

# A Role for the Clock Gene *Per1* in Prostate Cancer

Qi Cao,<sup>1,2</sup> Sigal Gery,<sup>1</sup> Azadeh Dashti,<sup>1</sup> Dong Yin,<sup>1</sup> Yan Zhou,<sup>2</sup> Jiang Gu,<sup>2,3</sup> and H. Phillip Koeffler<sup>1</sup>

<sup>1</sup>Cedars-Sinai Medical Center, Division of Hematology/Oncology, University of California at Los Angeles School of Medicine, Los Angeles, California; <sup>2</sup>Department of Pathology, School of Basic Medical Sciences, Peking University, Beijing, People's Republic of China; and <sup>3</sup>Shantou University Medical College, Shantou, People's Republic of China

## Abstract

**Circadian rhythms regulate diverse physiologic processes, including homeostatic functions of steroid hormones and their receptors. Perturbations of these rhythms are associated with pathogenic conditions, such as depression, diabetes, and cancer. Androgens play an important role in both normal development and carcinogenesis of the prostate. In the present study, we investigated a potential role for the core clock factor *Per1* in the pathogenesis of prostate cancer. Serum-shocked synchronized prostate cancer cells displayed disrupted circadian rhythms compared with the normal prostate tissue. Using Oncomine to perform a meta-analysis of microarray expression studies, we found that *Per1* is down-regulated in human prostate cancer samples compared with normal prostates. Reporter assays showed that *Per1* inhibited transactivation of the androgen receptor (AR) both in 293T cells overexpressing the AR and in the prostate cancer cell line LNCaP. Forced expression of *Per1* in LNCaP cells diminished the expression of known androgen-sensitive genes following stimulation with dihydrotestosterone. We showed that *Per1* physically interacted with AR; in addition, we found that *Per1* itself is regulated by androgens in prostate cancer cells. Overexpression of *Per1* in prostate cancer cells resulted in significant growth inhibition and apoptosis. Our results support the emerging role of circadian genes as key players in malignant transformation. Further elucidating the connections between clock genes and the AR pathway could benefit the development of new therapeutic strategies for prostate cancer as well as provide insights into chronotherapy as a way to optimize current therapies.** [Cancer Res 2009;69(19):7619–25]

## Introduction

Circadian rhythms reflect an internal timing system as exemplified by sleep/wake cycles. In mammals under the control of an intrinsic master clock, a multitude of physiologic processes and behavioral patterns are directed by circadian rhythms (1). The mammalian circadian system is composed of three components: the input pathways, the central pacemaker, and the output pathways. The input pathways transmit environmental signals to the central pacemaker, which coordinates the external signals with the central endogenous rhythm of the body (2). Of the environmental signals, light is the most powerful circadian

synchronizer. Light signals are captured by the retina and transmitted by the retinohypothalamic tract to the suprachiasmatic nucleus (3, 4). The suprachiasmatic nucleus, located in the anterior hypothalamus, acts as a master pacemaker generating neural and hormonal signals to peripheral clocks throughout the body (5, 6).

In the suprachiasmatic nucleus, circadian rhythms are generated by a set of core clock genes including period (*Per1-Per3*), casein kinase 1 $\epsilon$ , *Clock*, *Bmal1*, and *cryptochrome* (*Cry1-2*; ref. 2). *Clock* and *Bmal1*, two basic helix-loop-helix heterodimer transcriptional activators, are positive regulators that bind E-boxes in the promoter of various genes including *Per* and *Cry*. *Per* and *Cry* proteins form oligomers that move from the cytoplasm to the nucleus where they interfere with *Clock/Bmal1* activity, thereby forming the major negative circadian feedback loop. Further adding to the complexity, *Clock/Bmal1* heterodimers induce the expression of the nuclear orphan receptor Rev-erb $\alpha$ , resulting in the repression of the transcription of *Bmal1* through direct binding to a Rev-erb response element in the *Bmal1* promoter (7, 8). In addition, post-translational modification aids in regulating the circadian clock (9). Similar to the suprachiasmatic nucleus, the molecular clockwork in peripheral cells is composed of autoregulatory transcription-translation feedback loops orchestrated by the clock genes (10, 11). The peripheral oscillators, synchronized by the central clock, control the expression of downstream clock-controlled genes in a tissue-specific manner (12, 13).

Circadian rhythms influence many physiologic processes and pathologic conditions including cancer (14, 15). Epidemiologic studies have shown that disruption of normal circadian rhythm may increase the risk of developing various cancer types such as breast, prostate, colorectal, and endometrial cancers (16–21). Additionally, *Per1* and *Per2* have been reported to be deregulated in several human cancers (2). Furthermore, overexpression of either *Per1* or *Per2* in cancer cells inhibits their growth. Thus, *Per* genes may act as tumor suppressors.

Prostate cancer is the most frequently diagnosed and the second leading cause of cancer mortality in males in the United States (22). Androgens are necessary for male sexual differentiation and development as well as the maintenance of sexual organs in the adult. In addition, androgens contribute to the development and progression of age-associated pathologies including benign prostatic hyperplasia and prostate cancer. Androgen binds and activates the androgen receptor (AR), which acts as a transcription factor. Gene expression profiling studies show that ~1.5% to 4.3% of genes are either directly or indirectly regulated by androgens in prostate cancer cells (23). These AR-regulated genes have a notable effect on diverse cellular processes. The aim of this study was to investigate a possible association between the core clock gene, *Per1*, and the AR signaling pathway in prostate cancer.

## Materials and Methods

**Animals.** Male BALB/c mice (10 weeks old) were purchased from Vital River Laboratory Animal Technology. Mice were housed in a

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

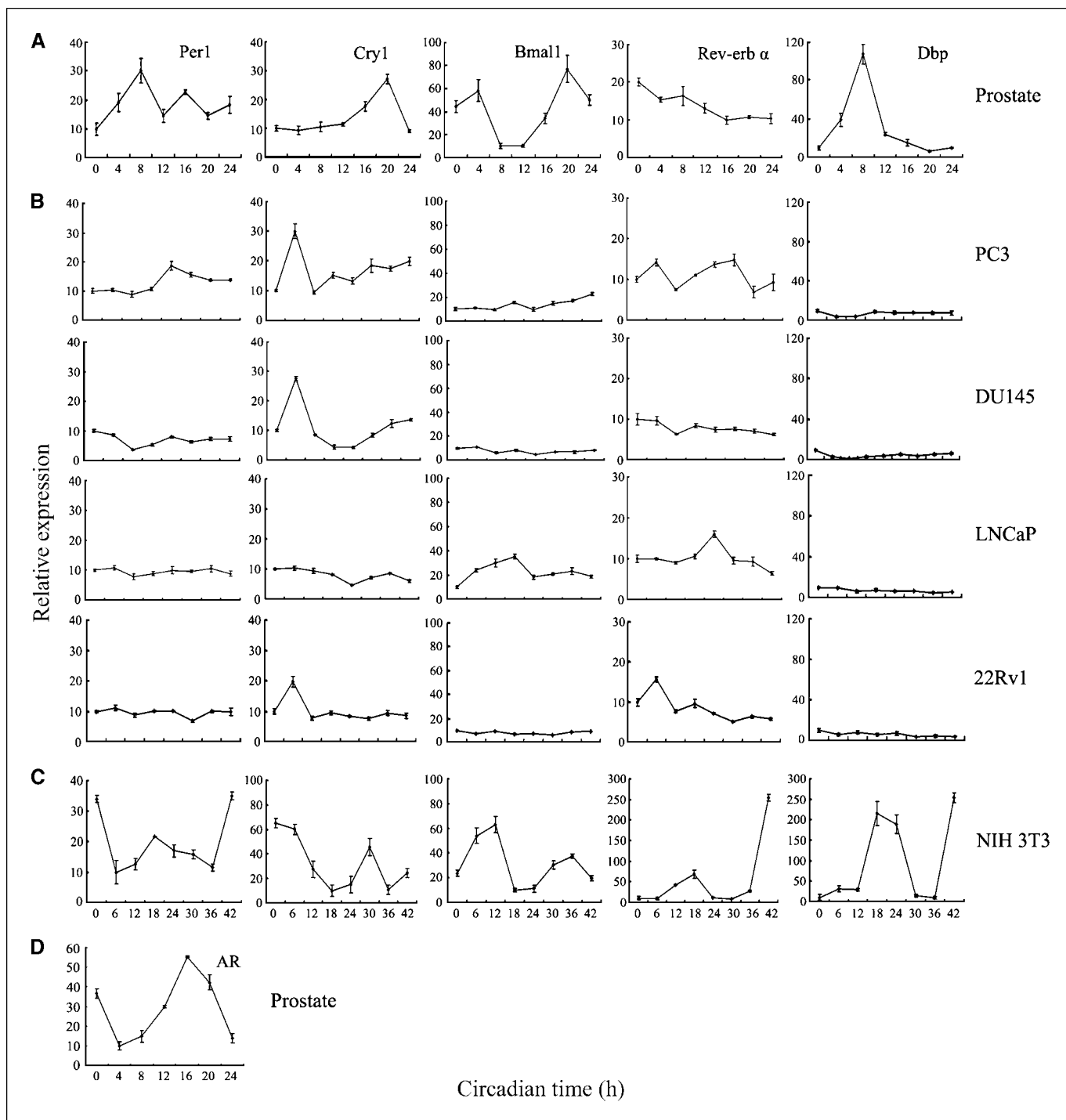
**Requests for reprints:** Sigal Gery, Cedars-Sinai Medical Center, Division of Hematology/Oncology, University of California at Los Angeles School of Medicine, 8700 Beverly Boulevard, Los Angeles, CA 90048. Phone: 310-423-4609; Fax: 310-423-0443; E-mail: Gerys@cshs.org; or Jiang Gu, Professor and Chair, Department of Pathology, Dean, Shantou University Medical College, Shantou, China 515041, E-mail: jianggudrive@gmail.com.

©2009 American Association for Cancer Research.  
doi:10.1158/0008-5472.CAN-08-4199

temperature-controlled room under a 12 h light/dark cycle, given free access to water, and fed *ad libitum* on a standard chow. All animal care and procedures were in accordance with the Institutional Animal Care and Use Committee guidelines. After being housed for 2 to 3 weeks, mice were sacrificed by cervical dislocation at 4 h intervals >24 h with one mouse at each time point for each group. These experiments were done three times. Prostate

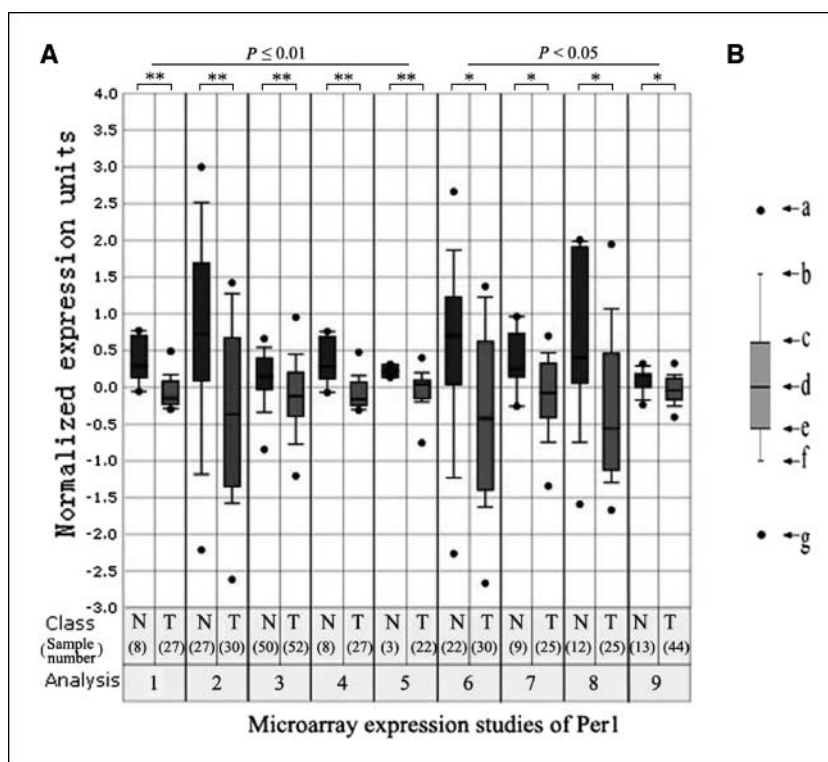
tissue was dissected, rapidly frozen in liquid nitrogen, homogenized in Trizol reagent (Invitrogen), and stored at  $-80^{\circ}\text{C}$  until RNA extraction. Total RNA for real-time PCR was extracted according to the manufacturer's protocol (Invitrogen).

**Reagents.** Dihydrotestosterone (DHT) was from Sigma-Aldrich. Per1 (N-20), AR (N-20), prostate-specific antigen (PSA; C-19), myc (9E10), and



**Figure 1.** Expression of clock genes in prostate tissue and prostate cancer cell lines. **A**, mice were entrained to 12 h light/dark cycles. Prostate tissue was collected at the indicated circadian times. Light was turned on and off at Zeitgeber 0 and 12, respectively. Levels of the indicated genes were quantified by real-time PCR. **B** and **C**, PC3, DU145, LNCaP, and 22Rv1 prostate cancer cells and NIH 3T3 normal fibroblasts were synchronized by serum shock. Samples were collected every 6 h for a total of 42 h. Expression of clock genes and *Dbp* were quantified by real-time PCR. Mean  $\pm$  SD of three measurements. **D**, mRNA levels of *AR* expression in prostate tissue at the indicated circadian times were quantified by real-time PCR. Mean  $\pm$  SD of triplicate samples.

**Figure 2.** Down-regulation of *Per1* in prostate tumors (*T*) compared with normal prostate tissue (*N*). *A*, data analysis was done with the Oncomine 3.0 database (24) using standard settings (Supplementary Table S1). *B*, explanation of box plot. *Dots*, maximum and minimum values (*a* and *g*, respectively); *whiskers*, 90th and 10th percentile values (*b* and *f*, respectively); and *horizontal lines*, 75th, 50th, and 25th percentile values (*c*, *d*, and *e*, respectively).



poly(ADP-ribose) polymerase (H-250) antibodies were from Santa Cruz Biotechnology.  $\beta$ -Actin antibody was from Sigma-Aldrich. Control IgG (normal rabbit IgG) was purchased from Santa Cruz Biotechnology.

**Oncomine.** Oncomine is a Web-based data-mining platform<sup>4</sup> (24). We compared *Per1* expression in prostate cancers and normal prostate tissues with  $P < 0.05$  in nine studies (Supplementary Table S1).

**Cell culture and transfection.** LNCaP and 22Rv1 human prostate cancer cells were grown in RPMI 1640 (Invitrogen) with 10% fetal bovine serum and 100 units/mL penicillin/streptomycin. 293T human embryonic kidney cell line as well as PC3 and DU145 human prostate cancer cell lines were cultured in DMEM (Invitrogen) with 10% fetal bovine serum and 100 units/mL penicillin/streptomycin. Transient transfection assays of the 293T cells were done using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For transfection of LNCaP, 22Rv1, and DU145 cells, TransIT-Prostate Transfection Kit (Mirus) was used following the manufacturer's protocol. PC3 cells were transfected using Nucleofector Technology (Amaxa Biosystems) with the Cell Line Nucleofector Kit-V according to the manufacturer's instructions.

**Plasmids and luciferase reporter assay.** myc-tagged mPer1 was a generous gift of Dr. David M. Virshup as described (25). ARE4-E4Luc, which has the multimerized four consensus androgen response elements (ARE) from the *PSA* promoter cloned upstream of the luciferase gene in the pGL3 vector (Promega), and *PSA* P/E-Luc, which has a 564-bp fragment of the *PSA* promoter with a 2.4-kb enhancer sequence (-5,322 to -2,925) cloned upstream of luciferase, were described previously (26). *mPer1*-7.2k luciferase was a kind gift of Dr. Hitoshi Okamura as described (27). Luciferase activity was measured with Dual-Luciferase Reporter 1000 Assay System (Promega) and was normalized by *Renilla* values. All transfection experiments were carried out in triplicate wells and repeated separately at least three times.

**Serum shock.** Serum shock of prostate cancer cells was done as described previously (28). PC3, DU145, LNCaP, 22Rv1, and NIH 3T3 cells were grown to 100% confluency. At time point 0 (unstimulated cells), cells were incubated with 50% horse serum (Life Technologies) in culture

medium and replaced with serum-free culture medium after 2 h of incubation. The cells were harvested at the indicated times and subjected to quantitative real-time reverse transcription-PCR analysis.

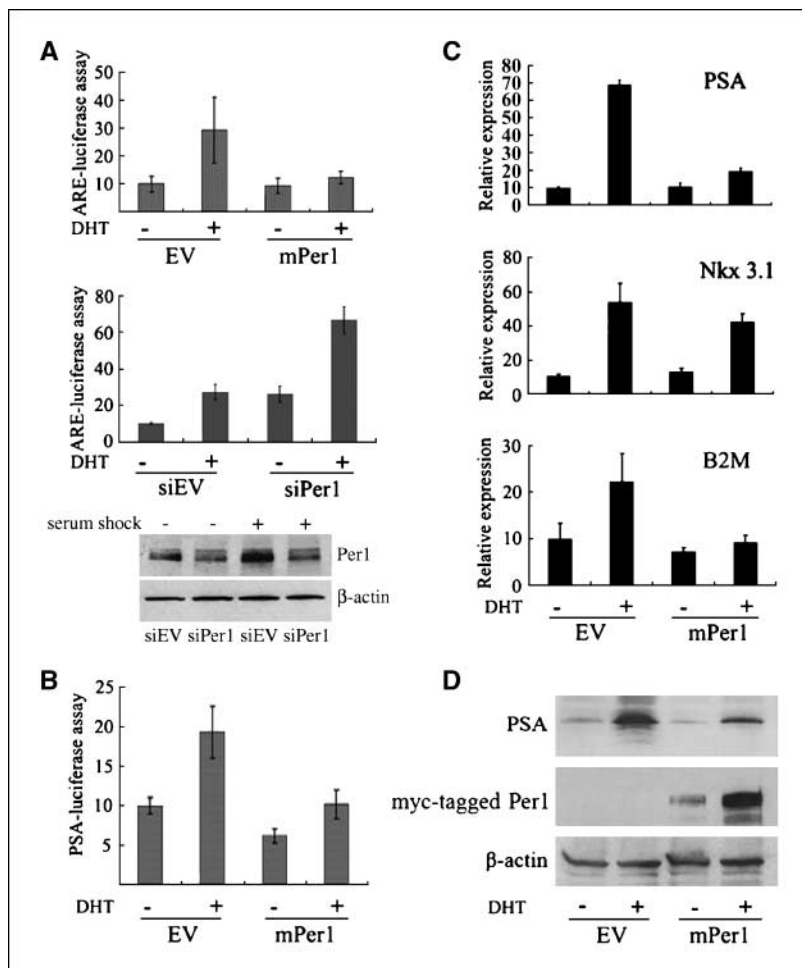
**RNA interference.** Small interfering RNA (siRNA) directed against *Per1* (5'-CGCUCGCCUGGCCAAUAAdTdT-3' and 5'-UUAUUGGCCAGGGC-GAGCGdGdG-3'; synthesized by Qiagen; ref. 29) and nonspecific control siRNA (Qiagen) were transfected into 293T using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

**Reverse transcription and quantitative real-time PCR.** Total RNA from cultured cells was extracted using Trizol reagent (Invitrogen). Two micrograms of RNA were processed directly to cDNA by reverse transcription with SuperScript III following the manufacturer's instructions (Invitrogen). PCR primers for each gene were designed using Real-time PCR Primer Design<sup>5</sup>; sequences used in this study were shown in Supplementary Table S2. We used SYBR Premix Ex Taq (Perfect Real-time; Takara Bio) for *PSA*, *Nkx 3.1*, *B2M*, *Cry1*, *Bmal1*, *Rev-erba*, *Dbp*, and *AR* quantitative real-time PCR with Applied Biosystems 7500 Real-time PCR System (Applied Biosystems) according to the manufacturer's instructions. The specificity of PCR products was checked on agarose gel. TaqMan probes were used in quantitative real-time PCR for *Per1* and *18S rRNA* as follows: *18S rRNA* probe 5'-AGCAGGCGCGCAAATTACCC-3' and *Per1* probe 5'-TCTA-CATTCGGAGCAGGCAGCCG-3'. These were purchased from Applied Biosystems and labeled with the reporter dye FAM in the 5' end and the quencher dye BHQ in the 3' end. Expression levels of *18S rRNA* were used as an endogenous reference.

**Western blotting and immunoprecipitation.** Cell lysates were prepared using the lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.5% NP-40]. For Western blotting, total cellular protein was separated on 4% to 15% Ready-to-Use SDS-PAGE gel (Bio-Rad) and transferred to nitrocellulose membranes, which were then incubated with the primary antibodies. After incubation with appropriate secondary antibodies, the immunoblots were developed using SuperSignal Western blotting kits (Pierce Biotechnology) and exposed to X-ray film according to the

<sup>4</sup> www.oncomine.org

<sup>5</sup> https://www.genscript.com/ssl-bin/app/primer



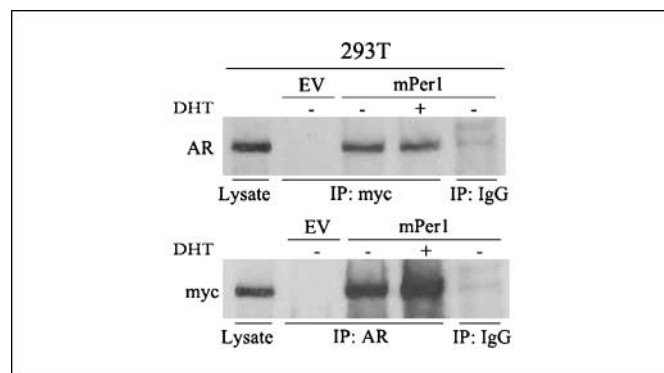
**Figure 3.** Per1 suppresses the transcriptional activity of AR. *A*, top, 293T cells were cotransfected with AR expression vector, ARE-luciferase reporter, and either empty vector (EV) or Per1 (*mPer1*) expression vector. Luciferase activity was measured either with or without exposure of cells to DHT (10 nmol/L, 24 h). *Middle*, 293T cells were cotransfected with ARE-luciferase construct and either with control siRNA (*siEV*) or Per1 siRNA (*siPer1*). Effectiveness of Per1 siRNA is shown by Western blot (*bottom*). *B*, LNCaP cells were cotransfected with PSA-luciferase construct and either empty vector or mPer1. *A* and *B*, luciferase activity was assayed with either DHT (10 nmol/L, 24 h) or diluent control. Results represent the fold increase of luciferase activity compared with untreated control cells. Mean  $\pm$  SD of triplicate samples. *C* and *D*, LNCaP cells were transfected with either empty vector or mPer1 and either untreated or treated with DHT. *C*, real-time PCR analysis. Mean  $\pm$  SD of triplicate samples. *D*, Western blot analysis.

manufacturer's protocol. Western blots were stripped between hybridizations with stripping buffer [10 mmol/L Tris-HCl (pH 2.3), 150 mmol/L NaCl]. For immunoprecipitation, 500  $\mu$ g total cell lysates were immunoprecipitated with 3  $\mu$ g of indicated antibodies at 4°C overnight. Immunocomplexes were captured with 20  $\mu$ L protein A/G gel slurry (Santa Cruz Biotechnology), and the samples were allowed to mix at 4°C for 2 h. The beads were washed three times.

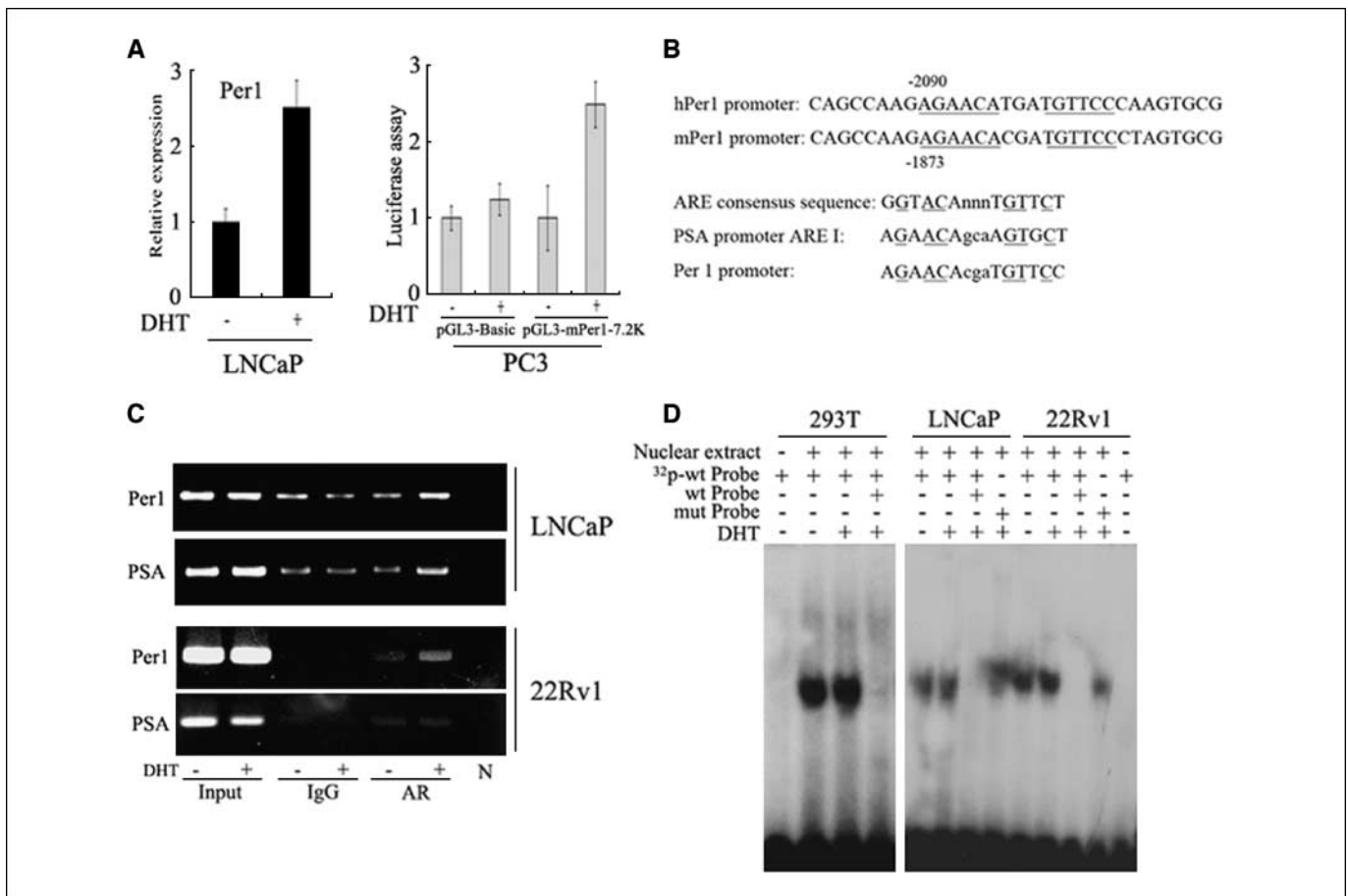
**Nuclear extracts and electrophoretic mobility shift analyses.** LNCaP cells were kept in RPMI 1640 without serum for 24 h and then treated either with or without DHT (10 nmol/L) for another 24 h. Nucleoplasmic proteins were extracted using CellLyte NuCLEAR Extraction Kit (Sigma-Aldrich). The nuclear extract (10  $\mu$ g) was incubated at a final volume of 20  $\mu$ L containing DNA-binding buffer [10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 20% (v/v) glycerol] and 1.5  $\mu$ g poly(deoxyinosinic-deoxycytidic acid) (Roche) for 10 min at room temperature and then incubated for 20 min at room temperature (30) with double-stranded <sup>32</sup>P-labeled (specific activity of ~300,000 cpm) *Per1*-ARE oligonucleotide (5'-CAAGAGAACATGATGTTCC-CAAG-3') and *Per1*-ARE mutant oligonucleotide (5'-CAAGAAAAAAT-GATGTTCCCAAG-3'). In cold competition experiments, unlabeled competitor *Per1*-ARE oligonucleotides ( $\times 100$ ) were incubated with nuclear extracts for 15 min on ice before the addition of the <sup>32</sup>P-labeled probe.

**Chromatin immunoprecipitation.** LNCaP or 22Rv1 cells were kept in RPMI 1640 without serum for 24 h, treated with 10 nmol/L DHT for 3 h, paraformaldehyde cross-linked, and sonicated. Chromatin immunoprecipitation assays were done using EZChIP kit according to the manufacturer (Millipore). The following *Per1* promoter-specific primers were used: 5'-GCAGATGGGAGTCTGAAAA-3' and 5'-GAGGCTGGAGAGACTGGAGA-3'. Primers for the *PSA* gene region ARE III were used as positive control (31).

**Analysis of apoptosis and cell death.** Cells were stained with propidium iodide and Annexin V-FITC (BD Biosciences). Briefly, after overexpression of either Per1 or empty vector, cells were selected in G418 (800  $\mu$ g/mL for LNCaP and 500  $\mu$ g/mL for PC3 and DU145) for >1 week, resuspended in PBS, and incubated with propidium iodide and Annexin V-FITC according to the manufacturer's instructions. Cells were analyzed by a Becton Dickinson FACScan flow cytometer using CellQuest software (Becton Dickinson).



**Figure 4.** Per1 interacts with AR. 293T cells were cotransfected with AR and either empty vector or myc-tagged mPer1 (*top*) or the cells were cotransfected with myc-tagged mPer1 and either empty vector or AR (*bottom*). Cells were either untreated or treated with DHT (10 nmol/L, 4 h). Lysates were immunoprecipitated (IP) and probed with the indicated antibodies. Cell lysates loaded directly were used to control for molecular size. Immunoprecipitation with IgG was used as negative control.



**Figure 5.** Per1 is regulated by AR. *A, left*, real-time PCR analysis of *Per1* expression. Mean  $\pm$  SD of triplicate samples. *Right*, reporter assay with *Per1*-luciferase construct. Cells were either untreated or treated with DHT (10 nmol/L, 24 h). Mean  $\pm$  SD of triplicate samples. *B, top*, sequence alignment of the murine and human *Per1* promoters. Potential ARE is underlined. *Bottom*, ARE I in the PSA promoter and the putative ARE in the *Per1* promoter are compared. *C*, chromatin immunoprecipitation assays. LNCaP and 22Rv1 cells were either untreated or treated with DHT (10 nmol/L, 3 h). *D*, electrophoretic mobility shift analysis. 293T cells transfected with AR and LNCaP and 22Rv1 cells were either untreated or treated with DHT (10 nmol/L, 24 h). Nuclear proteins were incubated with either wild-type or mutant labeled probes of the putative ARE region from *Per1* promoter. Cold competition assay was done using  $\times 100$  unlabeled wild-type probe.

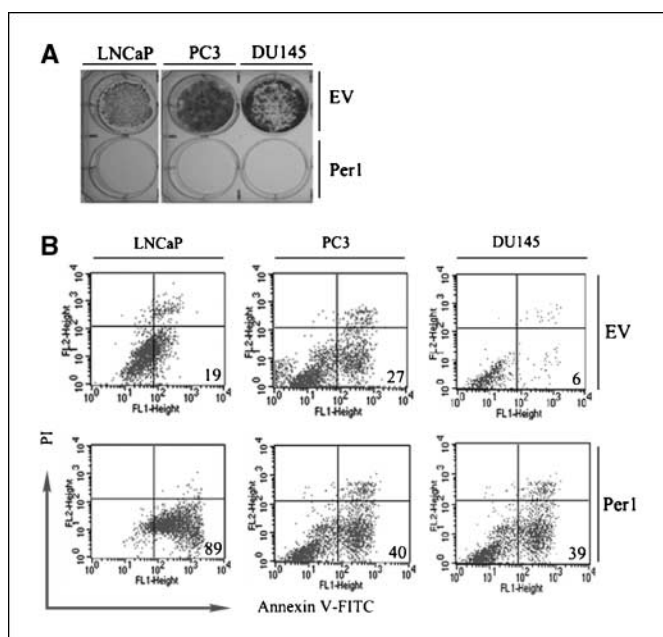
## Results

**Rhythmic expression of clock genes and AR in normal prostate.** To begin to understand the relationship between circadian rhythm in normal and cancer prostate cells, we surveyed the expression of major oscillator genes, *Per1*, *Cry1*, *Bmal1*, and *Rev-erb $\alpha$* , in normal prostate tissues by quantitative real-time PCR (Fig. 1A). Rhythmic expression of *Per1*, *Cry1*, *Bmal1*, and *Rev-erb $\alpha$*  as well as *Dbp* [a PAR leucine zipper transcription factor known to be a clock-controlled gene (32)] was noted. This result is in agreement with a recent study showing that core clock genes, *Per1*, *Per2*, and *Bmal1*, are rhythmically expressed in mouse prostate (33).

Rhythmic expression of core circadian genes can be induced in cultured cells by exposure to high concentration of serum (28, 29). To see whether core clock genes are rhythmically expressed in human prostate cancer cell lines, we examined their expression in PC3, DU145, LNCaP, and 22Rv1 cells (Fig. 1B). Quantitative real-time PCR showed that the pattern of the clock gene expression was disrupted in prostate cancer cell lines (Fig. 1B). Serum-shocked NIH 3T3 cells were used as positive control and, as expected (34), showed rhythmic expression of the clock genes (Fig. 1C). Additionally, we found that AR mRNA levels oscillate in prostate tissue of mice entrained to 12 h light/dark cycles (Fig. 1D).

**Down-regulation of *Per1* in prostate cancer tissue.** We performed *in silico* analysis of *Per1* expression in human normal prostate, benign prostatic hyperplasia, prostate carcinoma, and metastatic prostate cancer using microarray expression studies published in Oncomine (24). *Per1* expression was significantly lower in prostate cancer compared with normal prostate in either five ( $P < 0.01$ ) or nine ( $P < 0.05$ ) microarray expression studies (Fig. 2A; Supplementary Table S1). Similar analysis of *Per2* showed that *Per2* was also significantly down-regulated in either four ( $P < 0.01$ ) or six ( $P < 0.05$ ) microarray expression studies (data not shown).

**Per1 inhibits the transcriptional activity of AR.** To examine whether Per1 affects the AR transcriptional activity, we performed reporter assays. 293T cells were cotransfected with promoter-reporter vector containing ARE (ARE4-E4Luc), AR expression vector, and either the Per1 or empty vector and treated with DHT. Results showed that Per1 inhibited DHT-induced luciferase activity (Fig. 3A, top). Similarly, Per1 reduced luciferase activity in LNCaP cells (data not shown). In contrast, silencing of Per1 by siRNA increased the reporter activity  $\sim 3$ -fold in cultures either with or without DHT treatment (Fig. 3A, middle). Silencing of Per1 was confirmed by Western blotting (Fig. 3A, bottom). Per1 also decreased AR-mediated stimulation of the reporter vector PSA P/E-Luc in LNCaP cells (Fig. 3B).



**Figure 6.** Per1 inhibits growth and induces apoptosis in prostate cancer cell lines. LNCaP, PC3, and DU145 cells were transfected with either empty vector or Per1 expression vector followed by (A) colony formation assay and (B) Annexin V/propidium iodide staining. The percentage of early apoptotic cells is shown. Representative of three independent experiments.

Using the AR-positive prostate cancer cell line, LNCaP, we measured the affect of Per1 on endogenous expression of known AR target genes, *PSA*, *Nkx 3.1*, and *B2M*. LNCaP cells were transfected with either Per1 or control vector and cultured with or without DHT. Quantitative real-time PCR analysis showed that whereas expression of Per1 alone had little effect on these genes, it strongly inhibited DHT-mediated induction of *PSA* and *B2M* (3.5- to 2-fold, respectively) and modestly depressed *Nkx 3.1* (1.2- fold) expression compared with control cells (Fig. 3C). Per1 had a parallel effect on the protein level of PSA (Fig. 3D).

**Per1 interacts with AR.** To determine whether Per1 interacts with AR, 293T cells were cotransfected with myc-tagged mPer1 and either AR or empty vector. Immunoprecipitation experiments showed that Per1 is associated with AR (Fig. 4) and that DHT treatment does not affect the interaction.

**Per1 is induced by activated AR.** We hypothesized that activated AR may stimulate transcription of *Per1* as a feedback pathway. Indeed, *Per1* mRNA levels increased in LNCaP cells stimulated with DHT (Fig. 5A, left), showing that the human *Per1* gene is DHT-inducible in prostate epithelial cells. Similarly, reporter assays using a *mPer1*-luciferase vector in PC3 cells showed that activated AR induces *Per1* expression (Fig. 5A, right). Sequence analysis showed that the *Per1* promoter has a potential ARE binding site at -2,090 bp from the start site of *Per1* transcription and that these and the surrounding nucleotides are conserved between the human and mouse, suggesting their functional significance (Fig. 5B). The highest percentage of homology to the ARE consensus sequence is the sequence AGAACAAtgaTGTTC. Compared with the ARE consensus GGTA-ACAnnnTGTCT (35), these sequences are identical in six of the most essential positions (positions 2, 3, and 5 in each half-site, italicized). Chromatin immunoprecipitation assays using LNCaP and 22Rv1 cells detected the presence of AR at the same promoter

region of *Per1* following DHT treatment (Fig. 5C), showing that endogenous AR binds to the *Per1* promoter. Furthermore, electrophoretic mobility shift analyses showed protein-DNA binding between the ARE from the *Per1* promoter and nuclear extracts from 293T overexpressing AR as well as nuclear lysates from LNCaP and 22Rv1 prostate cancer cells (Fig. 5D). Specificity of binding was confirmed by competition with excess unlabeled ARE oligonucleotides, which successfully competed for the binding, whereas a mutated *Per1*-ARE probe was not an effective competitor (Fig. 5D).

**Per1 inhibits growth of prostate cancer cells.** To evaluate the effect of Per1 on proliferation of prostate cancer cells, we transfected PC3, DU145, and LNCaP cells with either Per1-Neo expression vector or Neo control vector and selected the cells with G418. Per1 induced profound growth inhibition in all three cell lines (Fig. 6A). In addition, apoptosis was measured by Annexin V/propidium iodide staining. Compared with vector controls, Per1-transfected LNCaP, PC3, and DU145 cells had 4.6-, 1.5-, and 7.0-fold increased level of apoptosis, respectively (Fig. 6B).

## Discussion

Despite tremendous efforts to improve our understanding of prostate cancer, its etiology remains largely unknown and it continues to be a major health problem. Older age, family history of the disease, and race are well-established risk factors for the disease. Recent epidemiologic data show that rotating-shift workers have a higher risk of prostate cancer compared with only day-shift or only night-shift workers (18, 19).

Oscillation of clock genes was found in various tissues including liver, skeletal muscle, and white and brown adipose tissues (36). Also, mammalian fibroblasts *in vitro* have functional clocks after their synchronization by either serum shock (37) or other stimuli. On the other hand, deregulated circadian rhythm has been associated with accelerated growth of malignant tumors (38). We found a clear circadian expression of the core clock genes, *Per1*, *Cry1*, *Bmal1*, and *Rev-erb $\alpha$* , in mouse prostate tissue. This result is in agreement with a recent report showing a similar expression profile of *Per1* and *Bmal1* in the prostate (33) as well as previously circadian patterns observed in the liver and muscle (36). In contrast, the rhythmic expression of these core clock genes was disrupted in prostate cancer cell lines. Interestingly, genetic variants of clock genes have been associated with risk of development of prostate cancer in a population-based study (39).

The suprachiasmatic nucleus synchronizes the peripheral oscillators by producing diffusible neural and hormonal molecules as well as directly targeting other regions of the brain, allowing animals to adapt their feeding, activity, and metabolism to predictable daily changes in the environment (2). Studies show circadian rhythms in lipid and glucose metabolism coupled with rhythmic expression of several nuclear receptors in metabolically active tissues (36). A testosterone circadian rhythm has been reported, which is usually absent in elderly men (40). Recently, it was shown that the mRNA levels of 28 of 49 murine nuclear receptors oscillate in metabolic tissues (36). In those metabolic tissues examined, AR did not show circadian expression. Remarkably, we found that *AR* mRNA levels oscillate in prostate tissue, suggesting that AR might link peripheral clocks to systemic hormonal regulation.

Using the Oncomine database, we found several studies in which *Per1* levels were significantly down-regulated in prostate cancer samples compared with normal prostate tissue. Down-regulation of *Per1* and other clock genes was previously reported in a variety of cancers including acute myeloid leukemia (41) as well as breast (42), lung (43), endometrial (44), and pancreatic (45) cancers. We showed that forced expression of *Per1* in prostate cancer cells inhibited AR transcriptional activity, including DHT stimulation of PSA. Conversely, silencing of *Per1* expression using siRNA was associated with increased transcriptional activity of AR. Also, *Per1* physically bound to AR. Thus, *Per1* appears to act as a negative regulator of AR in prostate cancer cells. In addition, we showed that activated AR increased the transcription of *Per1* associated with the recruitment of AR to the ARE of the *Per1* promoter.

Taken together, our results suggest that activated AR stimulates *Per1*, which in turn attenuates AR activity, thereby helping to maintain hormonal homeostasis. Furthermore, our data support

the hypothesis that disruption of circadian function may contribute to prostate tumorigenesis. Further elucidating the circadian-AR connections could pave the way for the development of novel therapeutic approaches.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

Received 11/6/08; revised 6/15/09; accepted 7/29/09; published OnlineFirst 9/14/09.

**Grant support:** NIH grant, H. and C. Koeffler funds, Inger Foundation, and China Scholarship Council.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. David M. Virshup for the myc-tagged mPer1 vector, Dr. Hitoshi Okamura for the mPer1-7.2k luciferase reporter, and Chunzhang Yang for help with the real-time PCR analysis.

## References

- Reppert SM, Weaver DR. Coordination of circadian timing in mammals. *Nature* 2002;418:935–41.
- Fu L, Lee CC. The circadian clock: pacemaker and tumour suppressor. *Nat Rev Cancer* 2003;3:350–61.
- Gooley JJ, Lu J, Chou TC, Scammell TE, Saper CB. Melanopsin in cells of origin of the retinohypothalamic tract. *Nat Neurosci* 2001;4:1165.
- Ruby NF, Brennan TJ, Xie X, et al. Role of melanopsin in circadian responses to light. *Science* 2002;298:2211–3.
- Liu C, Weaver DR, Strogatz SH, Reppert SM. Cellular construction of a circadian clock: period determination in the suprachiasmatic nuclei. *Cell* 1997;91:855–60.
- Yamazaki S, Numano R, Abe M, et al. Resetting central and peripheral circadian oscillators in transgenic rats. *Science* 2000;288:682–5.
- Albrecht U, Eichele G. The mammalian circadian clock. *Curr Opin Genet Dev* 2003;13:271–7.
- Brown SA, Ripperger J, Kadener S, et al. PERIOD1-associated proteins modulate the negative limb of the mammalian circadian oscillator. *Science* 2005;308:693–6.
- Gallego M, Virshup DM. Post-translational modifications regulate the ticking of the circadian clock. *Nat Rev Mol Cell Biol* 2007;8:139–48.
- Dunlap JC. Molecular bases for circadian clocks. *Cell* 1999;96:271–90.
- Shearman LP, Sriram S, Weaver DR, et al. Interacting molecular loops in the mammalian circadian clock. *Science* 2000;288:1013–9.
- Panda S, Antoch MP, Miller BH, et al. Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* 2002;109:307–20.
- Miller BH, McDearmon EL, Panda S, et al. Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation. *Proc Natl Acad Sci U S A* 2007;104:3342–7.
- Matsuo T, Yamaguchi S, Mitsui S, Emi A, Shimoda F, Okamura H. Control mechanism of the circadian clock for timing of cell division *in vivo*. *Science* 2003;302:255–9.
- Fu L, Pelicano H, Liu J, Huang P, Lee C. The circadian gene *Period2* plays an important role in tumor suppression and DNA damage response *in vivo*. *Cell* 2002;111:41–50.
- Hansen J. Increased breast cancer risk among women who work predominantly at night. *Epidemiology* 2001;12:74–7.
- Davis S, Mirick DK, Stevens RG. Night shift work, light at night, and risk of breast cancer. *J Natl Cancer Inst* 2001;93:1557–62.
- Kubo T, Ozasa K, Mikami K, et al. Prospective cohort study of the risk of prostate cancer among rotating-shift workers: findings from the Japan Collaborative Cohort study. *Am J Epidemiol* 2006;164:549–55.
- Conlon M, Lightfoot N, Kreiger N. Rotating shift work and risk of prostate cancer. *Epidemiology* 2007;18:182–3.
- Schernhammer ES, Laden F, Speizer FE, et al. Night-shift work and risk of colorectal cancer in 'the Nurses' Health Study. *J Natl Cancer Inst* 2003;95:825–8.
- Viswanathan AN, Hankinson SE, Schernhammer ES. Night shift work and the risk of endometrial cancer. *Cancer Res* 2007;67:10618–22.
- Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. *CA Cancer J Clin* 2009;59:225–49.
- Dehm SM, Tindall DJ. Molecular regulation of androgen action in prostate cancer. *J Cell Biochem* 2006;99:333–44.
- Rhodes DR, Yu J, Shanker K, et al. ONCOMINE: a cancer microarray database and integrated data-mining platform. *Neoplasia* 2004;6:1–6.
- Vielhaber E, Eide E, Rivers A, Gao ZH, Virshup DM. Nuclear entry of the circadian regulator mPER1 is controlled by mammalian casein kinase I $\epsilon$ . *Mol Cell Biol* 2000;20:4888–99.
- Hisatake JI, Ikezoe T, Carey M, Holden S, Tomoyasu S, Koeffler HP. Down-regulation of prostate-specific antigen expression by ligands for peroxisome proliferator-activated receptor  $\gamma$  in human prostate cancer. *Cancer Res* 2000;60:5494–8.
- Yamaguchi S, Mitsui S, Miyake S, et al. The 5' upstream region of mPer1 gene contains two promoters and is responsible for circadian oscillation. *Curr Biol* 2000;10:873–6.
- Balsalobre A, Damiola F, Schibler U. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* 1998;93:929–37.
- Cai W, Rambaud J, Teboul M, et al. Expression levels of estrogen receptor beta are modulated by components of the molecular clock. *Mol Cell Biol* 2008;28:784–93.
- Zoubeidi A, Zardan A, Beraldi E, et al. Cooperative interactions between androgen receptor (AR) and heat-shock protein 27 facilitate AR transcriptional activity. *Cancer Res* 2007;67:10455–65.
- Shang Y, Myers M, Brown M. Formation of the androgen receptor transcription complex. *Mol Cell* 2002;9:601–10.
- Lopez-Molina L, Conquet F, Dubois-Dauphin M, Schibler U. The DBP gene is expressed according to a circadian rhythm in the suprachiasmatic nucleus and influences circadian behavior. *EMBO J* 1997;16:6762–71.
- Bebas P, Goodall CP, Majewska M, Neumann A, Giebultowicz JM, Chappell PE. Circadian clock and output genes are rhythmically expressed in extrastriatal ducts and accessory organs of mice. *FASEB J* 2009;23:523–33.
- Marquez S, Crespo P, Carlini V, et al. The metabolism of phospholipids oscillates rhythmically in cultures of fibroblasts and is regulated by the clock protein PERIOD1. *FASEB J* 2004;18:519–21.
- Cleutjens KB, van Eekelen CC, van der Korput HA, Brinkmann AO, Trapman J. Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. *J Biol Chem* 1996;271:6379–88.
- Yang X, Downes M, Yu RT, et al. Nuclear receptor expression links the circadian clock to metabolism. *Cell* 2006;126:801–10.
- Nagoshi E, Saini C, Bauer C, Laroche T, Naef F, Schibler U. Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillators pass time to daughter cells. *Cell* 2004;119:693–705.
- Filipski E, King VM, Li X, et al. Host circadian clock as a control point in tumor progression. *J Natl Cancer Inst* 2002;94:690–7.
- Chu LW, Zhu Y, Yu K, et al. Variants in circadian genes and prostate cancer risk: a population-based study in China. *Prostate Cancer Prostatic Dis* 2008;11:342–8.
- Seidman SN. Androgens and the aging male. *Psychopharmacol Bull* 2007;40:205–18.
- Gery S, Gombart AF, Yi WS, Koeffler C, Hofmann WK, Koeffler HP. Transcription profiling of C/EBP targets identifies *Per2* as a gene implicated in myeloid leukemia. *Blood* 2005;106:2827–36.
- Chen ST, Choo KB, Hou MF, Yeh KT, Kuo SJ, Chang JG. Deregulated expression of the *PER1*, *PER2* and *PER3* genes in breast cancers. *Carcinogenesis* 2005;26:1241–6.
- Gery S, Komatsu N, Kawamata N, et al. Epigenetic silencing of the candidate tumor suppressor gene *Per1* in non-small cell lung cancer. *Clin Cancer Res* 2007;13:1399–404.
- Yeh KT, Yang MY, Liu TC, et al. Abnormal expression of period 1 (*PER1*) in endometrial carcinoma. *J Pathol* 2005;206:111–20.
- Pogue-Geile KL, Lyons-Weiler J, Whitcomb DC. Molecular overlap of fly circadian rhythms and human pancreatic cancer. *Cancer Lett* 2006;243:55–7.

# Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

## A Role for the Clock Gene *Per1* in Prostate Cancer

Qi Cao, Sigal Gery, Azadeh Dashti, et al.

*Cancer Res* 2009;69:7619-7625. Published OnlineFirst September 14, 2009.

<b>Updated version</b>	Access the most recent version of this article at: doi: <a href="https://doi.org/10.1158/0008-5472.CAN-08-4199">10.1158/0008-5472.CAN-08-4199</a>
<b>Supplementary Material</b>	Access the most recent supplemental material at: <a href="http://cancerres.aacrjournals.org/content/suppl/2009/09/10/0008-5472.CAN-08-4199.DC1">http://cancerres.aacrjournals.org/content/suppl/2009/09/10/0008-5472.CAN-08-4199.DC1</a>

<b>Cited articles</b>	This article cites 44 articles, 15 of which you can access for free at: <a href="http://cancerres.aacrjournals.org/content/69/19/7619.full#ref-list-1">http://cancerres.aacrjournals.org/content/69/19/7619.full#ref-list-1</a>
-----------------------	--

<b>Citing articles</b>	This article has been cited by 8 HighWire-hosted articles. Access the articles at: <a href="http://cancerres.aacrjournals.org/content/69/19/7619.full#related-urls">http://cancerres.aacrjournals.org/content/69/19/7619.full#related-urls</a>
------------------------	---

<b>E-mail alerts</b>	<a href="#">Sign up to receive free email-alerts</a> related to this article or journal.
----------------------	--

<b>Reprints and Subscriptions</b>	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a> .
-----------------------------------	--

<b>Permissions</b>	To request permission to re-use all or part of this article, use this link <a href="http://cancerres.aacrjournals.org/content/69/19/7619">http://cancerres.aacrjournals.org/content/69/19/7619</a> . Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.
--------------------	--