A Role for the Clock Gene Per1 in Prostate Cancer

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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 downregulated 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 1α, 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-erba, resulting in the repression of the transcription of Bmal1 through direct binding to a Rev-erba 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/).

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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°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...
poly(ADP-ribose) polymerase (H-250) antibodies were from Santa Cruz Biotechnology. β-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 (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 instructions. For transfection of LNCaP, 22Rv1, and NIH 3T3 cells were done using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**Plasmids and luciferase reporter assay.** 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 364-bp promoter sequence (−5,322 to −2,925) cloned upstream of luciferase, were used previously (26). mPer1-2.2k 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 luciferase. 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) for 4 h. 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′-CGCGUCGCCGCGCCAAUAATGTT-3′ and 5′-UAAGUUGCCAGGGAGAGCGG-3′; synthesized by Qagen; ref. 29) and nonspecific control siRNA (Qagen) 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; 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′-AGGAGACCCGCAAAATTACCCTG-3′ and Per1 probe 5′-CTTCAACCTTTTCGGAGCAGGACCGC-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 manufacturer’s instructions.

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).

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1. [www.oncomine.org](http://www.oncomine.org)
2. [https://www.genscript.com/ssl-bin/app/primer](https://www.genscript.com/ssl-bin/app/primer)
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 μg total cell lysates were immunoprecipitated with 3 μg of indicated antibodies at 4°C overnight. Immunocomplexes were captured with 20 μL protein A/G gel slurry (Santa Cruz Biotechnology), and the samples were allowed to mix at 4°C for 2h. 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. Nucleoplastic proteins were extracted using CelLytic NuCLEAR Extraction Kit (Sigma-Aldrich). The nuclear extract (10 μg) was incubated at a final volume of 20 μL 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 μg poly(deoxyinosinic-deoxycytidic acid) (Roche) for 10 min at room temperature and then incubated for 20 min at room temperature (30) with double-stranded 32P-labeled (specific activity of ~300,000 cpm) Per1-ARE oligonucleotide (5'-CAAGAGAACATGATGTTCCCAAG-3') and Per1-ARE mutant oligonucleotide (5'-CAAGAAAAAATGATGTTCCCAAG-3'). In cold competition experiments, unlabeled competitor Per1-ARE oligonucleotides (>100) were incubated with nuclear extracts for 15 min on ice before the addition of the 32P-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'-GCAGATGGGAGTCTCGAAAAA-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 μg/mL for LNCaP and 500 μg/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).
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-erbα, in normal prostate tissues by quantitative real-time PCR (Fig. 1A). Rhythmic expression of Per1, Cry1, Bmal1, and Rev-erbα 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 (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 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).
Sequence analysis showed that the functional significance (Fig. 5B) are conserved between the human and mouse, suggesting their transcription and that these and the surrounding nucleotides homology to the ARE consensus sequence is the sequence italicized. Chromatin immunoprecipitation assays using LNCaP most essential positions (positions 2, 3, and 5 in each half-site, AC) showed that activated AR induces gene is DHT-inducible in prostate epithelial cells. Similarly, Per1A, left Per1 expression compared with control cells (Fig. 3C). Per1 had a parallel effect on the protein level of PSA (Fig. 3D). 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 effect 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 AGAACAtgaTGTTC. Compared with the ARE consensus GGTACAnnnTGGTCT (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-erbα, 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 the 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.

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