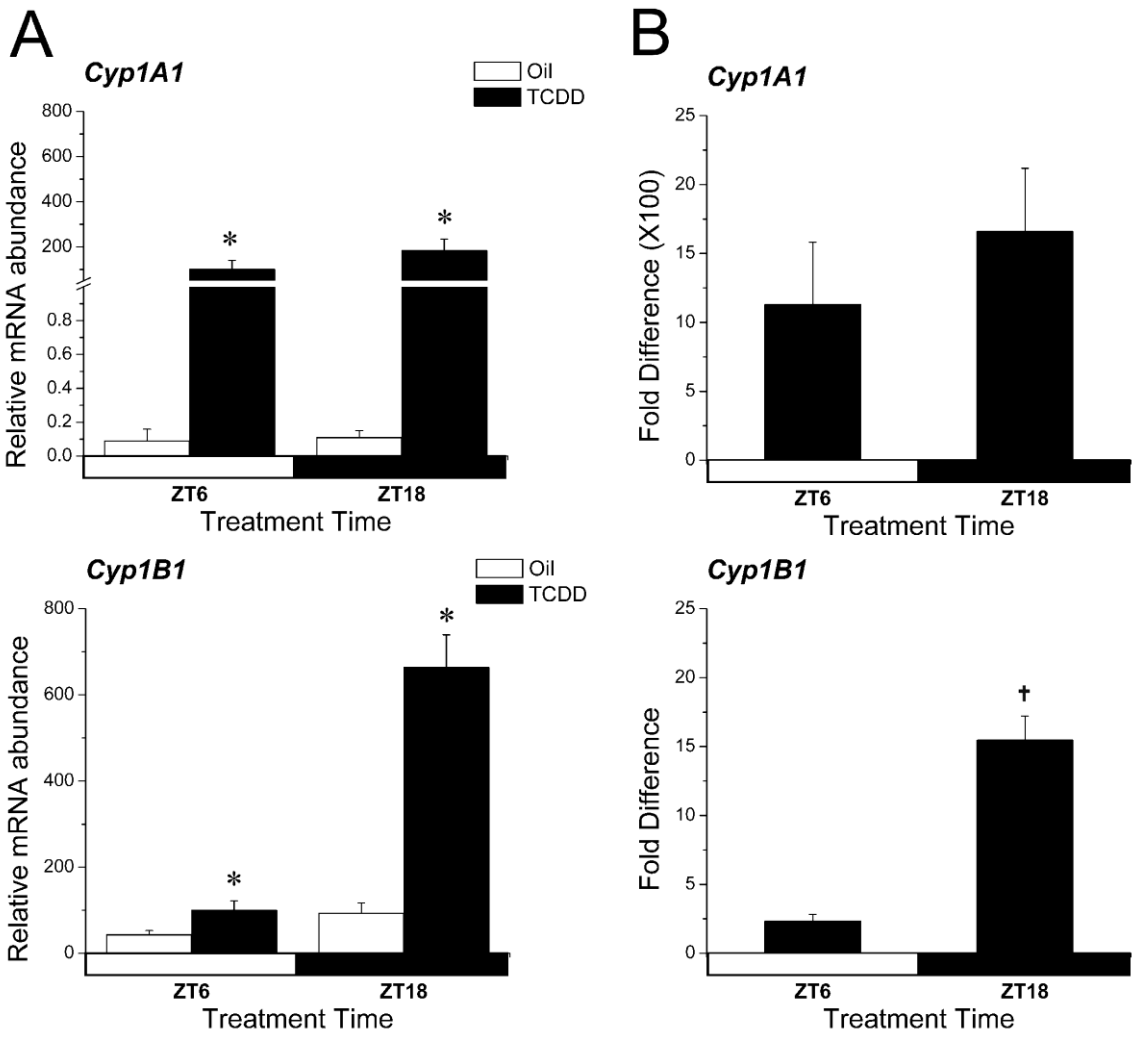


Fig. 15. Effects of treatment time on TCDD-mediated induction of *p450* genes in the *Per1*^{ldc}/*Per2*^{ldc} mouse mammary gland. For *Cyp1A1* and *Cyp1B1*, the relative mRNA abundance (A) and fold differences (B) in their expression after TCDD treatment during the daytime (ZT 6) and nighttime (ZT 18) were analyzed in the mammary glands of *Per1*^{ldc}/*Per2*^{ldc} mice. Data are expressed as the mean (±SEM) for each experimental group. The plotted values for the relative mRNA abundance correspond to the ratios of *Cyp1A1* or *Cyp1B1*/*CypA* mRNA signal that were adjusted in relation to the average for *Per1*^{ldc}/*Per2*^{ldc} mice exposed to TCDD at ZT6, which was arbitrarily set at 100. The values for fold differences in TCDD-induced *Cyp1A1* expression are represented at x100. Asterisks denote treatment times, in which TCDD induced significant (p<0.05) increases in *Cyp1A1* and *Cyp1B1* expression within the mammary gland relative to that observed in oil-treated controls. For each treatment time, fold differences in *p450* gene expression between the TCDD- and oil-treated groups were determined by normalizing all values to the average of oil-treated controls, which was arbitrarily set at 1.

DISCUSSION

Previous studies have linked the PAS genes, *Per1*, *Per2*, *Bmal1* and *Clock*, not only with the generation of circadian rhythms but also with the regulation of various non-clock functions. Mice with deletions or mutations of these genes exhibit alterations in the circadian regulation of locomotor activity (Bae et al., 2001; Reppert and Weaver, 2002) in conjunction with a myriad of other physiological or behavioral disturbances including decreased body weight, shortened life span, increased tendon calcification (McDearmon et al., 2006), premature aging, tissue hyperplasia (Fu et al., 2002; Lee, 2006), increased alcohol consumption (Spanagel et al., 2005) and altered responses to other drugs of abuse (Kondratov et al., 2007). Consistent with these observations, the present study revealed that targeted disruption of the *Per* genes modifies mammary gland responses to the environmental toxin, TCDD. It is interesting that the inductive effects of TCDD on expression of the cytochrome *p450* genes, *Cyp1A1* and *Cyp1B1*, were potentiated in mammary glands and in primary cultures of mammary cells from $Per1^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$, but not $Per2^{ldc}$, mice. Similar to primary analyses of these mutant mice indicating that the *Per1* and *Per2* genes influence different molecular processes but are indispensable for normal clock function (Zheng et al., 1999; Shearman et al., 2000; Bae et al., 2001), our findings suggest that *Per1* plays a distinct role in modulating TCDD activation of the AhR signaling pathway.

The mechanism by which the *Per* genes interact with components of the AhR signaling pathway and influence its activation by TCDD is currently unknown. However, a possible explanation is that the potentiation of TCDD-induced *Cyp1A1* and *Cyp1B1*

expression in the mammary gland is associated with the altered function of the circadian clockworks in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mice. Similar to the findings of Bae et al. (2001), these mutant mice exhibited arrhythmic patterns of wheel-running activity after 1-2 weeks of exposure to constant darkness (data not shown). Because up to 10% of the transcriptome is clock-controlled in peripheral tissues (Duffield, 2003) and some of these genes with oscillatory profiles are essential elements of critical biochemical processes mediating drug metabolism and responses to xenobiotic agents (Gachon et al., 2006; Menger et al., 2007), the disruptive effects of the *Per1* mutation on circadian clock function may extend to the rhythmic regulation of the AhR signaling pathway in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mice. This hypothesis is indirectly supported by the present observations that *AhR* expression and TCDD-mediated induction of *p450* genes in the mammary gland are marked by diurnal variation. In the mammary glands of WT mice, *AhR* mRNA levels are lower and TCDD-induced *Cyp1A1* and *Cyp1B1* expression is greater during the night than during the day. Because the diurnal variation in the TCDD-mediated *p450* induction in the mammary gland is inversely related to the temporal pattern of *Per1* gene expression, in which tissue mRNA levels peak during the day and remain low throughout the night (Metz et al., 2006), the disruption of *Per1* expression and rhythmicity in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mice may be responsible for the potentiated activity of the AhR signaling pathway in response to this toxin. This hypothesis is partially supported by the observation that the diurnal oscillation in the TCDD-induced *Cyp1A1* expression demonstrated in wild type animals is abolished in the *Per1*^{ldc}/*Per2*^{ldc} mice. Our results show that when *Per1* and *Per2* genes are disrupted

in vivo, the inductive effect of TCDD on *Cyp1A1* expression remains at a high level through out the day. The day/night variation in the TCDD-induced expression of *Cyp1B1*, however, appears to be enhanced in the *Per1*^{ldc}/*Per2*^{ldc} mice, which indicates that the mechanism regulating the day/night variation of different AhR target genes could be variable.

A related explanation for the present findings is that the disruption of *Per1* gene expression or clock function in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mice may indirectly mediate the potentiation of TCDD-induced *p450* expression in the mammary gland, perhaps by altering the levels and/or circadian cycles of hormones that influence the AhR signaling pathway. The potential involvement of *Per*-mediated hormonal changes in the altered TCDD responses in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mice is compatible with the observations that steroid hormones modulate AhR signaling *in vivo* (Gorski et al., 1988; Christou et al., 1995; Prough et al., 1996) and that steroid hormone levels and cycles are altered in *Per1*-deficient mice (Dallmann et al., 2006). However, the results of our *in vitro* study do not appear to support this possibility because the potentiation of TCDD-induced *Cyp1A1* and *Cyp1B1* expression persists in mammary cultures from these mutant mice despite the absence of hormonal signals that occur *in vivo*.

Alternatively, the potentiation of TCDD-induced *p450* gene expression in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mice may not be associated with the disruption of the circadian clockworks but instead related to changes in *Per* gene interactions with specific components of the AhR signaling pathway. Our findings raise the possibility that *Per1* may directly inhibit TCDD activation of the AhR signaling pathway. This inhibition

could occur via interactions between *Per1* and PAS gene components of the AhR signaling pathway at several different levels. Because the function of PER1 in regulating circadian rhythmicity is distinctly mediated through its interactions with other PAS proteins in the feedback loop (Bae et al., 2001), PER1 may similarly interact with the PAS proteins, AhR and ARNT, and perhaps inhibit their dimerization. *Per1* may also directly influence the AhR signaling pathway by inhibiting the binding of AhR:ARNT complexes to the DREs of target genes. This hypothesis is corroborated by the observation that *Drosophila* PER impedes the formation and DNA binding activity of AhR:ARNT complexes by dimerizing with AhR and ARNT via the PAS domain (Lindebro et al., 1995). Our *in vitro* results suggest that *AhR* expression is another prospective target for *Per1* in down-regulating TCCD-mediated activation of the AhR signaling pathway because *AhR* mRNA expression in mammary gland cells derived from $Per1^{ldc}$, $Per2^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ mutant mice was substantially higher than that found in WT cultures. Further analysis will be necessary to specifically determine whether the *Per* genes modulate TCDD-mediated induction of *p450* gene expression by inhibiting *AhR* expression, the formation of AhR:ARNT heterodimers or the binding of these complexes with DREs.

In summary, our data indicate that the targeted disruption of *Per1* potentiates the inductive effects of TCDD on *p450* gene expression in the mammary gland *in vivo* and *in vitro*. Because the induced expression of the *p450* genes, *Cyp1A1* and *Cyp1B1*, has been associated with increased cancer risk (Schrenk, 1998), this finding may have further implications for the involvement of the *Per* genes in carcinogenesis. Previous

studies have shown that *Per2* suppresses tumor development by regulating responses to DNA damage (Fu et al., 2002). Moreover, human breast cancer tissue is distinguished by *Per1* promoter methylation and associated alterations in PER1 protein levels relative to that found in adjacent normal cells (Chen et al., 2005). Together with the present evidence for diurnal fluctuations in *AhR* expression and TCDD-induced *Cyp1A1* and *Cyp1B1* expression within the mammary gland, these observations suggest that the *Per* genes, perhaps via their function in the circadian clockworks, may play an important role in regulating responses to environmental toxins and in modulating their carcinogenic effects.

CHAPTER IV

INHIBITION OF PERIOD GENE EXPRESSION ALTERS THE INDUCTIVE EFFECTS OF DIOXIN ON THE AHR SIGNALING PATHWAY IN THE MOUSE LIVER

INTRODUCTION

The liver is the primary center for detoxifying xenobiotics such as drugs and environmental contaminants ingested by vertebrate species. Liver xenobiotic metabolism is often associated with the generation of intermediate metabolites that can produce hepatotoxicity and deleterious effects on other tissues throughout the body. Among xenobiotics metabolized by the liver, polycyclic aromatic hydrocarbons (PAHs) are especially toxic. PAHs are a class of ubiquitous environmental contaminants found in grilled foods, vehicle exhaust, asphalt, hazardous waste sites and in smoke from cigarettes, residential wood burning and waste incinerators. PAHs are immunosuppressive, teratogenic and carcinogenic in laboratory animal. Some PAHs such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) have been classified as potential human carcinogens.

Members of the Per-Arnt-Sim (PAS) family of transcription factors, the aryl hydrocarbon receptor (*AhR*) and AhR nuclear translocator (*Arnt*), are critical components of the signaling pathway responsible for the mutagenic effects of PAHs in the liver. Similar to other PAS genes involved in sensing and adapting to environmental conditions, *AhR* partners with *Arnt* to initiate the signaling cascade that mediates the

transcriptional activation of xenobiotic metabolizing enzymes. Activation of this signaling pathway occurs when PAHs enter the cell and bind with AhR, which is complexed with 90 kD heat shock proteins (*Hsp90*) and the aryl hydrocarbon receptor interacting protein (*Aip*) (Mimura and Fujii-Kuriyama, 2003). In response to ligand binding, this complex dissociates, and PAH-bound AhR translocates from the cytoplasm to the nucleus and associates with ARNT. AhR:ARNT heterodimers bind to the xenobiotic response elements (XRE) in promoter regions of target genes and induce their expression. The primary targets of PAH-induced AhR signaling are cytochrome *p450* enzymes of the A and B subfamily including *Cyp1a1*, *Cyp1a2* and *Cyp1b1*. Induction of cytochrome *p450*s triggers oxidation of PAHs to reactive metabolites. If not rendered less reactive or suitable for excretion through conjugation by phase II detoxifying enzymes such as glutathione *S*-transferases and UDP- glucuronosyltransferases, these oxidative PAH metabolites may form DNA adducts capable of producing mutations and increased cancer risk.

Similar to AhR and ARNT, other PAS proteins predominantly function as heterodimeric pairs consisting of a sensor protein complexed with a general binding partner. Through such interactions, PAS proteins regulate a wide range of biological processes including morphogenesis and circadian rhythms. The role of PAS gene interactions in the regulation of circadian rhythms is particularly well-developed. Auto-regulatory feedback loops consisting of the PAS genes, *Clock*, brain and muscle ARNT-like protein 1 (*Bmal1*), Period 1 (*Per1*) and *Per2*, form core elements of the circadian clock mechanism in mammals. The dimerization of CLOCK and BMAL1 drives the

rhythmic transcription of the Period genes and the Cryptochrome genes (*Cry1* and *Cry2*) at the beginning of each day via the activation of E-box elements (Gekakis et al., 1998; Hogenesch et al., 1998; Jin et al., 1999). The PER and CRY proteins subsequently accumulate and form heterodimeric complexes that are translocated to the nucleus (Kume et al., 1999; Yagita et al., 2000). CRY proteins then close this loop by inhibiting CLOCK:BMAL1-mediated transcription. In turn, rhythmic increases in *Bmal1* transcription reset the clock and start the cycle anew. CLOCK and BMAL1 are not only core components of the circadian timekeeping mechanism, but heterodimers of these PAS proteins also mediate the regulation of clock-controlled outputs that provide for the rhythmic programming of downstream processes.

The multi-functional properties of the PAS domain in mediating ligand and DNA binding as well as interactions between PAS and non-PAS proteins have important implications for intercommunication between different PAS protein-regulated pathways through a variety of mechanisms including competition for binding partners (Woods and Whitelaw, 2002), functional interference (Moffett et al., 1997), direct interaction (Hogenesch et al., 1998) and transcriptional regulation (Chilov et al., 2001). Functional interactions between PAS protein-mediated processes are indicated in recent studies suggesting that molecular components of the circadian clock may also play a role in the regulation of toxin metabolism by the PAS gene, *AhR*. The results of these studies demonstrate that: 1) *Drosophila* PER forms dimers with AhR and ARNT via the PAS domain, and this process interferes with the DNA binding activity of AhR:ARNT heterodimers (Lindebro et al., 1995); 2) BMAL1 interacts with AhR (Hogenesch et al.,

1997); and 3) *Per1* is involved in regulating responses of the AhR to its protypical ligand, TCDD (Qu et al., 2007). Therefore, the objective of the present study was to further examine the function of the clock genes, *Per1* and *Per2*, in TCDD-induced activation of AhR signaling in the liver. Experiments were conducted to determine whether the effects of TCDD on hepatic expression of cytochrome *p450*s and other known biological markers in the AhR signaling pathway are altered following: 1) targeted disruption of *Per1* and/or *Per2 in vivo*; and 2) inhibition of these genes *in vitro* via infection with small interfering RNA (siRNA) constructs.

MATERIALS AND METHODS

Animals

Experimental subjects were female wild type (WT) 129/sv mice (N=38) purchased from Charles River (Wilmington, MA) and $Per1^{ldc}$, $Per2^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ mutant mice (each N=18) derived from breeding pairs generously provided by Dr. David Weaver (University of Massachusetts Medical School, Worcester, MA). Establishment and characterization of these transgenic mice has been described previously (Bae et al., 2001). Animals were maintained in the vivarium at Texas A&M University System Health Science Center under a standard 12h light: 12h dark cycle (LD 12:12; lights-on at 0600 hr) with access to food and water *ad libitum*. Procedures used in this study were approved by the University Laboratory Animal Care Committee at Texas A&M University.

Experiment 1: Effects of Targeted Disruption of *Per1, Per2* and *Per1/Per2* on TCDD-Induced Responses of the AhR Signaling Pathway in the Mouse Liver *in vivo*

Responses of the AhR signaling pathway were examined in 8-week-old female mice treated with TCDD (provided by Dr. Stephen Safe, Texas A&M University School of Veterinary Medicine, College Station, TX) at a dose of 10ug/kg body weight. Previous studies showed a single dose of 5ug/kg TCDD or higher for 24 hours significantly induces hepatic *Cyp1A1* expression in mice (Narasimhan et al., 1994). In the current study, animals received an intraperitoneal injection of vehicle (corn oil) or TCDD about 6 hours after lights-on in the LD12:12 cycles (1200 hr). Twenty-four hours after the treatment, animals were sacrificed by cervical dislocation at 1200 hr and liver tissues were collected in RNA Stabilization Reagent (RNA later, Qiagen, Valencia, CA) for later extraction of total RNA. For each tissue sample, approximately 30 mg of liver tissue was homogenized and processed for extraction of total cellular RNA using the RNeasy Mini Kit (Qiagen). The the final RNA pellet was subjected to on-column DNase digestion (Qiagen), suspended in 100µl RNase-free water, and then stored at –80°C.

Experiment 2: Effects of siRNA Inhibition of *Per1* or *Per2* Expression on TCDD-Induced Responses of the AhR Signaling Pathway in Hepa1c1c7 Cells

Hepa1c1c7 cells, derived from mouse hepatoma, (provided by Dr. Yanan Tian, Texas A&M University School of Veterinary Medicine, College Station, TX) were cultured on 6-well-plates in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine (Invitrogen) and 10% FBS at 37°C

in a humidified incubator with 5% CO_2. Hairpin siRNA-encoding oligonucleotide sequences targeting mouse *Per1* or *Per2* gene and a scrambled version of these sequences were designed at Ambion Inc. (Austin, TX). The sequences of the siRNA oligonucleotides are listed in Table 4. BLAST searches were performed on all oligonucleotides to avoid effects on nonspecific targets. Oligonucleotides encoding these sequences were synthesized at Integrated DNA Technologies (Coralville, IA), and cloned into the pSilencer 5.1-U6 Retro vector (Ambion). Retrovirus production was established by transfecting HEK-293T cells (obtained with permission from Dr. Gary Nolan, Stanford University, Stanford, CA) with pSilencer 5.1-U6 Retro vectors containing hairpin siRNA or scrambled control-encoding oligonucleotides using Lipofectamine 2000 (Invitrogen). Following transfection for 24 hours, HEK-293T cells were switched into regular growth medium (DMEM with 2 mM L-glutamine and 10% FBS) and incubated for 48 hours to produce the virus-conditioned medium, which was then filtered (0.45um cellulose acetate) to remove dissociated cells, frozen in liquid nitrogen and stored at –80°C. Hepa1c1c7 cells were subjected to 2 rounds of infection for 16 and 24 hours at 32℃ with retrovirus-conditioned medium supplemented with 4ug/ml polybrene. Stably-infected cells were selected by treatment with puromycin (4μg/ml) for 2-3 days, performed in parallel on mock-infected cultures of Hepa1c1c7 cells. Based on fluorescence microscopy, the infection efficiency of the retroviral constructs was variable (30-50%) in Hepa1c1c7 cells. Hepa1c1c7 cells infected with siCon or siRNA constructs were cultured on 6-well plates and at confluence were treated with vehicle (N=3; DMSO) or 10nM TCDD (N=3) for 24 hours. After treatment,

cultures were collected by trypsinization and total RNA was extracted using RNeasy mini kit [Qiagen].

TABLE 4
Oligonucleotide sequences of siRNA

Gene		siRNA sequences
Per1	siPer1a	Sense: 5'-GCAUAUCACAUCCGAAUACTT-3' Anti-sense: 5'-GUAUUCGGAUGUGAUAUGCTC-3'
	siPer1b	Sense: 5'-GCUCUUCAUUGAAUCUCGGTT-3' Anti-sense: 5'-CCGAGAUUCAAUGAAGAGCTG-3'
Per2	siPer1a	Sense: 5'-CGGGUGUCCUAAGACAUUCTT-3' Anti-sense: 5'-GAAUGUCUUAGGACACCCGTG-3'
	siPer1b	Sense: 5'-GGAAGAUAUCUUUCAUCAUTT-3' Anti-sense: 5'-AUGAUGAAAGAUAUCUUCCTG-3'

Western Blot Analysis

siRNA inhibition of PER1 and PER2 protein levels in Hepa1c1c7 cells was assessed by Western blot analysis using the Novex Western Transfer Apparatus (Invitrogen). Total cellular protein was extracted from individual cultures of stably-infected Hepa1c1c7 cells (N>3) using mammalian protein extraction reagent (M-PER; Pierce Biotechnology, Rockford, IL) supplemented with a protease inhibitor cocktail and PMSF (Sigma-Aldrich, St. Louis, MO). Sample protein content was measured by the bicinchoninic acid method (Pierce, Rockford, IL). The samples were boiled in sodium dodecyl sulfate sample buffer and loaded at 25 μg protein per lane onto 7.5% Tris-Tricine gels. Following separation at 25 mA for 1 hour and 45 minutes, proteins were transferred onto 0.45 μm nitrocellulose membranes (Invitrogen) and blocked at room temperature for 1 hour with 5% non-fat dried milk in Tris-buffered saline (TBS; 20 mM Tris, 137 mM NaCl)-Tween (0.1%). With interceding rinses in TBS-Tween, membranes were probed overnight at 4^{o}C with rabbit anti-PER1 (2ug/ml; Alpha Diagnostic International, San Antonio, Texas), chicken anti-PER2 (1ug/ml Chemicon) or monoclonal mouse anti β-actin (1:5000; Sigma-Aldrich) followed by a 1-hour incubation at 22°C with HRP-conjugated donkey anti-rabbit IgG for PER1, rabbit anti-chicken IgY for PER2 or goat anti-mouse IgG for β-actin (All at 1:10,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Immunoreactive signal for PER1, PER2 or β-actin was generated using enhanced chemiluminescence (ECL) reagent (Pierce Biotechnology) and detected on film (Biomax; Kodak, Rochester, NY). Optical density measurements for size-appropriate bands were obtained with ImageJ

software (National Institute of Health, USA) using β-actin as an internal control for differences in total protein content.

Quantitative RT-PCR Analysis

Quantification of relative mRNA abundance was performed using SYBR-Green real-time PCR technology (Applied Biosystems, Inc. [ABI], Foster City, CA) as described previously (Metz et al., 2006). Total RNA (1ug) was reverse transcribed using Superscript II (Invitrogen) and random hexamers. For each sample, the cDNA equivalent to 1.25ng total RNA per 12.5ul reaction was amplified in an ABI 7500 Fast Real-time PCR System using 9600 emulation modes. To control for differences in sample RNA content, cyclophilin A (CypA) or β-actin was amplified from the same samples. Primer sequences for PCR amplification of target and control genes are listed in Table 2.

The comparative C_T method was used to calculate the relative mRNA abundance for a given target gene. Using this method, the amount of target gene mRNA in each sample was normalized first to corresponding *CypA* or β-*actin* mRNA levels, and then relative to a calibrator consisting of pooled cDNA from multiple samples that was analyzed on each reaction plate.

Statistical Analysis

In Experiment 1, two-way analyses of variance (ANOVAs) were first applied to the data with treatment (vehicle vs. TCDD) and genotype (WT, $Per1^{ldc}$, $Per2^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$) as two independent variables. If significant main effects of treatment were identified, planned comparisons using independent *t*-tests were applied to compare gene expression between control and TCDD groups of the same genotype. Expression of

a given gene was evaluated separately within the control and TCDD groups using one-way ANOVA and, if required, Fisher's least significant difference (LSD) post hoc analyses to determine whether genotype had a significant effect on basal levels and TCDD-induced changes in mRNA abundance. In Experiment 2, the data were analyzed using two-way ANOVAs with treatment (vehicle vs. TCDD) and siRNA construct (siCon, siPer1a, and siPer1b, or siCon, siPer2a, and siPer2b) as two independent variables. If significant main effects of treatment were observed, planned comparisons using independent *t*-tests were applied to compare gene expression between vehicle- and TCDD-treated cells infected with the same siRNA construct. Expression of a given gene was again evaluated separately within each of these treatment groups using one-way ANOVA and, if required, Fisher's least significant difference (LSD) post hoc analyses to determine whether siRNA inhibition of *Per1* or *Per2* expression had a significant effect on basal levels and TCDD-induced changes in mRNA abundance. The α value was set at 0.05 for all statistical analyses.

RESULTS

Experiment 1: Effects of Targeted Disruption of *Per1, Per2* and *Per1/Per2* on TCDD-Induced Responses of the AhR Signaling Pathway in the Mouse Liver *in vivo*

WT, $Per1^{ldc}$, $Per2^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ mutant mice (Bae et al., 2001) were examined for differences in hepatic expression and TCDD-mediated regulation of key components of the AhR signaling pathway, *AhR*, *Arnt*, *Cyp1A1* and *Cyp1B1*. Similar to previous analysis of hepatic responses to TCDD (Narasimhan et al., 1994), *Cyp1A1*

expression in the liver was low in all vehicle-treated mice but was consistently increased after TCDD treatment. In both WT and mutant mice, *Cyp1A1* mRNA levels in the liver were significantly ($p<0.05$) in the TCDD-treated group than in vehicle controls (Fig. 16A). However, genotype-based differences in hepatic *Cyp1A1* expression were evident within both the vehicle and TCDD treatment groups. Among vehicle-treated animals, the basal levels of *Cyp1A1* mRNA in the liver were significantly ($p<0.05$) higher in $Per1^{ldc}$ mutant mice than in WT animals. Following TCDD treatment, hepatic *Cyp1A1* expression in $Per1^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ mutant mice was significantly ($p<0.05$) and about 2 times higher than that found in WT animals.

TCDD treatment similarly induced hepatic expression of another *p450* gene in the AhR signaling pathway, *Cyp1B1*. In all WT and mutant mice, TCDD had significant effect in increasing *Cyp1B1* expression in the liver ($p<0.05$) relative to the low levels observed in vehicle controls (Fig. 16B). No genotype-based differences were observed in either the basal expression or TCDD-mediated induction of *Cyp1B1* mRNA in the liver.

In addition to *p450* genes, two primary regulators of hepatic responses to TCDD, *AhR* and *Arnt* were analyzed for evidence of treatment- and genotype-based differences. For both *AhR* and *Arnt*, mRNA levels in the liver were comparable among all vehicle-treated mice (Fig. 16C-D). Moreover, there was no significant effect of either TCDD or genotype on *AhR* and *Arnt* mRNA expression in the liver.

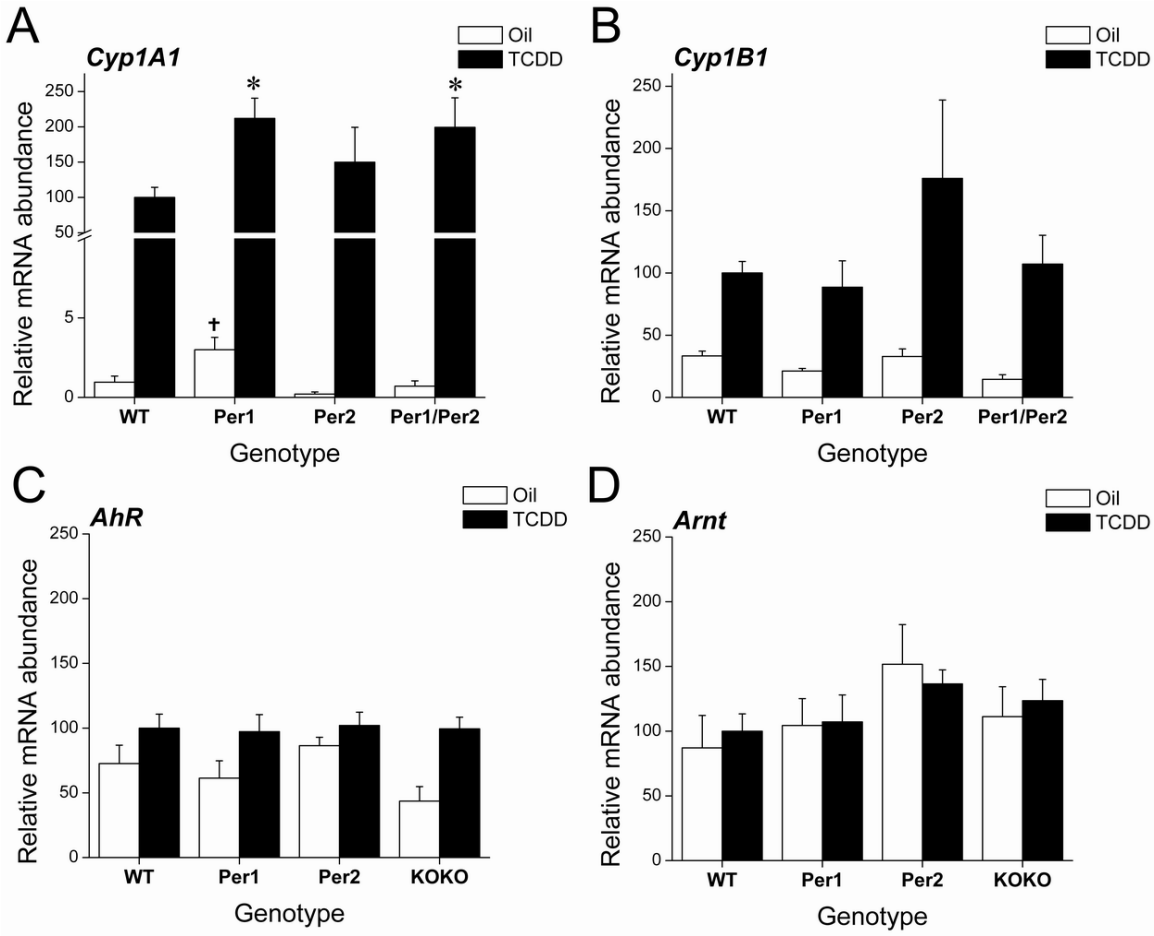


Fig. 16. Effects of targeted mutations of *Per1* (*Per1*^{ldc}), *Per2* (*Per2*^{ldc}), and *Per1*/ *Per2* (*Per1*^{ldc}/*Per2*^{ldc}) on the expression and TCDD-induced responses of *p450* genes in the mouse liver. For *Cyp1A1* (A), *Cyp1B1* (B), *AhR* (C) and *Arnt* (D), the relative mRNA abundance was analyzed in the liver of WT, *Per1*^{ldc}, *Per2*^{ldc}, and *Per1*^{ldc}/*Per2*^{ldc} mice following treatment with vehicle (oil) or TCDD. Data are expressed as the mean (±SEM) for each experimental group. The plotted values for the relative mRNA abundance correspond to the ratios of species-specific *Cyp1A1, Cyp1B1*, *AhR* or *Arnt*/*CypA* mRNA signal that were adjusted in relation to the average for TCDD-treated WT mice, which was arbitrarily set at 100. Regardless of genotype, TCDD exposure produced significant increases in *Cyp1A1* and *Cyp1B1* expression within the liver. Asterisks denote genotype comparisons of TCDD-treated mice in which *Cyp1A1* expression in the liver was significantly greater (p<0.05) than that observed in WT animals; The dagger denotes significant difference from the vehicle treated WT animals.

Experiment 2: Effects of siRNA inhibition of *Per1* or *Per2* Expression on TCDD-Induced Responses of the AhR Signaling Pathway in Hepa1c1c7 cells

To further determine whether altered *Per1* and *Per2* expression affects liver responses to TCDD, *in vitro* experiments were conducted in parallel to examine the influence of siRNA-mediated inhibition of these genes on TCDD-mediated regulation of AhR signaling in the mouse hepatoma cell line, Hepa1c1c7. Effects of all siRNA constructs on *Per1* or *Per2* in Hepa1c1c7 cells were first confirmed by real-time RT-PCR (Fig. 17A and 17C). In Hepa1c1c7 cells infected with siRNA constructs, *Per1* and *Per2* mRNA levels were respectively decreased to 15-36 % (Fig. 17A) and 37-46% (Fig. 17C) of the values found in scrambled control (siCon)-infected cells. These siRNA-mediated decreases in the expression of targeted mRNAs were associated with a corresponding inhibition of PER1 (Fig. 17B) and PER2 (Fig. 17D) protein levels. Western blot analysis revealed that PER1 and PER2 protein levels in *Per1* and *Per2* siRNA-treated Hepa1c1c7 cells were respectively reduced to 59-68% and 12-26% of those found in siCon-infected cultures. Importantly, β-actin protein levels in siRNA-treated Hepa1c1c7 cultures were comparable to those observed in cells infected with siCon constructs.

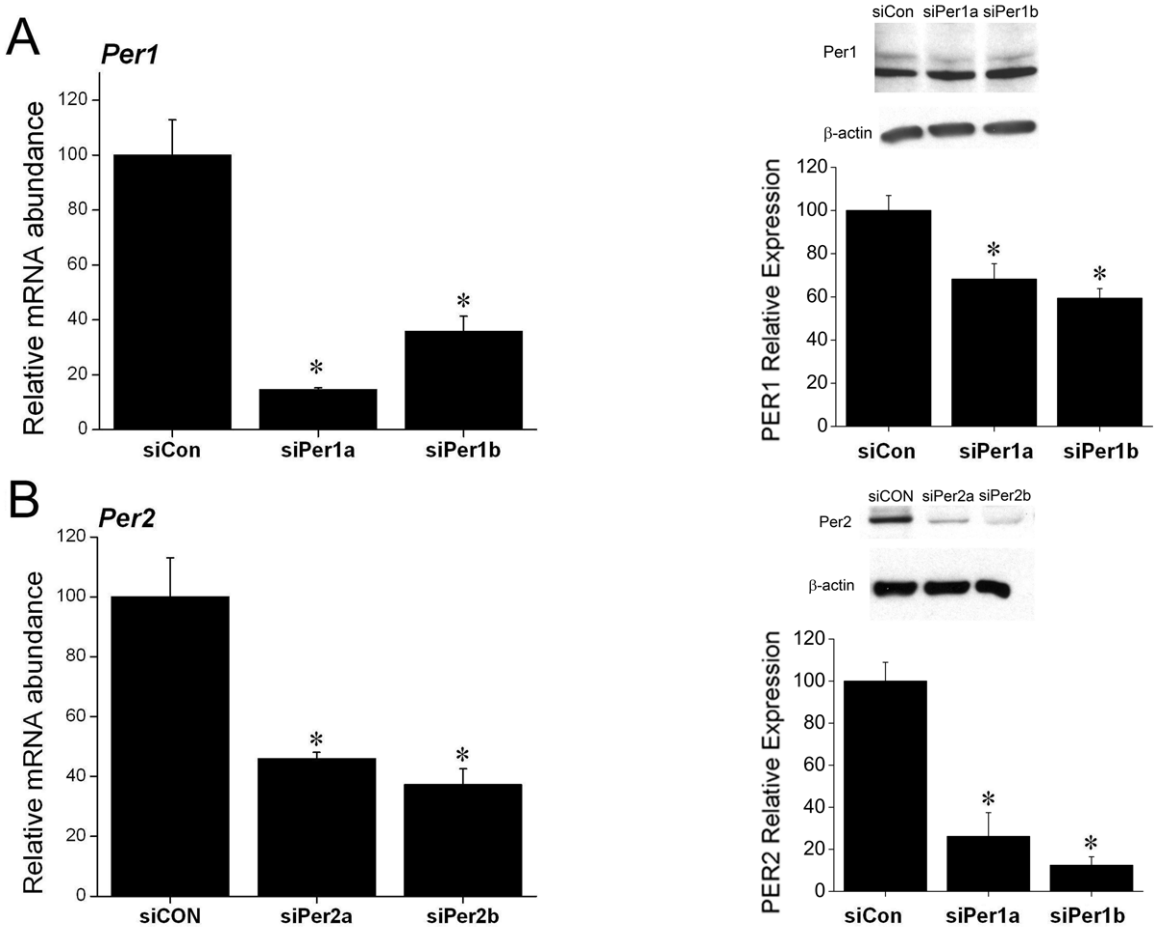


Fig. 17. Effects of siRNA inhibition of *Per1* (A) and *Per2* (B) on the expression and TCDD-induced responses of *p450* genes in Hepa1c1c7 cells. Panels on the left presnt Real-Time RT-PCR data of *Per1* and *Per2* mRNA abundance in siRNA-treated Hepa1c1c7 cells. Relative mRNA abundance of *Per1* (A) or *Per2* (B) was measured using Real-Time RT-PCR analysis in cells infected with siCon and siRNA constructs. Data are expressed as the mean (±SEM) for each treatment group. The plotted values for the relative mRNA abundance correspond to the ratios of target gene/ *β-actin* mRNA signal that were adjusted in relation to the average for the siCon-infected cells, which was arbitrarily set at 100. Righe panels present Western blot data of PER1 (A) or PER2 (B) protein levels in siRNA-treated Hepa1c1c7 cells. Representative western blot results are depicted. Densitometric analysis of gels was utilized to quantify relative PER protein levels. Bars represent the mean (± SEM) determinations. The plotted values for the relative protein expression present the ratios of target gene/ β-actin protein signal that were adjusted in relation to the average for the siCon infected cells, which was arbitrarily set at 100. Asterisks denote values for mRNA abundance or protein levels in siRNA-infected cells that were significantly decreased (p<0.05) in comparison with those observed in siCon-infected cells.

The expression and TCCD-mediated regulation of *Cyp1A1*, *Cyp1B1*, *AhR* and *Arnt* were assessed in siCon- and siRNA-infected Hepa1c1c7 cells to determine whether this inhibition of *Per1* or *Per2* expression alters toxin responses of the AhR signaling pathway. Following vehicle treatment, basal levels of *Cyp1A1* expression were observed in all Hepa1c1c7 cultures infected with scrambled control or siRNA constructs targeting *Per1* or *Per2* (Fig. 18A). In comparison with these vehicle control cultures, all TCDD-treated Hepa1c1c7 cells were marked by significant increases ($p<0.05$) in *Cyp1A1* mRNA expression (Fig. 18A). Differences in the TCDD induced *Cyp1A1* levels were evident in association with the siRNA-mediated inhibition of *Per1* or *Per2* expression. Consistent with our analysis of mouse liver *in vivo*, TCDD-induced *Cyp1A1* expression was significantly higher ($p<0.05$) in both groups of *Per1* siRNA-infected Hepa1c1c7 cells than in cultures infected with siCon constructs. In contrast to the effect of *Per1* inhibition, the TCDD induced *Cyp1A1* mRNA levels were significantly lower ($p<0.05$) in *Per2* siRNA-infected Hepa1c1c7 cells than in control cultures. The TCDD-mediated induction of *Cyp1A1* expression in the *Per1* and *Per2* siRNA infection groups were respectively increased by about 30% and decreased by 30-40% relative to siCon-infected controls.

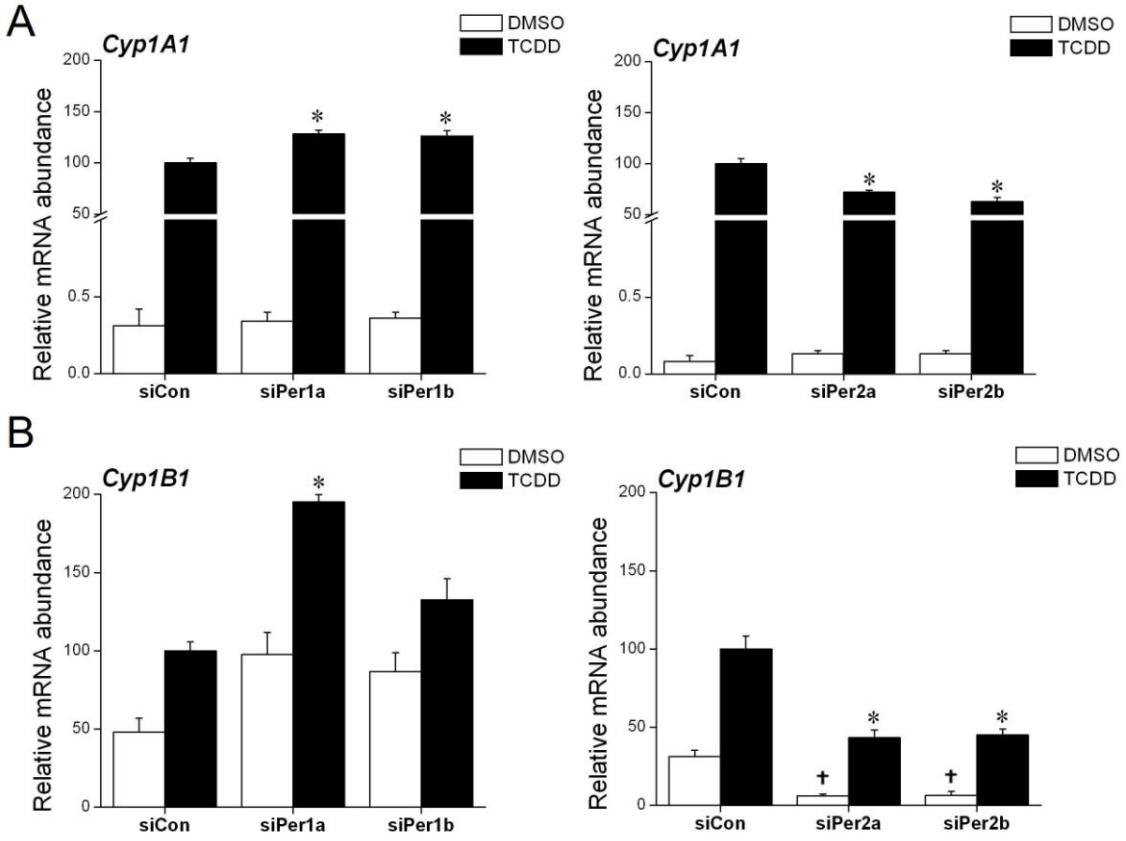


Fig. 18. Effects of siRNA inhibition of *Per1* or *Per2* on the expression and TCDD-induced responses of *p450* genes in Hepa1c1c7 cells. For *Cyp1A1* (A) and *Cyp1B1* (B), the relative mRNA abundance was analyzed in cells infected with siCon and siRNA constructs targeting *Per1* or *Per2*. Data are expressed as the mean (±SEM) for each experimental group. The plotted values for the relative mRNA abundance correspond to the ratios of *Cyp1A1* or *Cyp1B1*/*β-actin* mRNA signals that were adjusted in relation to the average for TCDD-treated siCon cells, which was arbitrarily set at 100. Regardless of siRNA treatment, TCDD significantly increases *Cyp1A1* and *Cyp1B1* expression in all cells ($p<0.05$). Daggers and asterisks respectively denote comparisons of DMSO-treated controls and TCDD-exposed cultures in which mRNA abundance in siRNA-infected cells was significantly different ($p<0.05$) from that found in siCon-infected cells.

Cyp1B1 expression was relatively low but variable among vehicle control cultures (Fig. 18B). Following vehicle treatment, *Cyp1B1* mRNA levels in *Per1* siRNA-infected Hepa1c1c7 cells were higher but not significantly different than those found in scrambled control cultures. Further comparison of vehicle-treated groups revealed that *Cyp1B1* expression in *Per2* siRNA-infected cells was significantly lower ($p<0.05$) than the basal levels observed in siCon-infected cultures. TCDD-mediated induction of *Cyp1B1* expression in Hepa1c1c7 cells followed the same general pattern as *Cyp1A1*. Relative to vehicle treatment, TCDD had a significant effect in inducing *Cyp1B1* expression in both siCon-and siRNA-infected Hepa1c1c7 cells ($p<0.05$). However, this TCDD-induced *Cyp1B1* expression in siRNA-infected Hepa1c1c7 cells was differentially modulated by the inhibition of *Per1* or *Per2* expression. Among TCDD-treated cultures, *Cyp1B1* mRNA levels were significantly higher ($p<0.05$) in Hepa1c1c7 cells infected by one of the siRNA constructs targeting *Per1* (siPer1a) but were significantly lower ($p<0.05$) in cells infected with *Per2* siRNA constructs than those observed in siCon-infected controls. The *Cyp1B1* expression following TCDD treatment in the siPer1a and the two *Per2* siRNA groups were respectively increased by about 100% and decreased by about 60% relative to controls.

The expression and TCDD-induced responses of *AhR* and *Arnt* in Hepa1c1c7 cells were also differentially affected by *Per* gene siRNA infection. Among vehicle-treated cultures, *AhR* (Fig. 19A) and *Arnt* (Fig. 19B) expression were comparable in all siCon- and *Per1* siRNA-infected cells but were altered by siRNA inhibition of *Per2* expression. Following vehicle treatment, *AhR* mRNA levels in both groups of *Per2* siRNA-infected Hepa1c1c7 cells were significantly lower ($p<0.05$) than those found in siCon cultures. Significant decreases in *Arnt* expression ($p<0.05$) were also observed among vehicle-treated cultures of cells infected with the siPer2a construct. *AhR* and *Arnt* expression in TCDD-treated Hepa1c1c7 cells were similarly influenced by siRNA inhibition of *Per1* or *Per2*. In TCDD-treated cultures, *AhR* and *Arnt* mRNA levels were not affected by siRNA inhibition of *Per1* but were significantly decreased ($p<0.05$) in *Per2* siRNA-infected Hepa1c1c7 cells. Following TCDD treatment, *AhR* and *Arnt* expression in *Per2* siRNA-infected cultures were respectively decreased to 42-47% and to 36-67% of the levels found siCon-infected cells.

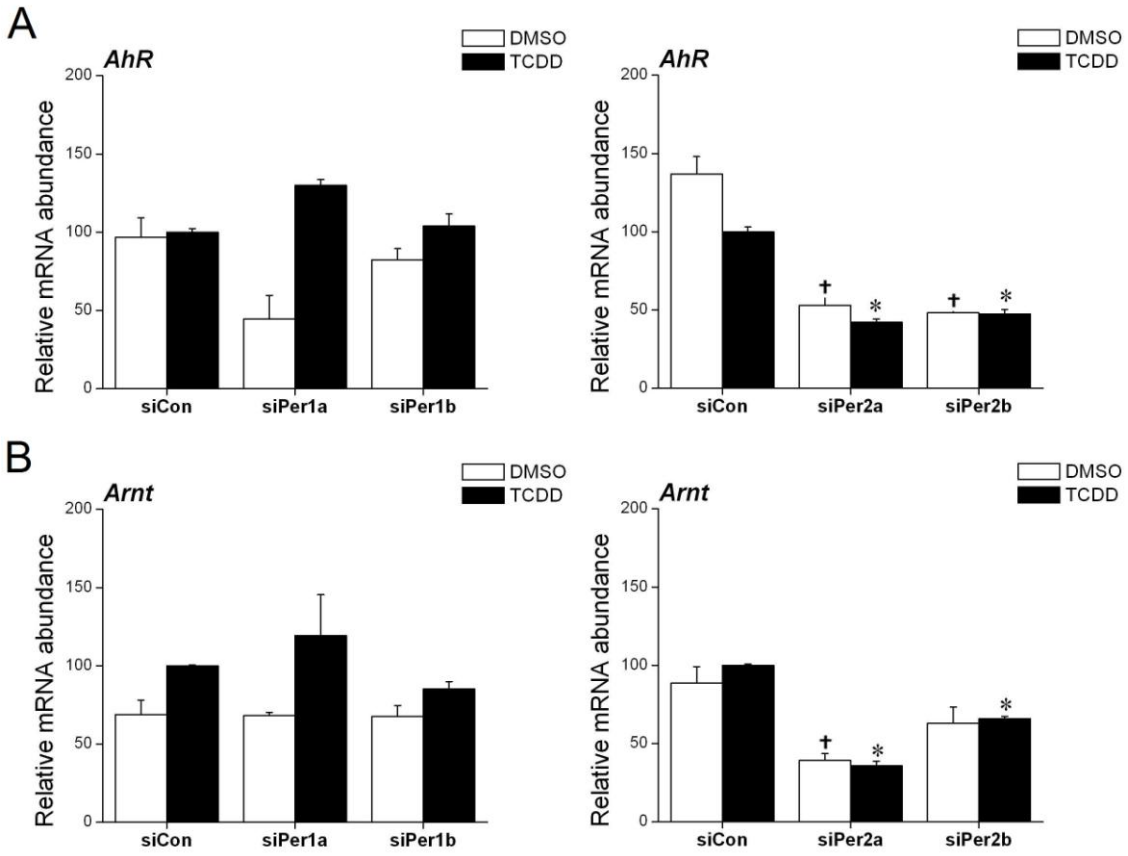


Fig. 19. Effects of siRNA inhibition of *Per1* or *Per2* on core components of the AhR signaling pathway, *AhR* and *Arnt*, in Hepa1c1c7 cells. For *AhR* (A) and *Arnt* (B), the relative mRNA abundance was analyzed in cells infected with siCon and siRNA constructs targeting *Per1* or *Per2*. Data are expressed as the mean (±SEM) for each experimental group. The plotted values for the relative mRNA abundance correspond to the ratios of *AhR* or *Arnt*/*β-actin* mRNA signals that were adjusted in relation to the average for TCDD-treated siCon cells, which was arbitrarily set at 100. Daggers and asterisks respectively denote comparisons of DMSO-treated controls and TCDD-exposed cultures in which *AhR* or *Arnt* mRNA abundance in siRNA-infected cells was significantly different ($p<0.05$) from that found in siCon-infected cells.

DISCUSSION

The impact of the circadian clock on drug and toxin metabolism has been reiterated in a number of studies demonstrating that the toxicity and efficacy of drugs varies depending on the time of administration (Levi and Schibler, 2007). This diurnal variation in the responses to drug treatment is consistent with evidence indicating that the functional activity of many drug metabolizing enzymes are rhythmically regulated by the circadian clock. For example, the activity of the hepatic P450 monooxygenase has been shown to vary between day and night, and this diurnal fluctuation is abolished in rats with complete lesions of the SCN (Furukawa et al., 1999). In addition, three PAR-domain basic leucine zipper (PAR bZip) proteins DBP, TEF, and HLF, which are output mediators of the circadian clock, have been shown to control the expression of drug metabolizing enzymes, such as cytochrome P450 enzymes, carboxylesterases and constitutive androstane receptor (CAR) (Gachon et al., 2006). Despite the wealth of descriptive observations on the rhythmic variation in drug/toxin responses and the activity of metabolizing enzymes, the present study provides the first indication that specific molecular components of the underlying clockworks are involved in regulating toxin responses pathways in the liver. Our results demonstrate that the targeted disruption or inhibition of *Per* gene expression alters AhR-mediated responses to TCDD in the mouse liver. The inductive effects of TCDD on expression of the cytochrome *p450* genes, *Cyp1A1* and *Cyp1B1*, were increased in $Per1^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$, but not in $Per2^{ldc}$ mice. *In vitro* experiments yielded similar implications for *Per1* involvement in the TCDD-induced activation of the AhR signaling pathway. In Hepa1c1c7 cells,

siRNA inhibition of *Per1* increased the inductive effect of TCDD on expression of these p450 genes. It is interesting that siRNA inhibition of *Per2* had the opposite effect, producing decreases in the TCDD-mediated induction of *Cyp1A1* and *Cyp1B1* expression. This difference in the effects of *Per1* and *Per2* inhibition on the AhR signaling pathway provides further evidence for functional distinctions between these two clock genes. In the clockworks, the *Per1* and *Per2* genes are indispensable for normal timekeeping function but influence different processes in the molecular clockworks (Zheng et al., 1999; Shearman et al., 2000; Bae et al., 2001). In this regard, the targeted disruption of *Per2*, but not *Per1*, has been shown to abolish SCN rhythms of clock gene expression (Bae et al., 2001). *Per1* and *Per2* also appear to have different functions in the regulation of non-clock processes because sensitization to cocaine is abolished in $Per1^{brd}$ mice but is enhanced in mutant mice with targeted disruption of *Per2* (Abarca et al., 2002). Nevertheless, our findings collectively support the role of *Per1* in modulating TCDD activation of AhR signaling pathways in the liver.

Despite increasing evidence for the functional link between the *Per* genes and the AhR signaling pathway, it is unclear how *Per1, Per2*, or other clock genes modulate responses of this pathway to TCDD. Similar to the analysis of the mammary gland in the previous chapter, it seems unlikely that the altered TCDD responses of the liver in $Per1^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ mice are caused by *Per*-mediated hormonal disturbances, because siRNA inhibition of *Per1* produced comparable changes in TCDD-mediated induction of *Cyp1A1* and *Cyp1B1* in Hepa1c1c7 cultures despite the absence of glucocorticoids or other steroid hormones that have been shown to modulate activation

of the AhR signaling pathway *in vivo* (Gorski et al., 1988; Christou et al., 1995; Prough et al., 1996).

As discussed previously in relation to the mammary gland studies, it is possible that the *Per* genes may modulate hepatic responses to TCDD by directly interacting with specific components of the AhR signaling pathway. In this regard, *Per* genes may modulate TCDD-mediated regulation of the AhR signaling pathway in the liver by influencing receptor levels, the formation of AhR:ARNT heterodimers or the binding of these complexes with the XRE. Current evidence for decreased *AhR* mRNA expression in Hepa1c1c7 cultures following *Per2* siRNA inhibition raises the possibility that *Per2* may regulate the AhR signaling pathway by modulating receptor levels. Although it is unknown whether the *Per* proteins directly affect the formation and DNA binding activity of AhR:ARNT heterodimers in the mammalian liver, *Drosophila* PER has been shown to form dimers with mammalian AhR and ARNT via the PAS domain *in vitro* and impedes the formation and DNA binding activity of AhR/ARNT complexes (Lindebro et al., 1995). Future studies will be necessary to identify the mechanism by which the *Per* genes modulate the TCDD-mediated regulation of the AhR signaling in the liver.

The current study showed that the core clock gene, namely *Per1*, is negatively involved in the AhR mediated toxin response pathway. Our data showed that the disruption of *Per1* leads to hyper induction of *p450* expression in liver following the exposure of TCDD, a prototype of PAHs. Animals with targeted disruption of clock genes and clock controlled genes demonstrate increased susceptibility to environmental

stressors, including genotoxic stressors and toxic pollutants (Gachon et al., 2006; Lee, 2006). Because the hyper induction *p450* has been shown to further promote harmful effects of environmental toxins on the organism (Chen et al., 2002; Knerr and Schrenk, 2006), the present study presents the possible link between hypersensitivity of clock gene mutant animal to environmental stressors and certain core component of the circadian feedback loops, namely *Per1*.

CHAPTER V

INHIBITION OF PERIOD GENE EXPRESSION ALTERS THE AHR MEDIATED *CYP1A1* TRANSCRIPTION

INTRODUCTION

Observations in Chapters III and IV indicate that the targeted disruption of the clock gene *Per1*, but not *Per2*, potentiates the TCDD-induced *p450* gene expression in mouse mammary gland and liver *in vivo*. This finding is complemented by *in vitro* data, demonstrating that siRNA-mediated inhibition of *Per1* similarly increases TCDD-induced *p450* expression in Hepa1c1c7 cells. Although these observations suggest that *Per1* may function as a negative or inhibitory factor in regulating the TCDD-mediated activation of the AhR signaling pathway, the mechanism by which *Per1* exerts this influence is unknown.

Based on previous studies, TCDD activation of the AhR signaling pathway consists of multiple key steps that ultimately lead to the induction of target genes. Briefly, the ligand-activated AhR is translocated from the cytoplasm into the nucleus, where it dimerises with ARNT. AhR:ARNT dimers and co-activators then bind to the XRE regulatory region of target genes, most of which encode drug metabolizing enzymes such as the cytochrome P450s. Because changes in any of these and other components of the AhR signaling pathway may alter *p450* gene induction, the modulating activity of the *Per* genes on TCDD-induced activation of this pathway could occur at a number of different levels. The *Per* genes may influence AhR signaling in

response to TCDD by changing: 1) endogenous levels of AhR and ARNT, 2) the stability of the AhR/chaperones complex in the cytoplasm, 3) the rate of AhR shuttling from the cytoplasm to the nucleus, 4) dimer formation between AhR and ARNT, 5) the binding properties of AhR, ARNT and other trans-acting elements on regulatory regions of target genes, 6) the binding property of RNA Polymerase II (Pol II) and the target gene Transcription Start Site (TSS), which controls the level of transcription, 7) post-transcriptional regulation of *p450* genes, 8) the feed-back inhibition by the AhR Repressor (AhRR), or 9) the rate of ligand-induced degradation of AhR (Fujii-Kuriyama and Mimura 2005; Pollenz and Dougherty, 2005). Moreover, the levels for TCDD-mediated regulation of the AhR signaling pathway are often interrelated. For example, decreases in the formation of AhR:ARNT dimers cause a reduction in the recruitment efficiency of AhR:ARNT complexes onto the XRE, which in turn hinders the binding of other trans-activating elements to the *Cyp1A1* regulatory regions and leads to lower transcription activity of the Pol II at the gene TSS. Therefore, *Per1* and *Per2* may influence the AhR signaling pathway at multiple levels.

Molecular studies of the feedback loops comprising the mammalian clockworks indicate that PER1 and PER2 function as transcription factors of other core components. Previous studies revealed that PER1 inhibits CLOCK:BMAL1 activation of clock gene transcription via E-box sites (Sangoram et al., 1998). PER2, on the other hand, is thought to function as a positive regulator of *Bmal1* expression. PER2 may regulate *Bmal1* directly as a transcription coactivator or indirectly by repressing the expression of the orphan nuclear receptor *Rev-erbα*, which is a negative regulator of *Bmal1* transcription

(Preitner et al., 2002; Shearman et al., 2000). In conjunction with the results from our previous studies, these observations suggest that the *Per* genes may modulate TCDD-mediated responses of the AhR signaling pathway by regulating the AhR activated transcription of target genes such that *Per* genes may influence the AhR binding with the XRE of target genes and therefore affect the gene transcription by Pol II. This hypothesis is corroborated by the observation that over-expression of *Drosophila* PER blocks AhR binding with the XRE and inhibits the ligand induced transcription of target genes (Lindebro et al., 1995). To test this hypothesis, we investigated the effects of siRNA inhibition of *Per1* or *Per2* on AhR-mediated *Cyp1A1* transcription in Hepa1c1c7 cells. We applied chromatin immunoprecipitation (CHIP) assay to measure the binding levels of AhR with the XRE as well as Pol II with the *Cyp1A1* TSS. Based on the results of our *in vitro* studies, siRNA inhibition of *Per1* and *Per2* is expected to respectively increase and decrease the AhR mediated *p450* transcription in response to TCDD. Therefore, we predict that the inhibition of *Per1* is likely to be associated with higher levels of TCDD-induced AhR/XRE and Pol II/TSS binding, while the inhibition of *Per2* is expected to decrease the AhR/XRE and Pol II/TSS association in response to TCDD treatment.

MATERIALS AND METHODS

CHIP Assay

Hepa1c1c7 cells were infected with viral media containing siRNA (siCON, siPer1a, siPer1b, siPer2a or siPer2b) as described in Chapter IV. Cells were passed onto T75 flasks and stably-infected cells were selected in the presence of puromycin for 48 hours. Cells were exposed to 10nM TCDD or vehicle (DMSO) for 5 hours. After treatment, cells were incubated in regular growth media containing 1% formaldehyde for 10 minutes at room temperature so as to provide for the cross-linking of proteins. This reaction was stopped by adding glycine (final concentration = 0.125 M). Cells were then rinsed twice with ice-cold PBS supplemented with proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μg/mL aprotinin and 1 μg/mL pepstatin A [Sigma-Aldrich, St. Louis, MO]), removed with cell scrappers and pelleted via centrifugation. Cell pellets were resuspended in SDS lysis buffer (Upstate) containing proteinase inhibitors and sonicated on ice so as to yield DNA fragments of 200-1000 base pairs. Cell debris was removed via centrifugation at 13,000 rpm for 10 minutes at 4°C. An equal volume (200ul) of supernatant from each sample was set aside and incubated with 0.2M NaCl at 65°C over night. DNA was extracted using QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and used as input for later steps. The rest of the supernatant was processed using the CHIP Assay Kit (Upstate, Temecula, CA). The manufacturer's protocols were used with minor modifications. For each CHIP reaction, 200ul of cell lysate was diluted in 1800ul CHIP Dilution Buffer containing proteinase inhibitors. Samples were pre-cleared by incubation with rabbit IgG (Sigma-Aldrich)

bound Protein A Agarose/Salmon Sperm DNA beads at 4 °C for 1 hour and then centrifuged at 13,000rpm for 5 minutes. The supernatant was collected and incubated with either rabbit IgG or rabbit polyclonal antibodies against AhR [Biomol, Plymouth Meeting, PA] or RNA polymerase II [Santa Cruz Biotechnology, Santa Cruz, CA]) at 4 °C over night. The antibody/histone complex was isolated by incubation with 60ul Protein A Agarose/Salmon Sperm DNA beads at 4 °C for 1 hour. Beads were collected by centrifugation at 700rpm for 1 minute at 4 °C and the pellets were washed once with Low Salt Immune Complex Wash Buffer, once with High Salt Immune Complex Wash Buffer, once with LiCl Immune Complex Wash Buffer and twice with TE Buffer for 5 minutes at 4 °C. To elute the histone complex, the beads were incubated twice with 250ul elution buffer (1%SDS, 0.1M NaHCO3) at room temperature for 15 minutes with vigorous shaking and centrifuged at 13,000 rpm for 15 seconds. The eluded supernatant was incubated with 0.2M NaCl at 65°C over night to reverse the cross-linking. Samples were then subjected to RNase A (Invitrogen, Carlsbad, California) and Proteinase K digestion (Promega, Madison, WI). DNA was extracted using QIAquick PCR Purification Kit and concentrated by ethanol-precipitation. DNA pellets were resuspended in TE buffer (Qiagen) and analyzed in parallel with input DNA using regular Polymerase Chain Reaction (PCR) and quantitative Real-Time PCR amplification.

Regular PCR was carried out for 30 cycles using the following primers: 5'-GCCGAGCATCGCACGCAAACC-3' (-1219) and 5'-GGATCCACGCGAGACA GCAGG-3' (-814) for AhR antibody immunoprecipitated samples; 5'-

TATCCGGTATGGCTTCTTGC-3' (-155) and 5'-CTCACCTTGGGCTGTAAGGA-3' (+92) for RNA Pol II antibody immunoprecipitated samples. Serially diluted input DNA (1/250, 1/1000 and 1/4000) were used to determine the linear range of PCR reaction for each primer pair. PCR products were then analyzed by 2% agarose gel electrophoresis with ethidium bromide staining (1ug/ml). Optical density (OD) measurements were obtained with ImageJ software (National Institute of Health). OD values from serially diluted input DNA were used to a construct standard curve for a given group, from which the relative amounts of the immunoprecipitated DNA were calculated.

DNA samples were also analyzed by real-time PCR using the following primers: 5'-AGAGGATGGAGCAGGCTTAC-3' (-918) and 5'-GCAGGCAACACAGAGA AGTC-3' (-743) for AhR antibody immunoprecipitated samples; 5'-GAGCACTCCCTA AGGCTGTC-3' (-110) and 5'-GAAGGCACCACCACCTTTAT-3' (-9) for RNA Pol II antibody immunoprecipitated samples. Real-time PCR analysis was conducted using modifications of protocols that have been established previously (Aparicio et al., 2004).

Briefly, 6ul of template DNA (either 1/5 dilution of the immunoprecipitated sample [IP] or 1/1000 dilution of the input) per 15ul reaction was amplified in an ABI 7500 Fast Real-time PCR System using 9600 emulation modes. For each amplification product, the ^{NET}Ct was determined using the formula $^{NET}Ct = Ct(Input) - Ct(IP)$ (Aparicio et al., 2004). Relative occupancy (F) of the protein (P) of interest was calculated as $F(P) = 1.9E[^{NET}Ct(P)]$. The IgG background was calculated as $F(IgG) = 1.9E[^{NET}Ct(IgG)]$. Finally, the net occupancy of the protein (P) of interest equals to [F(P) –F(IgG)] (Aparicio et al., 2004). The base value of 1.9 is a consistent (±0.05) approximation of the amplification efficiency.

RESULTS

In the present experiment, we evaluated the effects of siRNA inhibition of *Per1* and *Per2* on the AhR mediated *Cyp1A1* transcription activity. Such evaluation was done by using the CHIP assay to measure binding levels of AhR with the XRE as well as Pol II with the *Cyp1A1* TSS. Data from the present experiment were derived from single samples in each treatment group and thus only reflect trends with regard to how *Per1* and *Per2* inhibition affects *p450* gene transcription.

We first evaluated the AhR activation of *Cyp1A1* transcription by measuring the relative occupancy of the AhR protein on the XRE in the regulatory region of the *Cyp1A1* gene (Fig. 20). Consistent with its effects in previous studies, TCDD increased AhR binding to the XRE in siCON-infected Hepa1c1c7 cells (Fig. 20A) (Tian et al., 2003). Such increased AhR/XRE binding is also consistent with results from Chapter IV, which showed that TCDD treatment increases the *Cyp1A1* mRNA abundance. A similar increase in the AhR occupancy on the XRE also occurred in siPer1b- and siPer2b-infected cells, but not in cultures treated with the siPer1a and siPer2a constructs. The level of AhR/XRE association in vehicle-treated cells is comparable among all cells, except that the basal level in siPer1b-infected cells is lower than that of the siCON infected cells (Fig. 20A). TCDD-induced AhR/XRE binding is altered by the siRNA-mediated inhibition of the *Per* genes. In TCDD-treated Hepa1c1c7 cells, the siRNA inhibition of *Per1* or *Per2* reduced the AhR activation of the *Cyp1A1* transcription, as indicated by the decreased level of TCDD-induced AhR/XRE association, compared to that observed in siCON-infected cells (Fig. 20A). These results are consistent with those obtained by real-time PCR analysis (Fig. 20B), except that in the vehicle-treated controls the basal level of AhR/XRE binding was lower in the siPer2b- than siCON-infected cells.

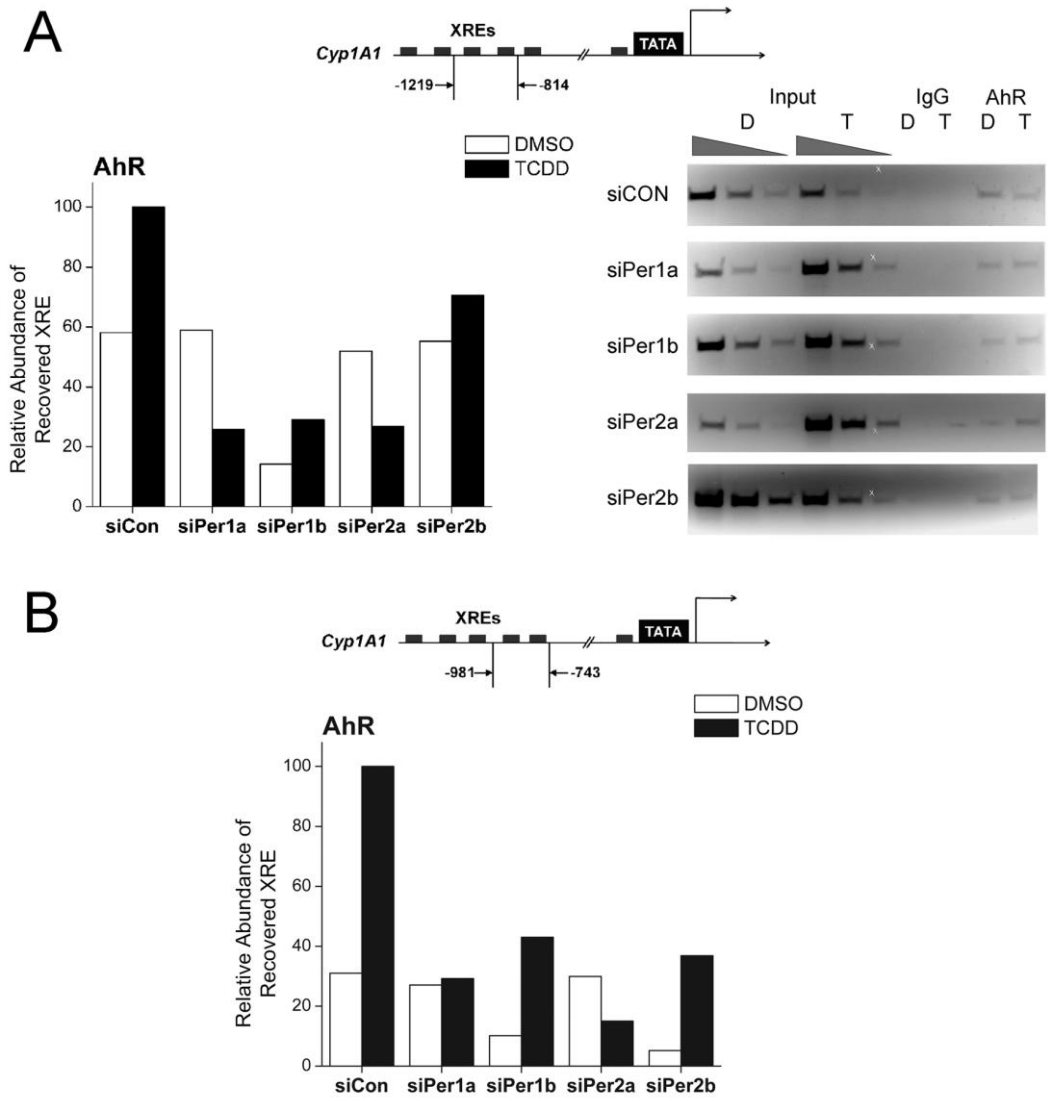


Fig. 20. Effects of siRNA inhibition of *Per1* or *Per2* on the association of AhR with the XRE of *Cyp1A1* gene. CHIP assay was performed to evaluate the XRE occupancy by AhR in Hepa1c1c7 cells following vehicle and TCDD treatment. The relative amount of *Cyp1A1* DNA recovered from the CHIP assay represents the relative binding level of AhR on the XRE and was analyzed by regular PCR (A) and Real-Time PCR (B). The location of the multiple XREs and PCR amplified regions are schematically illustrated on top of each panel. The gel picture (A) shows regular PCR products from serially diluted input DNA as well as DNA from IgG IP and AhR IP. Types of siRNA constructs used to infect cells are indicated on the side of the gel picture. Optical densities (OD) were calculated from the regular PCR gel image (A) using methods explained in Materials and Methods. Real-Time PCR assay (B) presents the level of DNA recovered from AhR IP relative to 1/1000 diluted input DNA. Plotted values for both regular (A) and Real-Time PCR (B) were adjusted in relation to TCCD-treated siCon infected cells, which were arbitrarily set at 100.

The relative level of Pol II recruitment onto the *Cyp1A1* TSS was also examined in the current experiment (Fig. 21). As expected, TCDD treatment increased the transcription activity of Pol II at the *Cyp1A1* TSS in siCON-infected cells, which is indicated by the increased level of Pol II binding with the *Cyp1A1* TSS (Fig. 21A) (Fretland et al, 2004; Tian et al., 2003). A comparable increase in Pol II recruitment onto the gene TSS was observed in Hepa1c1c7 cells infected with siRNA constructs targeting *Per1* or *Per2* (Fig. 21A). Among vehicle-treated cells, siRNA inhibition of the *Per* genes increases the basal level of the *Cyp1A1* transcription, as demonstrated by the elevated levels Pol II/TSS binding in siPer1b- and siPer2a-infected cultures, but not in siPer1a- and siPer2b-infected cells. Comparison of TCDD-treated cells indicates that siRNA inhibition of *Per1* or *Per2* both produce a reduction in the TCDD-induced levels of *Cyp1A1* transcription such that the relative occupancy of Pol II on the gene TSS is lower in the *Per* gene inhibited cells than in the siCON infected cells (Fig. 21A). Similar results were obtained with real-time PCR analysis, except that TCDD did not increase Pol II/TSS binding in siPer2a-infected cells and the basal level of Pol II/TSS binding is lower in siPer2b- than siCON-infected cells (Fig. 21B).

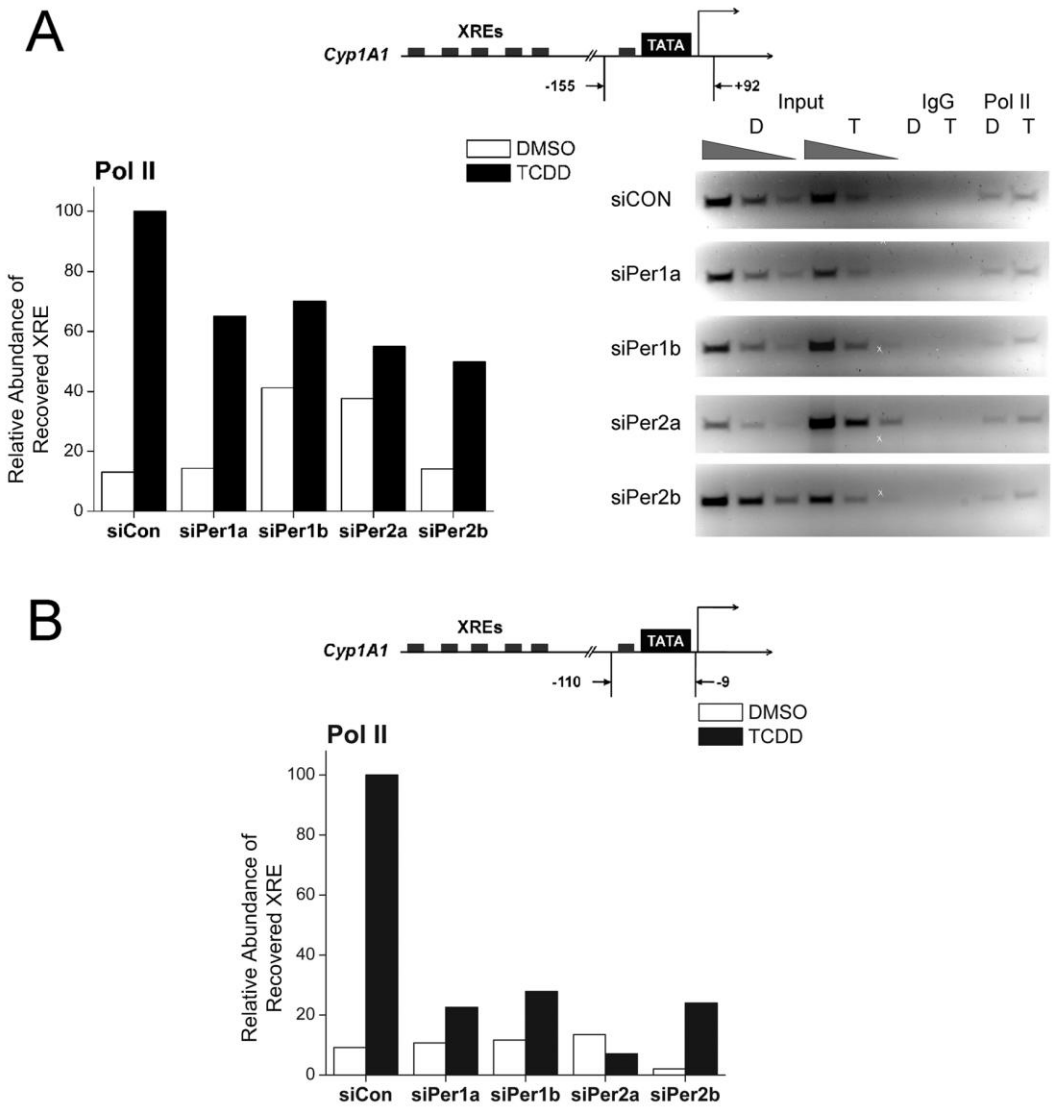


Fig. 21. Effects of siRNA inhibition of *Per1* or *Per2* on the association of RNA Pol II with the *Cyp1A1* TSS. CHIP assay was performed to evaluate the *Cyp1A1* TSS occupancy by Pol II in Hepa1c1c7 cells following vehicle and TCDD treatment. The relative amount of *Cyp1A1* DNA recovered from the CHIP assay represents the relative binding level of Pol II on the TSS and was analyzed by regular PCR (A) and Real-Time PCR (B). The location of the *Cyp1A1* TSS and PCR amplified regions are schematically illustrated on top of each panel. The gel picture shows regular PCR products from serially diluted input DNA as well as DNA from IgG IP and Pol II IP. Types of siRNA constructs used to infect cells are indicated on the side of the gel picture. Optical densities (OD) were calculated from the regular PCR gel image (A) using methods explained in Materials and Methods. Real-Time PCR assay (B) presents the level of DNA recovered from Pol II IP relative to 1/1000 diluted input DNA. Plotted values for both regular (A) and Real-Time PCR (B) were adjusted in relation to TCCD-treated siCon infected cells, which were arbitrarily set at 100.

DISCUSSION

Based on observations in the previous chapter, demonstrating that TCDD-induced *Cyp1A1* expression in Hepa1c1c7 cells is increased by siRNA inhibition of *Per1* and decreased by *Per2* repression, we predicted that *Per1* is a negative factor, while *Per2* is a positive factor in the responses of the AhR signaling pathway to TCDD *in vitro*. Therefore, we expected the CHIP assay to reveal that the AhR-initiated *Cyp1A1* transcription is higher in the *Per1* siRNA-infected cells and lower in *Per2* siRNA-infected cells relative to scrambled control (siCON)-infected cultures. Because there was only one replicate per experimental group, the current data were not subjected to statistical analysis and therefore only reflect trends of the observation. Our results indicated the potential regulation of *Per1* and *Per2* in the AhR activation of *Cyp1A1* transcription by showing that levels of TCDD-induced AhR/XRE and Pol II/TSS binding are altered in *Per1* or *Per2* inhibited Hepa1c1c7 cells, although the direction of the alterations were not entirely as expected. The present results demonstrate that the siRNA inhibition of *Per1* and *Per2* both decrease the TCDD-induced recruitment of AhR and Pol II onto the AhR target gene, *Cyp1A1*. Thus, these findings support the proposed role of *Per2* as a positive regulator in the TCDD-mediated activation of AhR target gene transcription *in vitro*.

Our current observation indicates that *Per1* could modulate the AhR signaling pathway at multiple levels other than the AhR recruitment onto the XRE. Therefore, the siRNA inhibition of *Per1* causes alterations at different levels, some inhibitory and some stimulatory, which add up together and eventually lead to increased TCDD-induced

Cyp1A1 mRNA abundance. For example, *Per1* may affect post-transcriptional regulation of AhR target genes, perhaps by modulating mRNA degradation, which has been identified as an important step in the regulation *p450* gene expression (Lee and Safe, 2001; Ciolino and Yeh, 1999). PER1 may also affect the degradation of AhR protein, which occurs shortly after the ligand binding. The inhibition of this degradation process has been shown to cause the super-induction of *Cyp1A1* in response to TCDD treatment (Pollenz, 2002). Another possible scenario by which PER1 modulates the AhR signaling pathway is that PER1 may influence the expression of the AhR repressor (*AhRR*), an AhR target gene, which negatively feeds back and inhibits the AhR/ARNT dimer formation as well as the *Cyp1A1* transcription. The next chapter will discuss in detail these possible mechanisms for *Per1* function in the TCDD-mediated regulation of the AhR signaling pathway.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSIONS

In mammals, the master pacemaker in the SCN and local oscillators in other brain regions and in peripheral tissues are responsible for the regulation of circadian rhythms. Because this timekeeping system imposes circadian rhythmicity on many biochemical and physiological processes, it is not surprising that the toxicity and efficacy of drugs vary rhythmically based on the time of administration. Clinical evidence indicates that certain medications, such as anti-hypertension medications, are more effective when administered at a particular time of day. For example, evening doses of valsartan or nifedipine are more effective than morning administration in treating high blood without adverse effects (Hermida et al., 2007). Chronotherapeutics also have important implications in improving the efficacy and reducing the toxicity of chemotherapy. Laboratory research indicates that the toxicity and efficacy of over 30 anticancer drugs vary by more than 50% depending on the treatment time (Lévi et al., 2007). In clinical trials, the chronotherapeutic delivery of oxaliplatin, a drug used in the treatment of colorectal cancer, improves drug tolerance by five-fold and produces a two-fold increase in anti-tumor activity relative to that observed with constant-rate delivery of the medicine (Lévi et al., 2007). Furthermore, chronopharmacokinetic studies have revealed 24-hour rhythms in processes that determine drug disposition including absorption, distribution, metabolism, and elimination of drugs (Bruguerolle, 1998). These chronotherapeutic and chronopharmacological observations are compatible with

evidence indicating that the levels of drug metabolizing enzymes, such as cytochrome P450s, fluctuate between day and night (Furukawa et al., 1999).

Despite the wealth of evidence for these daily rhythms of drug efficacy and toxicity, it is not known how the circadian clock interacts with drug response pathways at the molecular level or whether the canonical clock genes or their configuration in the molecular feedback loop are necessary for the generation of these rhythms. The PAS genes comprise key elements of the circadian clock mechanism as well as the signaling pathway mediating the effects of xenobiotics, including drugs and environmental toxins. Therefore, the working hypothesis underlying most of the present research was that PAS protein components of the clockworks may interact with other proteins in this family such as AhR and ARNT and thus may modulate xenobiotic responses. Because *Drosophila* PER interacts with mammalian AhR and ARNT and impedes the activation of the AhR signaling pathway (Lindebro et al., 1995), this hypothesis was tested by determining whether targeted disruption or inhibition of the clock genes, *Per1* and *Per2*, alters the TCDD-mediated regulation of the AhR signaling pathway in both the mouse mammary gland and liver.

Based on our current results, the circadian clock gene *Per1* appears to function as a negative or inhibitory factor in regulating the TCDD-mediated activation of the AhR signaling pathway. Our results showed that the targeted disruption of *Per1* leads to increased activation of the AhR signaling pathway by dioxin. We measured the activity of the AhR signaling pathway by analyzing the mRNA abundance of its two target genes, *Cyp1A1* and *Cyp1B1*. Similar results were observed in mouse mammary and liver, with

both showing increases in TCDD-induced *Cyp1A1* and *Cyp1B1* expression in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mice, but not in *Per2*^{ldc} animals. Similar results were also observed in *in vitro* experiments using mammary primary cells derived from mutant and wild type mice and in Hepa1c1c7 cells subjected to siRNA-mediated inhibition of *Per1* or *Per2*.

The present study provides the first evidence for the involvement of specific clock genes in toxin response pathways. It indicates that a PAS gene in the circadian clockworks, namely *Per1*, is also involved in the regulation of toxin responses. Future studies are needed to determine whether other PAS genes (i.e., *Clock* and *Bmal1*) and even non-PAS genes (i.e., *Cry1* and *Cry2*) in the clock feedback loops are also involved in mediating TCDD responses of the AhR signaling pathway. The established *Clock*$^{\Delta 5\text{-}6}$, *Bmal1*$^{-/-}$, *Cry1*$^{-/-}$, *Cry2*$^{-/-}$, and *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mutant mice would provide an opportunity to address these possibilities *in vivo*, while gene silencing via RNA interference would again be useful for *in vitro* analyses. An important implication of our results is that similar to its function as a negative element in the clock feedback loops, *Per1* inhibits TCDD-induced activation of the AhR signaling pathway. Given that *Clock* and *Bmal1* function on the positive limb of the circadian feedback loop and induce the transcription of clock genes, it is possible that these two clock genes may also act as positive regulators of the AhR signaling pathway. Therefore, the targeted disruption of *Clock* or *Bmal1* may decrease TCDD-mediated induction of AhR target gene expression. It will also be important to address the possible involvement of *Cry1* and *Cry2* in the AhR signaling pathway. Although these genes do not belong to the PAS family, *Cry1* and *Cry2* are important negative elements of the clock feedback loops. In comparison with

the PER proteins, CRY1 and CRY2 have a stronger inhibitory influence in the negative limb of the molecular clockworks. Therefore, *Cry1* and *Cry2* may also function as negative regulators of TCDD-induced transcriptional activity in the AhR signaling pathway, and the targeted disruption of the *Cry* gene(s) may enhance TCDD-induced *p450* expression.

Our current discovery provides new insights into the involvement of circadian clock genes in non-clock biological pathways. Previous studies demonstrated that clock gene mutation results in disrupted locomoter rhythms, in association with malfunctions of non-clock biological processes such as development, metabolism, reproduction, and tumorigenesis (Bunger et al., 2005; Dallmann et al., 2006; Fu et al., 2002; Gery et al., 2005; Kondratov et al., 2007; Lee, 2005; Miller et al., 2004; Rudic et al., 2004; Turek et al., 2005). Changes in these non-clock processes among clock gene mutant animals could be due to the disruption of the circadian clock per se. It may also reflect certain regulative roles of clock genes that are outside the clock. One way to distinguish between these two possibilities is to investigate if different types of clock gene mutants have a certain health defect in common. For example, one study showed that although the mutation of *Per* genes and *Cry* genes both disrupt the mammalian circadian clock, only *Per* mutant but not *Cry* mutant animals demonstrated increased susceptibility to cancer (Fu and Lee, 2003; Gauger and Sancar, 2005). $Cry1^{-/-}/Cry2^{-/-}$ mutant animals and cells are indistinguishable from wild type in terms of their sensitivity to ionizing radiation and UV radiation and ionizing radiation-induced DNA damage checkpoint responses (Gauger and Sancar, 2005; van der Horst et al., 1999; Vitaterna et al., 1999).

Therefore, the *Per* genes probably inhibit tumorigenesis through pathways that are outside the circadian clock. These pathways include regulating ATM-Chk1/Chk2 DNA damage response pathways and stimulating apoptosis (Gery et al, 2007; Hua et al, 2007) and functioning as a mediator of estrogen's regulation on mammary gland development and tumorigenesis (Gery et al, 2007). It is unknown if the *Per* genes regulate the toxin responses through a unique pathway that is independent of the clock, or if the *Per* genes influence the AhR signaling pathway through the rhythmic regulation of the clock. Future study needs to investigate the AhR signaling pathway following TCDD treatment in other clock gene mutant animals such as *Clock*, *Bmal*, and *Cry* gene mutants. Because responses to drugs and toxins vary daily, it is likely that the circadian clock has important roles in regulating the drug response pathways. Therefore, clock gene mutant animals with the disrupted biological clock are highly likely to demonstrate alterations in drug response pathways, such as the AhR signaling pathway.

On a global level, because drug toxicity and efficacy are known to show circadian variation, it would not be surprising if one of the molecular pathways mediating the toxin responses, the AhR signaling pathway, is under the regulation of the clock. In fact, microarray studies indicate that 3% of rhythmic genes in SCN2.2 cells are associated with defense and detoxification function (Menger et al., 2005). This percentage is even higher in cells derived from peripheral tissues. When rhythmic gene expression is initiated in NIH/3T3 mouse fibroblast cells by forskolin treatment, 13% of the rhythmically expressed genes were shown related to defense and detoxification (Menger et al., 2007). Our *in vivo* study of the mouse mammary gland indicates diurnal

fluctuation in the TCDD-mediated activation of AhR target genes. Therefore, the altered responses of AhR signaling pathway to TCDD in the *Per1* mutant mice could be due to perturbations in rhythmic regulation of key components in this pathway. This hypothesis is indirectly supported by the observation that diurnal variation in the TCDD-induced *Cyp1A1* expression is abolished in the mammary glands of $Per1^{ldc}/Per2^{ldc}$ mice.

Because *Per1* and *Per2* are both core components of the interlocked circadian feedback loops, the disruption of the *Per* genes alters the expression of other genes at the transcriptional and/or translational level (Bae et al., 2001; Jin et al., 1999; Sangoram et al., 1998). Therefore, the disruption of *Per1* may affect the AhR signaling pathway indirectly through alterations in other clock genes or clock-controlled genes. Immunoprecipitation analysis may be applied to determine whether the PER proteins as well as other clock PAS proteins directly interact with key regulators of the AhR signaling pathway.

It is currently unknown how *Per* gene disruption or inhibition alters responses of the AhR signaling pathway to TCDD. An inhibitory effect of PER on AhR-mediated *Cyp1A1* transcription has been observed in previous studies showing that the ectopic expression of *Drosophila* PER represses the recruitment of the AhR onto the XRE enhancer of the *Cyp1A1* gene (Lindebro et al., 1995). This observation is consistent with the current finding that the siRNA inhibition of *Per1* increases the TCDD-induced expression of *Cyp1A1* in Hepa1c1c7 cells. Therefore, we hypothesized that the mammalian *Per1* normally modulates pathway responses by inhibiting the AhR binding with the XRE and therefore reducing the *Cyp1A1* transcription. To test this hypothesis,

we used the CHIP assay to examine the basal and TCDD-induced level of AhR/XRE and Pol II/promoter association following siRNA inhibition of *Per1* or *Per2*. Our observations, however, do not support the proposed inhibitive role of *Per1* on TCDD-induced AhR recruitment onto the XRE.

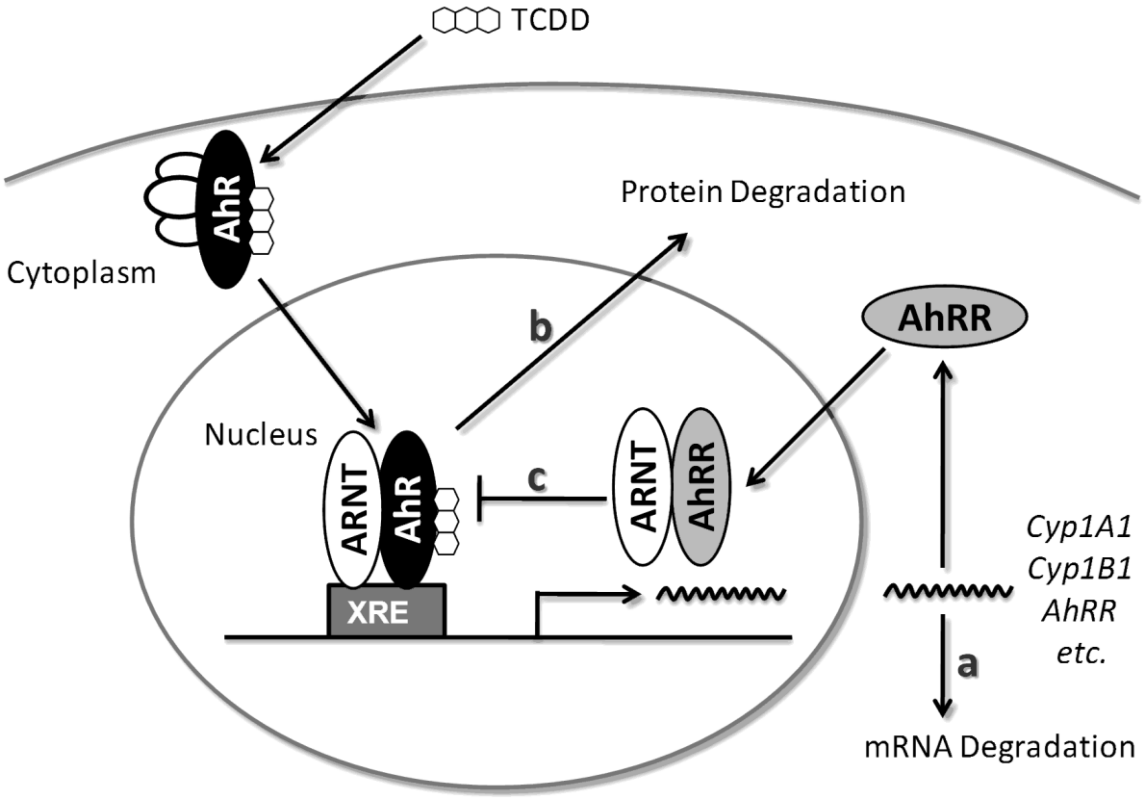


Fig. 22. The siRNA inhibition of *Per1* could modulate the AhR signaling pathway at multiple levels. (a) The post-transcriptional degradation of the *Cyp1A1* mRNA, (b) the ligand-induced degradation of the AhR protein, and (c) the feedback repression from the AhR Repressor.

The incongruity between this prediction and observations from the CHIP assay suggests that *Per1* may modulate the AhR signaling pathway at multiple levels (Fig. 22). Among different factors that modulate the expression of AhR target genes, the post-transcriptional regulation is particularly important (Fig. 22). Post-transcriptional events, such as mRNA decay, are known to affect *Cyp1A1* mRNA abundance. For example, inhibitors of the AhR signaling pathway, such as the adrenal steroid hormone dehydroepiandrosterone (DHEA) and resveratrol, do not affect TCDD-induced *Cyp1A1* transcription, but accelerate *Cyp1A1* mRNA degradation *in vitro* and therefore inhibit the activation of the AhR signaling pathway (Lee and Safe, 2001; Ciolino and Yeh, 1999). Similar post-transcriptional regulation of gene expression is also evident in pathways regulated by other PAS proteins. The regulation of hypoxia response pathway by the hypoxia inducible factor 1 alpha (HIF1α) provides a good example, because the induction of HIF1α target genes, such as the vascular endothelial growth factor (VEGF), is largely related to increased mRNA stability (Gu et al., 2000; Stein et al., 1995; Dibbens et al., 1999). Although the clock genes *Per1* and *Per2* have not been directly linked to the regulation of RNA stability, clock-controlled genes, such as *Nocturnin*, which encodes a polyA ribonuclease, have been shown to modulate mRNA stability (Baggs and Green, 2003; Oishi et al., 2003; Wang et al., 2001). Future studies are necessary to determine whether *Cyp1A1* mRNA stability is altered following *Per1* inhibition in Hepa1c1c7 cells.

Cyp1A1 expression is also regulated by processes that terminate activation of the AhR signaling pathway such as the degradation of the AhR protein (Fig. 22). Rapid

ubiquitin-mediated degradation of AhR has been shown to occur following ligand binding (Pollenz, 2002). Moreover, blockade of AhR degradation increases the expression of AhR target genes (Pollenz, 2002). At present, there is no evidence for *Per1* function in the degradation of AhR, but *Per1* is involved in regulating the stability of other PAS proteins. The role of *Per1* in regulating the degradation of PER2 is supported by the observation that the light-triggered degradation of PER2 protein in the SCN is significantly decreased in $Per1^{brd}$ than in wild-type mice (Masubuchi et al., 2005). To investigate possible involvement of *Per1* in regulating AhR stability, it will be necessary to compare the rates of TCDD-induced AhR degradation between normal and *Per1*-inhibited cells.

Another key event that occurs during the termination phase of the AhR signaling pathway is the AhR:ARNT-mediated induction of the PAS gene, AhR Repressor (AhRR) (Mimura et al., 1999). The *AhRR* gene contains the XRE that mediates its induction in response to TCDD. AhRR forms dimers with ARNT via the PAS domain. AhRR thus provides for competitive inhibition of AhR:ARNT heterodimers and the negative feedback regulation of the AhR signaling pathway (Fig. 22). Evidence for AhRR function in the termination phase of the AhR signaling pathway is derived from the finding that over-expression of AhRR blocks the recruitment of AhR:ARNT onto the XRE and inhibits the AhR:ARNT activation of gene transcription (Mimura et al., 1999). This feedback mechanism involving PAS proteins closely resembles the molecular feedback loop composed of clock PAS proteins such as CLOCK, BMAL1 and the PERIOD proteins. Although there is currently no evidence for the role of *Per1* in

modulating AhRR levels, the TCDD-induced *AhRR* expression could be enhanced by the inhibition of *Per1*, because other AhR target genes, such as the *p450s*, are up-regulated in this experimental group. Future study will be necessary to determine whether siRNA inhibition of *Per1* alters the balance between the transcriptional activation of AhR target genes and AhRR feedback control on the AhR signaling pathway.

It is well known that the circadian clock is important in the health and well-being of an organism. Early awareness about the importance of circadian rhythms was established in studies examining the physiological consequences caused by the perturbations in clock function. Temporary disturbances in the temporal organization of circadian rhythms caused by, for example, jet lag, usually lead to transient symptoms, such as fatigue, reduced alertness and loss of sleep at night, which typical resolve as the rhythms are resynchronized with the environment and each other (Dunlap et al., 2004). However, the constant disruption of the sleep/wake cycle has been shown to cause long-term health problems. Epidemiologic studies on a large number of night and rotating shift workers indicate that the incidence in sleep-wake disorders and cardiovascular disease is increased in these populations (Moore-Ede and Richardson, 1985). Lately, studies on shift workers have revealed a correlation between disturbances of the circadian clock and tumorigenesis such as colon cancer and breast cancer (Haus and Smolensky, 2006). The relation between the clock and the risk of cancer is also supported by rodent studies demonstrating that the disruption of circadian rhythms by constant light exposure promotes diethylnitrosamine-induced hepatocarcinogenesis (van den Heiligenberg et al., 1999). Considerable effort has since been invested in examining

the relation between the clock and tumorigenesis. Observations from multiple studies suggest that clock genes, such as *Per1* and *Per2*, may function as tumor suppressors both at the molecular level and through their circadian regulation of global processes in the organism (Fu and Lee, 2003). Because tumor development is largely due to the toxic and carcinogenic effects of environmental toxins, pathways mediating toxin responses present potential targets, through which the circadian clock could inhibit tumor development. A recent study conducted by Gachon and colleagues demonstrated the regulation of clock on the response to drugs and toxins through the function of clock controlled PAR bZIP transcription factors, namely DBP, HLF and TEF. PAR bZip triple knockout mice are hypersensitive to environmental toxins and deficient in detoxification (Gachon et al., 2006). Our study provides the first evidence that suggests the involvement of a core circadian clock gene, namely *Per1*, in toxin responses of the AhR signaling pathway. Because AhR mediates the toxic and carcinogenic effects of polynuclear aromatic hydrocarbons (PAHs), the function of the clock gene *Per1* could contribute to the tumor suppression by the clock as well as the circadian regulation of drug efficacy and toxicity.

REFERENCES

Abarca C, Albrecht U, and Spanagel R (2002) Cocaine sensitization and reward are under the influence of circadian genes and rhythm. *Proc Natl Acad Sci USA* **99:**9026-30.

Allen G, Rappe J, Earnest DJ, and Cassone VM (2001) Oscillating on borrowed time: diffusible signals from immortalized suprachiasmatic nucleus cells regulate circadian rhythmicity in cultured fibroblasts. *J Neurosci* **21:**7937-43.

Allen G, Farnell Y, Bell-Pedersen D, Cassone VM, and Earnest DJ (2004) Effects of altered Clock gene expression on the pacemaker properties of SCN2.2 cells and oscillatory properties of NIH/3T3 cells. *Neuroscience* **127:**989-999.

Alvarez JD, Chen D, Storer E, and Sehgal A (2003) Non-cyclic and developmental stage-specific expression of circadian clock proteins during murine spermatogenesis. *Biol Reprod* **69:**81-91.

Aparicio OM, Geisberg JV, and Struhl K (2004) Chromatin immunoprecipitation for determining the association of proteins with specific genomic sequences in vivo, in *Current Protocols in Molecular Biology* (Ausubel FA, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, and Struhl K eds) pp 21.3.1–21.3.32, John Wiley & Sons, New York.

Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, and Weaver DR (2001) Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. *Neuron* **30:** 525-536.

Baggs JE and Green CB (2003) Nocturnin, a deadenylase in Xenopus laevis retina: a mechanism for posttranscriptional control of circadian-related mRNA. *Curr Biol* **13:**189-98.

Ball RK, Friis RR, Schoenenberger CA, Doppler W, and Groner B (1988) Prolactin regulation of beta-casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line. *EMBO J* **7:**2089-2095.

Balsalobre A, Damiola F, and Schibler U (1998) A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* **93:**929-37.

Barnett KR, Tomic D, Gupta RK, Miller KP, Meachum S, Paulose T, and Flaws JA (2007) The aryl hydrocarbon receptor affects mouse ovarian follicle growth via

mechanisms involving estradiol regulation and responsiveness. *Biol Reprod* **76:**1062-70.

Barouki R, Coumoul X, and Fernandez-Salguero PM (2007) The aryl hydrocarbon receptor, more than a xenobiotic-interacting protein. *FEBS Lett* **581:**3608-15.

Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL, and Zoran MJ (2005) Circadian rhythms from multiple oscillators: lessons from diverse organisms. *Nat Rev Genet* **6:**544-56.

Benedict JC, Lin TM, Loeffler IK, Peterson RE, and Flaws JA (2000) Physiological role of the aryl hydrocarbon receptor in mouse ovary development. *Toxicol Sci* **56:**382-8.

Berger JJ and Daniel CW (1982) Diurnal rhythms in developing ducts of the mouse mammary gland. *J Exp Zool* **224:**115-118.

Bruguerolle B (1998) Chronopharmacokinetics. *Current Status Clin Pharmacokinet* **35:**83-94.

Buchi KN, Moore JG, Hrushesky WJ, Sothern RB, and Rubin NH (1991) Circadian rhythm of cellular proliferation in the human rectal mucosa. *Gastroenterology* **101:**410-415.

Bunger MK, Walisser JA, Sullivan R, Manley PA, Moran SM, Kalscheur VL, Colman RJ, and Bradfield CA (2005) Progressive arthropathy in mice with a targeted disruption of the Mop3/Bmal-1 locus. *Genesis* **41:**122-32.

Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, and Bradfield CA (2000) Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* **103:**1009-17.

Cermakian N, Monaco L, Pando MP, Dierich A, and Sassone-Corsi P (2001) Altered behavioral rhythms and clock gene expression in mice with a targeted mutation in the Period1 gene. *EMBO J* **20:**3967-74.

Chen ST, Choo KB, Hou MF, Yeh KT, Kuo SJ, and Chang JG (2005) Deregulated expression of the PER1, PER2 and PER3 genes in breast cancers. *Carcinogenesis* **26:** 1241-1246.

Chen SY, Wang LY, Lunn RM, Tsai WY, Lee PH, Lee CS, Ahsan H, Zhang YJ, Chen CJ, and Santella RM (2002) Polycyclic aromatic hydrocarbon-DNA adducts in liver tissues of hepatocellular carcinoma patients and controls. *Int J Cancer* **99:**14-21.

Chen ZH, Hurh YJ, Na HK, Kim JH, Chun YJ, Kim DH, Kang KS, Cho MH, and Surh YJ (2004) Resveratrol inhibits TCDD-induced expression of CYP1A1 and CYP1B1

and catechol estrogen-mediated oxidative DNA damage in cultured human mammary epithelial cells. *Carcinogenesis* **25:**2005-2013.

Chilov D, Hofer T, Bauer C, Wenger RH, and Gassmann M (2001) Hypoxia affects expression of circadian genes PER1 and CLOCK in mouse brain. *FASEB J* **15:**2613-2622.

Christou M, Savas U, Schroeder S, Shen X, Thompson T, Gould MN, and Jefcoate CR (1995) Cytochromes CYP1A1 and CYP1B1 in the rat mammary gland: cell-specific expression and regulation by polycyclic aromatic hydrocarbons and hormones. *Mol Cell Endocrinol* **115:**41-50.

Ciolino HP and Yeh GC (1999) The steroid hormone dehydroepiandrosterone inhibits CYP1A1 expression in vitro by a post-transcriptional mechanism. *J Biol Chem* **274:**35186-90.

Crews ST and Fan CM (1999) Remembrance of things PAS: regulation of development by bHLH-PAS proteins. *Curr Opin Genet Dev* **9:**580-587.

Dallmann R, Touma C, Palme R, Albrecht U, and Steinlechner S (2006) Impaired daily glucocorticoid rhythm in Per1Brd mice. *J Comp Physiol* **192:**769-775.

DeBruyne JP, Noton E, Lambert CM, Maywood ES, Weaver DR, and Reppert SM (2006) A clock shock: mouse CLOCK is not required for circadian oscillator function. *Neuron* **50:**465-77.

Dibbens JA, Miller DL, Damert A, Risau W, Vadas MA, and Goodall GJ (1999) Hypoxic regulation of vascular endothelial growth factor mRNA stability requires the cooperation of multiple RNA elements. *Mol Biol Cell* **10:**907-19.

Duffield GE (2003) DNA microarray analyses of circadian timing: the genomic basis of biological time. *J Neuroendo* **15:**991-1002.

Dunlap JC, Loros JJ, and Decoursey PJ (2004) *Chronobiology: Biological Timekeeping*. Sinauer Associates, Sunderland, Ma.

Earnest DJ and Sladek CD (1987) Circadian vasopressin release from perifused rat suprachiasmatic explants in vitro: effects of acute stimulation. *Brain Res* **422:**398-402.

Fernandez-Salguero P, Pineau T, Hilbert DM, McPhail T, Lee SS, Kimura S, Nebert DW, Rudikoff S, Ward JM, and Gonzalez FJ (1995) Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. *Science* **268:**722-726.

Fretland AJ, Safe S, and Hankinson O (2004) Lack of antagonism of 2,3,7,8-tetrachlorodibenzo-p-dioxin's (TCDDs) induction of cytochrome P4501A1 (CYP1A1)

by the putative selective aryl hydrocarbon receptor modulator 6-alkyl-1,3,8-trichlorodibenzofuran (6-MCDF) in the mouse hepatoma cell line Hepa-1c1c7. *Chem Biol Interact* **150:**161-70.

Fu L and Lee CC (2003) The circadian clock: pacemaker and tumour suppressor. *Nat Rev Cancer* **3:**350-61.

Fu L, Pelicano H, Liu J, Huang P, and Lee C (2002) The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo. *Cell* **111:**41-50.

Fujii-Kuriyama Y, and Mimura J (2005) Molecular mechanisms of AhR functions in the regulation of cytochrome P450 genes. *Biochem Biophys Res Commun* **338:** 311-7.

Furness SG, Lees MJ, Whitelaw ML (2007) The dioxin (aryl hydrocarbon) receptor as a model for adaptive responses of bHLH/PAS transcription factors. *FEBS Lett.* **581:**3616-25.

Furukawa T, Manabe S, Watanabe T, Sehata S, Sharyo S, Okada T, and Mori Y (1999) Daily fluctuation of hepatic P450 monooxygenase activities in male rats is controlled by the suprachiasmatic nucleus but remains unaffected by adrenal hormones. *Arch Toxicol* **73:**367-72.

Gachon F, Olela FF, Schaad O, Descombes P, and Schibler U (2006) The circadian PAR-domain basic leucine zipper transcription factors DBP, TEF, and HLF modulate basal and inducible xenobiotic detoxification. *Cell Metabolism* **4:**25-36.

Garcia MN, Barbeito CG andrini LA, and Badran AF (2001) Circadian rhythm of DNA synthesis and mitotic activity in tongue keratinocytes. *Cell Biol Int* **25:**179-183.

Gauger MA, Sancar A (2005) Cryptochrome, circadian cycle, cell cycle checkpoints, and cancer. *Cancer Res* **65:**6828-34.

Gekakis N, Staknis D, Nguyen HB, Davis FC, Wilsbacher LD, King DP, Takahashi JS, and Weitz CJ (1998) Role of the CLOCK protein in the mammalian circadian mechanism. *Science* **280:**1564-1569.

Gery S, Gombart AF, Yi WS, Koeffler C, Hofmann WK, and Koeffler HP (2005) Transcription profiling of C/EBP targets identifies Per2 as a gene implicated in myeloid leukemia. *Blood* **106:**2827-36.

Gery S, Komatsu N, Baldjyan L, Yu A, Koo D, and Koeffler HP (2006) The circadian gene per1 plays an important role in cell growth and DNA damage control in human cancer cells. *Mol Cell* **22:**375-82.

Gillette MU and Reppert SM (1987) The hypothalamic suprachiasmatic nuclei:circadian patterns of vasopressin secretion and neuronal activity in vitro. *Brain Res Bull* **19:**135-139.

Gorski JR, Lebofsky M, and Rozman K (1988) Corticosterone decreases toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in hypophysectomized rats. *J Toxicol Environ Health* **25:**349-360.

Granda TG, Liu XH, Smaaland R, Cermakian N, Filipski E, Sassone-Corsi P, and Levi F (2005) Circadian regulation of cell cycle and apoptosis proteins in mouse bone marrow and tumor. *FASEB J* **19:**304-306.

Griffin EA, Staknis D, and Weitz CJ (1999) Light-independent role of CRY1 and CRY2 in the mammalian circadian clock. *Science* **286:**768-771.

Gu YZ, Hogenesch JB, and Bradfield CA (2000) The PAS superfamily: sensors of environmental and developmental signals. *Annu Rev Pharmacol Toxicol* **40:**519-61.

Haus E and Smolensky M (2006) Biological clocks and shift work: circadian dysregulation and potential long-term effects. *Cancer Causes Control* **17:**489-500.

Hermida RC, Ayala DE, Smolensky MH, and Portaluppi F (2007) Chronotherapy in hypertensive patients: administration-time dependent effects of treatment on blood pressure regulation. *Expert Rev Cardiovasc Ther* **5:**463-75.

Hogenesch JB, Chan WK, Jackiw VH, Brown RC, Gu YZ, Pray-Grant M, Perdew GH, and Bradfield CA (1997) Characterization of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signaling pathway. *J Biol Chem* **28:**8581-8593.

Hogenesch JB, Gu YZ, Jain S, and Bradfield CA (1998) The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. *Proc Natl Acad Sci USA* **95:**5474-5479.

Honma S, Nakamura W, Shirakawa T, and Honma K (2004) Diversity in the circadian periods of single neurons of the rat suprachiasmatic nucleus depends on nuclear structure and intrinsic period. *Neurosci Lett* **358:**173-6.

Hua H, Wang Y, Wan C, Liu Y, Zhu B, Wang X, Wang Z, Ding JM (2007) Inhibition of tumorigenesis by intratumoral delivery of the circadian gene mPer2 in C57BL/6 mice. *Cancer Gene Ther* **14:**815-8.

Jin X, Shearman LP, Weaver DR, Zylka MJ, de Vries GJ, and Reppert SM (1999) A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. *Cell* **96:**57-68.

Kewley RJ, Whitelaw ML, and Chapman-Smith A (2004) The mammalian basic helix-loop-helix/PAS family of transcriptional regulators. *Int J Biochem Cell Biol* **36:**189-204.

King DP, Zhao Y, Sangoram AM, Wilsbacher LD, Tanaka M, Antoch MP, Steeves TD, Vitaterna MH, Kornhauser JM, Lowrey PL, Turek FW, and Takahashi JS (1997) Positional cloning of the mouse circadian clock gene. *Cell* **89:**641-53.

Kita Y, Shiozawa M, Jin W, Majewski RR, Besharse JC, Greene AS, and Jacob HJ (2002) Implications of circadian gene expression in kidney, liver and the effects of fasting on pharmacogenomic studies. *Pharmacogenetics* **12:**55-65.

Knerr S and Schrenk D (2006) Carcinogenicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in experimental models. *Mol Nutr Food Res* **50:**897-907.

Ko CH and Takahashi JS (2006) Molecular components of the mammalian circadian clock. *Hum Mol Genet* **15:**R271-7.

Kondratov RV, Chernov MV, Kondratova AA, Gorbacheva VY, Gudkov AV, and Antoch MP (2003) BMAL1-dependent circadian oscillation of nuclear CLOCK: posttranslational events induced by dimerization of transcriptional activators of the mammalian clock system. *Genes Dev* **17:**1921-32.

Kondratov RV, Gorbacheva VY, and Antoch MP (2007) The role of mammalian circadian proteins in normal physiology and genotoxic stress responses. *Curr Top Dev Biol* **78:**173-216.

Kume K, Zylka MJ, Sriram S, Shearman LP, Weaver DR, Jin X, Maywood ES, Hastings MH, and Reppert SM (1999) mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell* **98:**193-205.

Kwon I, Lee J, Chang SH, Jung NC, Lee BJ, Son GH, Kim K, and Lee KH (2006) BMAL1 shuttling controls transactivation and degradation of the CLOCK/BMAL1 heterodimer. *Mol Cell Biol* **26:**7318-30.

Lee CC (2005) The circadian clock and tumor suppression by Mammalian period genes. *Methods Enzymol* **393:**852-61.

Lee CC (2006) Tumor suppression by the mammalian Period genes. *Cancer Causes Control* **17:**525-530.

Lee JE and Safe S (2001) Involvement of a post-transcriptional mechanism in the inhibition of CYP1A1 expression by resveratrol in breast cancer cells. *Biochem Pharmacol* **62:**1113-24.

Levi F and Schibler U (2007) Circadian rhythms: mechanisms and therapeutic implications. *Annu Rev Pharmacol Toxicol* **47:**593-628.

Lévi F, Focan C, Karaboué A, de la Valette V, Focan-Henrard D, Baron B, Kreutz F, and Giacchetti S (2007) Implications of circadian clocks for the rhythmic delivery of cancer therapeutics. *Adv Drug Deliv Rev* **59:**1015-35.

Lindebro MC, Poellinger L, and Whitelaw ML (1995) Protein-protein interaction via PAS domains: role of the PAS domain in positive and negative regulation of the bHLH/PAS dioxin receptor-Arnt transcription factor complex. *EMBO J* **14:**3528-3539.

Masubuchi S, Kataoka N, Sassone-Corsi P, and Okamura H (2005) Mouse Period1 (mPER1) acts as a circadian adaptor to entrain the oscillator to environmental light/dark cycles by regulating mPER2 protein. *J Neurosci* **25:**4719-24.

Matsuo T, Yamaguchi S, Mitsui S, Emi A, Shimoda F, and Okamura H (2003) Control mechanism of the circadian clock for timing of cell division in vivo. *Science* **302:**255-259.

McClung CA, Sidiropoulou K, Vitaterna M, Takahashi JS, White FJ, Cooper DC, and Nestler EJ (2005) Regulation of dopaminergic transmission and cocaine reward by the Clock gene. *Proc Natl Acad Sci USA* **102:**9377-81.

McDearmon EL, Patel KN, Ko CH, Walisser JA, Schook AC, Chong JL, Wilsbacher LD, Song EJ, Hong HK, Bradfield CA, and Takahashi JS (2006) Dissecting the functions of the mammalian clock protein BMAL1 by tissue-specific rescue in mice. *Science* **314:**1304-1308.

Menger GJ, Allen GC, Neuendorff N, Nahm SS, Thomas TL, Cassone VM, and Earnest DJ (2007) Circadian profiling of the transcriptome in NIH/3T3 fibroblasts: comparison with rhythmic gene expression in SCN2.2 cells and the rat SCN. *Physiol Genomics* **29:**280-9.

Menger GJ, Lu K, Thomas T, Cassone VM, and Earnest DJ (2005) Circadian profiling of the transcriptome in immortalized rat SCN cells. *Physiol Genomics* **21:**370-81.

Metz RP, Qu X, Laffin B, Earnest D, and Porter WW (2006) Circadian clock and cell cycle gene expression in mouse mammary epithelial cells and in the developing mouse mammary gland. *Dev Dyn* **235:**263-271.

Michaud JL, Rosenquist T, May NR, and Fan CM (1998) Development of neuroendocrine lineages requires the bHLH-PAS transcription factor SIM1. *Genes Dev* **12:**3264-75.

Miller BH, McDearmon EL, Panda S, Hayes KR, Zhang J andrews JL, Antoch MP, Walker JR, Esser KA, Hogenesch JB, and Takahashi JS (2007) Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation. *Proc Natl Acad Sci USA* **104:**3342-7.

Miller BH, Olson SL, Turek FW, Levine JE, Horton TH, and Takahashi JS (2004) Circadian clock mutation disrupts estrous cyclicity and maintenance of pregnancy. *Curr Biol* **14:**1367-73.

Mimura J and Fujii-Kuriyama Y (2003) Functional role of AhR in the expression of toxic effects by TCDD. *Biochim Biophys Acta* **1619:**263-268.

Mimura J, Ema M, Sogawa K, and Fujii-Kuriyama Y (1999) Identification of a novel mechanism of regulation of Ah (dioxin) receptor function. *Genes Dev* **13:**20-5.

Moffett P, Reece M, and Pelletier J (1997) The murine Sim-2 gene product inhibits transcription by active repression and functional interference. *Mol Cell Biol* **17:**4933-4947.

Moore RY and Eichler VB (1972) Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Res* **42:**201-6.

Moore-Ede MC and Richardson GS (1985) Medical implications of shift-work. *Annu Rev Med* **36:**607-17.

Morse D, Cermakian N, Brancorsini S, Parvinen M, and Sassone-Corsi P (2003) No circadian rhythms in testis: Period1 expression is clock independent and developmentally regulated in the mouse. *Mol Endocrinol* **17:**141-151.

Muller WJ and Neville MC (2001) Introduction: signaling in mammary development and tumorigenesis. *J Mammary Gland Biol Neoplasia* **6:**1-5.

Nagoshi E, Saini C, Bauer C, Laroche T, Naef F, and Schibler U (2004) Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillators pass time to daughter cells. *Cell* **119:**693-705.

Nakamura TJ, Moriya T, Inoue S, Shimazoe T, Watanabe S, Ebihara S, Shinohara K (2005) Estrogen differentially regulates expression of Per1 and Per2 genes between central and peripheral clocks and between reproductive and nonreproductive tissues in female rats. *J Neurosci Res.* **82:**622-30.

Narasimhan TR, Craig A, Arellano L, Harper N, Howie L, Menache M, Birnbaum L, and Safe S (1994) Relative sensitivities of 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced Cyp1a-1 and Cyp1a-2 gene expression and immunotoxicity in female B6C3F1 mice. *Fundam Appl Toxicol* **23:**598-607.

Newman GC, Hospod FE, Patlak CS, and Moore RY (1992) Analysis of in vitro glucose utilization in a circadian pacemaker model. *J Neurosci* **12:**2015-2021.

Oishi K, Miyazaki K, Kadota K, Kikuno R, Nagase T, Atsumi G, Ohkura N, Azama T, Mesaki M, Yukimasa S, Kobayashi H, Iitaka C, Umehara T, Horikoshi M, Kudo T, Shimizu Y, Yano M, Monden M, Machida K, Matsuda J, Horie S, Todo T, and Ishida N (2003) Genome-wide expression analysis of mouse liver reveals CLOCK-regulated circadian output genes. *J Biol Chem* **278:**41519-27.

Okamura H, Miyake S, Sumi Y, Yamaguchi S, Yasui A, Muijtjens M, Hoeijmakers JH, and van der Horst GT (1999) Photic induction of mPer1 and mPer2 in cry-deficient mice lacking a biological clock. *Science* **286:**2531-4.

Pieper AA, Wu X, Han TW, Estill SJ, Dang Q, Wu LC, Reece-Fincanon S, Dudley CA, Richardson JA, Brat DJ, and McKnight SL (2005) The neuronal PAS domain protein 3 transcription factor controls FGF-mediated adult hippocampal neurogenesis in mice. *Proc Natl Acad Sci USA* **102:**14052-7.

Pollenz RS (2002) The mechanism of AH receptor protein down-regulation (degradation) and its impact on AH receptor-mediated gene regulation. *Chem Biol Interact* **141:**41-61.

Pollenz RS and Dougherty EJ (2005) Redefining the role of the endogenous XAP2 and C-terminal hsp70-interacting protein on the endogenous Ah receptors expressed in mouse and rat cell lines. *J Biol Chem* **280:**33346-56.

Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, and Schibler U (2002) The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* **110:**251-60.

Prough RA, Linder MW, Pinaire JA, Xiao GH, and Falkner KC (1996) Hormonal regulation of hepatic enzymes involved in foreign compound metabolism. *FASEB J* **10:**1369-1377.

Pullan SE and Streuli CH (1996) The mammary gland epithelial cell, in *Epithelial cell culture* (Harris A ed) pp 97-121, Cambridge University Press, Cambridge, UK.

Qu X, Metz RP, Porter WW, Cassone VM, and Earnest DJ (2007) Disruption of clock genes expression alters responses of the AhR signaling pathway in the mouse mammary gland. *Mol Pharmacol* **72:**1349-58.

Ralph MR, Foster RG, Davis FC, and Menaker M (1990) Transplanted suprachiasmatic nucleus determines circadian period. *Science* **247:**975-8.

Reddy AB, Wong GK, O'Neill J, Maywood ES, and Hastings MH (2005) Circadian clocks: neural and peripheral pacemakers that impact upon the cell division cycle. *Mutat Res* **574:**76-91.

Reppert SM and Weaver DR (2001) Molecular analysis of mammalian circadian rhythms. *Annu Rev Physiol* **63:**647-676.

Reppert SM and Weaver DR (2002) Coordination of circadian timing in mammals. *Nature* **418:**935-941.

Rudic RD, McNamara P, Curtis AM, Boston RC, Panda S, Hogenesch JB, and Fitzgerald GA (2004) BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. *PLoS Biol* **2:**e377.

Sangoram AM, Saez L, Antoch MP, Gekakis N, Staknis D, Whiteley A, Fruechte EM, Vitaterna MH, Shimomura K, King DP, Young MW, Weitz CJ, and Takahashi JS (1998) Mammalian circadian autoregulatory loop: a timeless ortholog and mPer1 interact and negatively regulate CLOCK-BMAL1-induced transcription. *Neuron* **21:**1101-13.

Scheving LE, Tsai TH, Pauly JE, and Halberg F (1983) Circadian effect of ACTH 1-17 on mitotic index of the corneal epithelium of BALB/C mice. *Peptides* **4:**183-190.

Schlezinger JJ, Liu D, Farago M, Seldin DC, Belguise K, Sonenshein GE, Sherr DH (2006) A role for the aryl hydrocarbon receptor in mammary gland tumorigenesis. *Biol Chem* **387:**1175-87.

Schrenk D (1998) Impact of dioxin-type induction of drug-metabolizing enzymes on the metabolism of endo- and xenobiotics. *Biochem Pharmacol* **15:**1155-1162.

Schwartz WJ and Zimmerman P (1991) Lesions of the suprachiasmatic nucleus disrupt circadian locomotor rhythms in the mouse. *Physiol Behav* **49:**1283-7.

Seagroves TN, Krnacik S, Raught B, Gay J, Burgess-Beusse B, Darlington GJ, and Rosen JM (1998) C/EBPbeta, but not C/EBPalpha, is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland. *Genes Dev* **12:**1917-1928.

Shearman LP, Sriram S, Weaver DR, Maywood ES, Chaves I, Zheng B, Kume K, Lee CC, van der Horst GT, Hastings MH, and Reppert SM (2000) Interacting molecular loops in the mammalian circadian clock. *Science* **288:**1013-9.

Shimba S, Ishii N, Ohta Y, Ohno T, Watabe Y, Hayashi M, Wada T, Aoyagi T, and Tezuka M (2005) Brain and muscle Arnt-like protein-1 (BMAL1), a component of the molecular clock, regulates adipogenesis. *Proc Natl Acad Sci USA* **102:**12071-6.

Shimizu Y, Nakatsuru Y, Ichinose M, Takahashi Y, Kume H, Mimura J, Fujii-Kuriyama Y, and Ishikawa T (2000) Benzo[a]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor. *Proc Natl Acad Sci USA* **97:**779-82.

Spanagel R, Pendyala G, Abarca C, Zghoul T, Sanchis-Segura C, Magnone MC, Lascorz J, Depner M, Holzberg D, Soyka M, Schreiber S, Matsuda F, Lathrop M, Schumann G, and Albrecht U (2005) The clock gene Per2 influences the glutamatergic system and modulates alcohol consumption. *Nat Med* **11:**35-42.

Stein I, Neeman M, Shweiki D, Itin A, and Keshet E (1995) Stabilization of vascular endothelial growth factor mRNA by hypoxia and hypoglycemia and coregulation with other ischemia-induced genes. *Mol Cell Biol* **15:**5363-8.

Stephan FK and Zucker I (1972) Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proc Natl Acad Sci USA* **69:**1583-6.

Storch KF, Lipan O, Leykin I, Viswanathan N, Davis FC, Wong WH, and Weitz CJ (2002) Extensive and divergent circadian gene expression in liver and heart. *Nature* **417:**78-83.

Tian Y, Ke S, Chen M, and Sheng T (2003) Interactions between the aryl hydrocarbon receptor and P-TEFb. Sequential recruitment of transcription factors and differential phosphorylation of C-terminal domain of RNA polymerase II at cyp1a1 promoter. *J Biol Chem* **278:**44041-8.

Tong Y, Guo H, Brewer JM, Lee H, Lehman MN, and Bittman EL (2004) Expression of haPer1 and haBmal1 in Syrian hamsters: heterogeneity of transcripts and oscillations in the periphery. *J Biol Rhythms* **19:**113-125.

Turek FW, Joshu C, Kohsaka A, Lin E, Ivanova G, McDearmon E, Laposky A, Losee-Olson S, Easton A, Jensen DR, Eckel RH, Takahashi JS, and Bass J (2005) Obesity and metabolic syndrome in circadian Clock mutant mice. *Science* **13:**1043-5.

van den Heiligenberg S, Deprés-Brummer P, Barbason H, Claustrat B, Reynes M, and Lévi F (1999) The tumor promoting effect of constant light exposure on diethylnitrosamine-induced hepatocarcinogenesis in rats. *Life Sci* **64:**2523-34.

van der Horst GT, Muijtjens M, Kobayashi K, Takano R, Kanno S, Takao M, de Wit J, Verkerk A, Eker AP, van Leenen D, Buijs R, Bootsma D, Hoeijmakers JH, and Yasui A (1999) Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature* **398:**627-30.

Vitaterna MH, Selby CP, Todo T, Niwa H, Thompson C, Fruechte EM, Hitomi K, Thresher RJ, Ishikawa T, Miyazaki J, Takahashi JS, and Sancar A (1999) Differential

regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. *Proc Natl Acad Sci USA* **96:**12114-9.

Wang Y, Osterbur DL, Megaw PL, Tosini G, Fukuhara C, Green CB, and Besharse JC (2001) Rhythmic expression of Nocturnin mRNA in multiple tissues of the mouse. *BMC Dev Biol* **1:**9-23.

Weaver DR (1998) The suprachiasmatic nucleus: a 25-year retrospective. *J Biol Rhythms* **13:**100-12.

Welsh DK, Logothetis DE, Meister M, and Reppert SM (1995) Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. *Neuron* **14:**697-706.

Woods SL and Whitelaw ML (2002) Differential activities of murine single minded 1 (SIM1) and SIM2 on a hypoxic response element. Cross-talk between basic helix-loop-helix/per-Arnt-Sim homology transcription factors. *J Biol Chem* **277:**10236-10243.

Xiao J, Li C, Zhu NL, Borok Z, and Minoo P (2003) Timeless in lung morphogenesis. *Dev Dyn* **228:**82-94.

Yagita K, Yamaguchi S, Tamanini F, van Der Horst GT, Hoeijmakers JH, Yasui A, Loros JJ, Dunlap JC, and Okamura H (2000) Dimerization and nuclear entry of mPER proteins in mammalian cells. *Genes Dev* **14:**1353-1363.

Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, Block GD, Sakaki Y, Menaker M, and Tei H (2000) Resetting central and peripheral circadian oscillators in transgenic rats. *Science* **288:**682-685.

Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr ED, Siepka SM, Hong HK, Oh WJ, Yoo OJ, Menaker M, and Takahashi JS (2004) PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci USA* **101:**5339-46.

Zghoul T, Abarca C, Sanchis-Segura C, Albrecht U, Schumann G, and Spanagel R (2007) Ethanol self-administration and reinstatement of ethanol-seeking behavior in Per1(Brdm1) mutant mice. *Psychopharmacology (Berl)* **190:**13-9.

Zheng B, Larkin DW, Albrecht U, Sun ZS, Sage M, Eichele G, Lee CC, Bradley A (1999) The mPer2 gene encodes a functional component of the mammalian circadian clock. *Nature.* **400:**169-73.

Zheng B, Albrecht U, Kaasik K, Sage M, Lu W, Vaishnav S, Li Q, Sun ZS, Eichele G, Bradley A, and Lee CC (2001) Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. *Cell* **105:**683-694.

Zylka MJ, Shearman LP, Weaver DR, and Reppert SM (1998) Three period homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. *Neuron* **20:**1103-10.

VITA

Name

Xiaoyu Qu

Address

Center for Research on Biological Clocks, Department of Biology

Texas A&M University, College Station, Texas 77843-3258

Education

B.S., Biology, Huazhong Agricultural University, Wuhan, China, 2002

Ph.D., Biology, Texas A&M University, College Station, TX, 2008

Publications

Qu X, Metz RP, Porter WW, Cassone VM, and Earnest DJ (2007) Disruption of clock gene expression alters responses of the aryl hydrocarbon receptor signaling pathway in the mouse mammary gland. *Mol Pharmacol*. **72:**1349-58

Metz RP, Qu X, Laffin B, Earnest D, and Porter WW (2006) Circadian clock and cell cycle gene expression in mouse mammary epithelial cells and in the developing mouse mammary gland. *Dev Dyn*. **235:**263-71

Allen GC, Qu X, and Earnest DJ (2005) TrkB-deficient mice show diminished phase shifts of the circadian activity rhythm in response to light. *Neurosci Lett*. **378:**150-5