

WOMEN IN CANCER THEMATIC REVIEW

Circadian rhythmicity and the influence of 'clock' genes on prostate cancer

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Abstract

The androgen receptor (AR) plays a key role in the development and progression of prostate cancer (CaP). Since the mid-1990s, reports in the literature pointed out higher incidences of CaP in some select groups, such as airline pilots and night shift workers in comparison with those working regular hours. The common finding in these 'high-risk' groups was that they all experienced a deregulation of the body's internal circadian rhythm. Here, we discuss how the circadian rhythm affects androgen levels and modulates CaP development and progression. Circadian rhythmicity of androgen production is lost in CaP patients, with the clock genes *Per1* and *Per2* decreasing, and *Bmal1* increasing, in these individuals. Periodic expression of the clock genes was restored upon administration of the neurohormone melatonin, thereby suppressing CaP progression. Activation of the melatonin receptors and the AR antagonized each other, and therefore the tumour-suppressive effects of melatonin and the clock genes were most clearly observed in the absence of androgens, that is, in conjunction with androgen deprivation therapy (ADT). In addition, a large-scale study found that high-dose radiation was more effective in CaP patients when it was delivered before 17:00 h, compared with those delivered after 17:00 h, suggesting that the therapy was more effective when delivered in synchrony with the patient's circadian clock. As CaP patients are shown to become easily resistant to new therapies, perhaps circadian delivery of these therapeutic agents or delivery in conjunction with melatonin and its novel analogs should be tested to see if they prevent this resistance.

Key Words

- ▶ circadian clock
- ▶ androgen receptor
- ▶ melatonin
- ▶ *per1*
- ▶ *bmal1*

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Introduction

The prostate is an androgen-dependent organ that relies on male hormones (androgens) for growth and maintenance. In disease states, androgens can stimulate oncogenes that promote cellular proliferation, thus increasing the chances of DNA copy errors. Rodent studies

showed that excessive androgen production can induce prostate cancer (CaP) (Brown *et al.* 1979) and this was later supported by human studies that explored long-term exposure to high levels of androgens (Gann *et al.* 1996). The androgen receptor (AR) is a member of the nuclear

steroid hormone receptor family that also includes the progesterone receptor and the oestrogen receptor (ER). The AR is responsible for mediating the effects of androgens, which are synthesized primarily by the testes and the adrenal cortex, but also other organs such as skin and ovaries. Androgens play an important role in the development and maintenance of the male reproductive tissues. The AR, in its inactive state, is located in the cytoplasm complexed with other proteins such as the heat shock proteins. It is activated by binding to androgens that cause the AR to translocate to the nucleus, where it functions as a transcription factor. The main androgens in humans are testosterone and its more active metabolite, 5- α -dihydrotestosterone (DHT), although other androgens such as dehydroepiandrosterone (DHEA), androstenedione, androstenediol and androsterone are also produced. Upon binding to androgens, the AR is freed from its protein complex and is allowed to move to the nucleus, binding target genes through their androgen response elements located in the promoter or enhancer regions. Many of the genes targeted by the AR are involved in the growth and survival of prostate cells; therefore, dysregulation of AR activity is strongly implicated in CaP development and progression.

Primary CaP is mostly treated with either prostatectomy or with radiation therapy, although watchful waiting is a common option. Approximately 30–40% of those thus treated will experience a recurrence and will be administered androgen deprivation therapy (ADT), which consists of leutenizing hormone-releasing hormone (LHRH) agonists or antagonists, or orchiectomy, to decrease the levels of circulating androgens. ADT is initially effective in >95% of patients, but many initial responders develop resistance to ADT and will eventually develop castration-resistant CaP (CRPC). The majority of castration-resistant tumours still express AR, and androgen-regulated genes such as prostate-specific antigen (PSA), indicating that the AR pathway is still active (Denmeade *et al.* 2003). CRPC patients will be further treated with chemotherapy, immunotherapy, radiotherapy or the use of androgen-signalling inhibitors: the AR inhibitor enzalutamide or the CYP17 inhibitor abiraterone acetate. However, the duration of efficacy of the inhibitors is short and eventually, most patients will develop resistance to these treatments as well.

A series of publications pointed to a pattern of CaP development in unique sets of conditions. A 1996 study in a cohort of 2740 Air Canada pilots showed that standardized incidence ratio (SIR) for CaP was

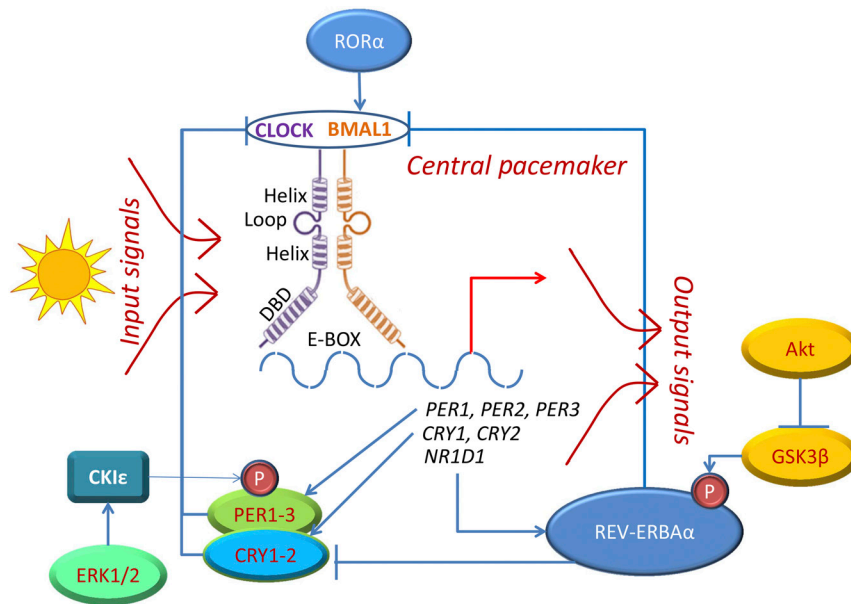
significantly increased among male pilots (SIR=1.87, 90% confidence interval (CI) 1.38–2.49) compared with the general population, although there was an overall decrease in cancer-related mortality for all sites of cancer (Band *et al.* 1996). Following this, in 2003, it was reported that a cohort of 10,051 male airline pilots from Nordic countries (Denmark, Finland, Iceland, Norway and Sweden) experienced an increase in the relative risk of CaP with increasing number of flight hours in long-distance aircrafts ($P=0.01$) (Pukkala *et al.* 2003).

In addition, continuing with this pattern, a 2006 study from Japan reported that among 14,052 working men in Japan, compared with day workers, rotating-shift workers were significantly at risk for CaP (relative risk=3.0, 95% CI: 1.2–7.7) (Kubo *et al.* 2006). This was borne out by a meta-analysis of 2,459,845 individuals from eight published studies which showed that night shift work was associated with a significantly increased risk of CaP (Rao *et al.* 2015). The association between night shift work and CaP risk was particularly strong for tumours with worse prognosis (Papantoniou *et al.* 2015a).

Based on the reports outlined in the last two paragraphs, a 2006 commentary hypothesized that 'both altered-lighted environments and genetic variations in genes responsible for maintaining circadian rhythms may result in deregulation of clock-associated biological processes, such as androgen expression, and consequently influence an individual's risk of prostate cancer' (Zhu *et al.* 2006). In support of a role for altered light environments in CaP, a study linking a cohort of 17,557 persons with visual impairment demonstrated decreased incidence of CaP by degree of visual impairment (Pukkala *et al.* 2006). These reports supported the validity of the chronodisruption hypothesis and indicated a role for the circadian rhythm in the development of CaP.

What is the circadian rhythm?

Circadian rhythms reflect daily oscillations in biological processes regulated by an internal timing system; for example, the sleep/wake cycles (Fu & Lee 2003). Circadian rhythm has a very important adaptive significance that greatly influences the behaviour and physiology of all living organisms from prokaryotes to humans. Other functions in the human body that are influenced by the circadian rhythms include thermoregulation, arterial pressure in systemic circulation, endocrine functions, the digestive system and immune response (Reppert & Weaver 2002). Disruption of circadian rhythms has been

**Figure 1**

Relationship between the various clock genes. Input signals such as sunlight activate the 8 known core clock genes: period (Per1–Per3), casein kinase I ϵ (CKI ϵ), Clock/NPAS2, Bmal1/ARNTL and cryptochrome (Cry1–2). Clock and Bmal1 are two basic helix-loop-helix (bHLH) transcription factors (each containing a bHLH region and a DNA-binding domain – DBD), which bind the E-box sequence (CACGTG) in the promoter of target genes as a heterodimer. Target genes include Per, Cry and NR1D1, which produces an orphan nuclear receptor protein REV-ERBA α . When REV-ERBA α is phosphorylated by GSK3 β , a downstream target of Akt, it transcriptionally represses target genes such as Bmal1 and Cry. In this way, REV-ERBA α balances against the effects of another orphan nuclear receptor ROR α , which transcriptionally upregulates Bmal1. Similarly, Per1 is regulated by phosphorylation by CKI ϵ , which is itself regulated by ERK kinases. Activated Per dimerizes with Cry to form a complex that disrupts the Clock-Bmal1 complex and prevents transcription. P denotes protein phosphorylation.

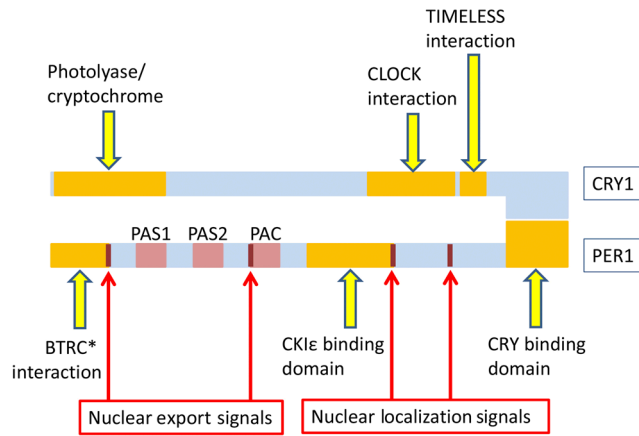
linked to various human ailments including insomnia, jet lag, stomach ailments, coronary heart attacks, depression and cancer (Reppert & Weaver 2002).

In mammals, the circadian system is made of three parts: the input pathways, the central pacemaker and the output pathways (Fig. 1). Environmental signals, such as external light, are transmitted via the input pathways to the central endogenous rhythm of the body, the central pacemaker, which transmits neural and hormonal signals to peripheral circadian systems throughout the body (output pathways) (Liu *et al.* 1997). The central pacemaker resides in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus, which is made up of a number of single-cell circadian oscillators. In a normal individual, the single-cell oscillators are synchronized and generate coordinated circadian outputs (Welsh *et al.* 1995). However, in disease states, this cycle is disordered and leads to disruption of various functions of the related organs.

Circadian rhythms of the central pacemaker are generated by 8 known clock genes: period (Per1–Per3), casein kinase I ϵ (CKI ϵ), Clock, Bmal1, and cryptochrome (Cry1–2) (Fu & Lee 2003). Proteins encoded by these genes form a molecular feedback loop that regulates the circadian rhythm (Ko & Takahashi 2006). Clock and Bmal1 are two basic helix-loop-helix (bHLH) transcription co-activators, which positively regulate the circadian cycle by forming a heterodimer that binds E-box sequences (typically CACGTG) in the promoter of target genes such as Per and Cry (Fig. 1). At the protein level, intracellular expression of Clock remains more or less constant throughout the

24-h period, whereas Bmal1 levels are upregulated at the beginning of the day and fall off as night approaches. At high levels of Bmal1, it forms a heterodimer with Clock, which binds to E-box sequences in the promoters of the Cry, Per and NR1D1 genes to activate transcription at the beginning of a circadian day. NR1D1 encodes the protein Rev-ErbA α , which is an orphan nuclear receptor that acts as a transcriptional repressor and participates in the circadian regulation of organs like the brain, liver, skeletal muscle and adipose tissue. It is known to suppress transcription of Bmal1 and the Cry genes. Rev-ErbA α is phosphorylated on the amino terminus by glycogen synthase kinase (GSK 3 β), which contributes to its protein stability. Bmal1 is coordinated by opposing effects of Rev-ErbA α and the RAR-related orphan receptor ROR α , likely to fine-tune its effect on the circadian rhythm (Fig. 1).

Per1 (rigui) is rhythmically transcribed in the SCN, with a period of approximately 24 h. It is also expressed in other organs, including the prostate. This rhythm is sustained in constant darkness, and with changing light cycles, which likely explains why the circadian rhythm is maintained even in humans with partial or complete visual impairment (Pukkala *et al.* 2006). The three Per genes encode proteins that contain PER-ARNT-SIM (PAS)- and PAC-domains (Fu & Lee 2003) (Fig. 2). All three contain a CKI ϵ -binding domain that allows interaction with this kinase and subsequent phosphorylation, and several nuclear localization and export sequences that allow the complex to enter and exit the nucleus as needed (Sun *et al.* 1997, Tei *et al.* 1997). Per2 has a similar



*Beta-Transducin Repeat Containing E3 ubiquitin protein ligase

Figure 2

General structure of core clock genes *Per* and *Cry*. The three *Per* genes encode proteins that contain the PER-ARNT-SIM (PAS) and the related PAC motifs and form oligomers with the *Cry* proteins to form functional DNA-binding complexes. *Per1* contains a BTRC interaction domain at the N terminal end. In addition, *Per* proteins also contain a phosphorylation domain where casein kinase 1 binds and phosphorylates these proteins. The very C terminus contains a specific domain that allows oligomerization with the *Cry* proteins. In addition, *Per2* contains a PPAR gamma-binding site between the CKIε- and CRY-binding sites that is not seen in *Per1* or *Per3*. *Per2* also has a stabilization domain upstream of the PAS motifs not seen in other *Per* proteins. All *Per* proteins, however, express nuclear localization and export sequences that allows them to enter and leave the nucleus as needed. The structure of the *Cry* proteins is much less complex. Both *Cry1* and *Cry2* contain a cryptochrome (flavoproteins that are sensitive to blue light) interaction domain, as well as a domain that allow them to interact with and inhibit the CLOCK/BMAL1 dimers. In addition, *Cry1* also contain a domain that allows interaction with TIMELESS (which negatively regulates CLOCK/BMAL-1 interactions), but this domain is not found in *Cry2*. (Based on <http://www.uniprot.org/>.)

function, but in addition, it was shown that *Per2* contains glucocorticoid response elements and can interact with PPARγ (So *et al.* 2009). *Cry1* and *Cry2* belong to a class of flavoproteins that in plants and insects induce sensitivity to blue light (Hsu *et al.* 1996). In mammals, they can also act in a light-independent manner as inhibitors of Clock-Bmal1 components of the circadian clock (Griffin *et al.* 1999) (Fig. 2).

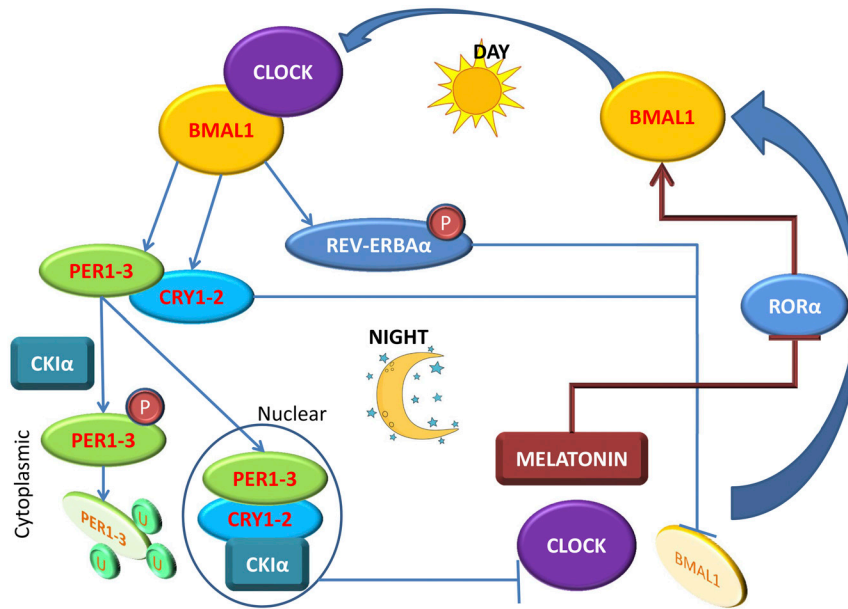
Per and *Cry* proteins form oligomers with each other in the cytoplasm, and the oligomeric structure likely stabilizes the complex. In particular, *Per2*, but not the other *Per* proteins, contain a stabilization sequence that may allow the stabilization of an oligomeric complex. As the day progresses, the oligomer complex translocate from the cytoplasm, where they are formed, to the nucleus. As night falls, the *Per/Cry* oligomer interfere with Clock/Bmal1 activity, thereby preventing further accumulation of the components of the oligomer.

Any *Per* protein accumulated in the cytoplasm is phosphorylated by CKIε, an event that leads to a conformational change that masks the proteins stabilization sequence in *Per2*. This makes the protein complex unstable and it is degraded by ubiquitylation likely by recruitment of the BTRC E3 ubiquitin ligases to the N-terminal region of *PER* proteins. Interaction with TIMELESS leads to disruption of the Clock/Bmal1-associated transcriptional complex and results in the inhibition of *Cry*, *Per* and *Rev-Erb* α transcription, and de-repression of *Bmal1* transcription (Reppert & Weaver 2001, 2002). The interacting positive and negative feedback loops of circadian genes ensure low levels of *Per* and *Cry*, and a high level of *Bmal1* at the beginning of a new circadian day (Fig. 3).

Regulation of the circadian clock by melatonin

The pineal gland in the human brain (so-called because it resembles a pine cone) is best known as a producer of the hormone melatonin, an indoleamine neurohormone derived from the neurotransmitter serotonin, which affects the modulation of sleep patterns in the circadian rhythm. Many of melatonin's biological effects are produced by the interactions of melatonin with its two receptors MT1 and MT2 (Boutin *et al.* 2005). Melatonin is a powerful antioxidant, and this function may be important in its interaction with the circadian rhythm. Serum melatonin levels are usually elevated nocturnally (80–120 pg/mL) compared with its expression during the day (2–20 pg/mL), MT1 and MT2 are G-protein coupled receptors (GPCR), which upon activation by melatonin binding, mediate the repression of RORα transcriptional activity, and hence blocks the expression of the clock gene *BMAL1* (Hill *et al.* 2009) (Fig. 3). During the day, as melatonin levels are degraded, *BMAL1* levels increase and resume binding to clock. Studies indicate improved sleep when melatonin is taken at the appropriate time for jet lag and shift work.

The U.S. Dietary Supplement Health and Education Act of 1994 allow synthetic melatonin to be sold as a dietary supplement; there have not been any reported cases of proven toxicity or overdose by melatonin; in fact, melatonin was found to prevent the cytotoxic effects of other drugs (Asghari *et al.* 2016, Esteban-Zubero *et al.* 2016), including chemotherapeutic drugs (Demir *et al.* 2015). Clinical studies indicate a role for melatonin as an adjuvant therapy for sleep disorders of circadian aetiology (jet lag, delayed sleep phase syndrome, sleep deterioration

**Figure 3**

24-h cycle of the circadian clock. The expression of Clock itself does not significantly vary during the 24-h cycle, but it is only one of few genes in this group that does not. Major regulation of the circadian clock is mediated by the orphan nuclear receptors ROR α and REV-ERBA α , which determine the expression of Bmal1 (also known as ARNTL), the key binding partner of Clock. During the day, the Clock-Bmal1 dimer transcribes genes that result in the synthesis of Per and Cry, as well as REV-ERBA α . As the day progresses, Per and Cry dimerizes and forms a stable complex that translocates to the nucleus. Any free Per remaining in the cytoplasm is phosphorylated by CKI α , which causes it to be ubiquitinated and degraded. As night falls, the Per/Cry oligomer complex in the nucleus disrupts the formation of the Clock-Bmal1 heterodimer, which then stops producing Per and Cry. In addition, REV-ERBA α is phosphorylated by GSK3 β , which stabilizes the protein, and allow it to transcriptionally repress the expression of Bmal1. Bmal1 levels are also suppressed when melatonin levels increase in the circulation, which inhibits the expression of ROR α that positively regulates Bmal1 expression. Decrease in Bmal1 further disrupts the Clock-Bmal1 complex and prevents the transcription of target genes, including Per and Cry. As REV-ERBA α levels in the cell, and melatonin levels in the circulation, decrease early in the morning, Bmal1 synthesis is resumed. Further, Per and Cry levels in the nucleus also fall off due to decreased synthesis, and Clock-Bmal1 complexes resume, which then allows the circadian clock to move forward. P denotes protein phosphorylation, U denotes protein ubiquitination.

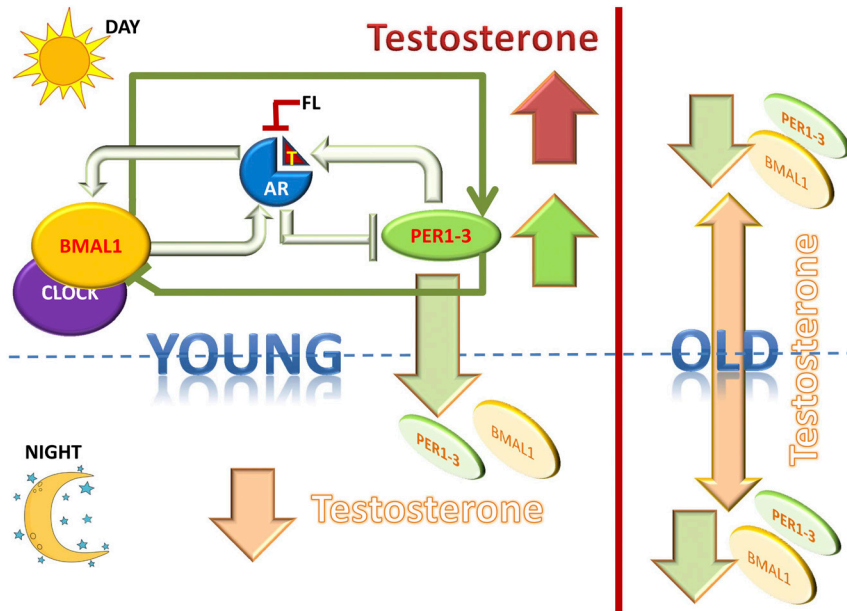
associated with ageing, etc.) (Sanchez-Barcelo *et al.* 2010). Melatonin seems to facilitate sleep in insomniac patients in some cases (Rohr & Herold 2002). However, the National Sleep Foundation warns that 'the correct dosage, method and time of day it is taken must be appropriate to the sleep problem'.

Relation between the circadian clock and androgens

Circadian rhythms have been shown to play an important role in the expression of androgens, and studies show high levels of testosterone production in early mornings in healthy men that decline as the day progresses leading to lower levels by the evening (Cooke *et al.* 1993). As a result, in the diagnosis of hypogonadism in males, it is recommended that testosterone levels be assessed in the morning before 00:10h. This decline was coincident with the initial rise in the concentration of cortisol (Cooke *et al.* 1993). Surprisingly, the early morning spike in testosterone

levels characteristic of young men was blunted in old age (Bremner *et al.* 1983). Additional studies indicated that an adrenal androgen-regulating system was impaired in the older subjects (Montanini *et al.* 1988), especially over the age of 70 (Diver *et al.* 2003). A comparison of young (3-month) and aged (18- and 24-month) rats showed that expression of several clock genes (Bmal1, Per1, Per2, Per3 and Rev-ErbA α) were reduced in the 24-month-old group compared with the younger groups (Baburski *et al.* 2016) (Fig. 4). These results seem to suggest that the reduced levels of the clock genes in aged males may be responsible for the loss of circadian rhythmicity observed in ageing.

In support of the above observations, delayed peak androgen production was identified in night shift workers compared with day workers (Papantoniou *et al.* 2015b), and may have contributed in part to the increased risk for CaP observed in night shift workers (Kubo *et al.* 2006, Papantoniou *et al.* 2015a, Rao *et al.* 2015). Significantly, the rhythmicity of Per2 mRNA levels in the blood depended on the period of activity of the subjects, therefore, it was

**Figure 4**

Circadian regulation of testosterone production in young and old men. Healthy young men demonstrate high levels of testosterone production in early mornings that decline as the day progresses leading to lower levels by the evening. During the day, Per complexes promote testosterone production, as did Bmal1; the produced testosterone binds to and activates the androgen receptor (AR), but as night falls, activated AR suppresses Per levels, thereby decreasing testosterone production and deactivates the AR. This in turn suppressed Bmal1 levels, further preventing Per expression. As Per levels increase once more the next morning, along with Bmal1 levels, testosterone production is renewed. However, several clock genes (Bmal1, Per1, Per2, Per3 and Rev-ErbA α) are reduced in older males compared with younger ones, resulting in the loss of circadian rhythmicity in testosterone synthesis observed in ageing males. T, testosterone.

higher in the morning for day shift workers, similar to the general population, but higher in the evening for night shift workers (Fang *et al.* 2015). This shift in Per2 levels was coincident with testosterone synthesis, suggesting strong association between androgen production and the expression of Per2. Additional reports showed that Bmal1 and Clock, but not Per2 and Nr1d1/Rev-ErbA α , are downregulated by testosterone deficiency (Kawamura *et al.* 2014). Thus, it is likely that Per2 drives testosterone levels. This observation was borne out by another study in rat prostate mesenchymal cells, which showed that neither testosterone nor DHT caused any change in Per2 activity (Yoshida *et al.* 2010). On the other hand, the same study showed that the anti-androgen flutamide, which is used for the treatment of advanced CaP, upregulated the amplitude of circadian Per2 oscillations in a dose-dependent manner (Yoshida *et al.* 2010). As flutamide binds to and inhibits the AR, it is possible that AR transcriptional activity, through activation of a downstream target of the AR, regulate Per2 activity and levels (Fig. 4).

A significant relation exists between other clock genes and testosterone levels as well. Studies in rodent models revealed that the AR is concentrated in the SCN core in male mice (Iwahana *et al.* 2008). Similar to Per2, Per1 also inhibited AR transactivation and diminished the expression of AR target genes following DHT stimulation; while Per1 itself is regulated by androgens in CaP cells (Cao *et al.* 2009). Both male and female Bmal1 knockout (K/O) mice are infertile (Alvarez *et al.* 2008). Male Bmal1 K/O mice had low testosterone and high luteinizing

hormone serum concentrations, suggesting a role for Bmal1 in androgen production, while in turn, Bmal1 and Clock are downregulated by testosterone deficiency (Kawamura *et al.* 2014). Importantly, Leydig cells, which produce testosterone, rhythmically express Bmal1 protein (Alvarez *et al.* 2008). Further evidence that the Bmal1-Clock heterodimer is involved in testosterone production and AR transactivation came from studies using mice homozygous for a dominant-negative allele of the Clock gene (Clock ($\Delta 19/\Delta 19$)), which have slightly but significantly decreased male fertility (Liang *et al.* 2013). Taken together, these reports point to a process by which feedback loops may exist between the AR axis and the clock genes in males (Fig. 4).

Clock genes and prostate cancer regulation

Clock genes are known to be directly involved in the regulation of tumorigenesis in the prostate and other organs. Per1 is downregulated in human CaP samples compared with normal prostates (Cao *et al.* 2009). On the other hand, overexpression of Per1 in CaP cells resulted in significant growth inhibition and apoptosis (Cao *et al.* 2009). Other studies found that Clock and Per2 protein levels were downregulated whereas Bmal1 protein levels were upregulated in CaP cells, compared with normal prostate cells (Jung-Hynes *et al.* 2010). Overexpression of Per2 reduced CaP cell growth and viability. Interestingly, melatonin treatment increased Per2 and Clock and reduced Bmal1, thereby causing resynchronization of oscillatory circadian rhythm genes

Table 1 Association of various single-nucleotide polymorphisms (SNP) in known circadian-related genes with susceptibility to CaP (Zhu *et al.* 2009) or with fatal CaP (Markt *et al.* 2015).

Clock gene	SNP associated with susceptibility to CaP	SNP associated with fatal CaP
PER1	rs885747 and rs2289591	rs2289591
PER2	rs7602358	rs10462023
PER3	rs1012477	
CRY1	rs12315175	rs7297614, rs1921126 and rs12315175
CRY2	rs2292912	
CK1 ϵ	rs1534891	
BMAL1 (ARNTL)	rs7950226	rs969485
CLOCK/INPAS2	rs11133373, rs1369481, rs895521, and rs17024926	rs3754674 and rs10206435

in CaP cells (Jung-Hynes *et al.* 2010). It appears that resynchronization of the clock genes may have tumour-suppressive effects in CaP cells.

Population-based genetic associations studies which genotyped single-nucleotide polymorphisms (SNP) in known circadian-related genes showed that at least one SNP in each of the core circadian genes was significantly associated with susceptibility to CaP (Zhu *et al.* 2009), and quite a few were associated with fatal CaP (Markt *et al.* 2015) (Table 1). However, there are variations in the strength of association between fatal CaP and these SNPs among different cohorts showing variability in different patient cohorts (Markt *et al.* 2015). Taken together, these reports illustrate a possible role of multiple clock genes in the regulation of CaP progression and development.

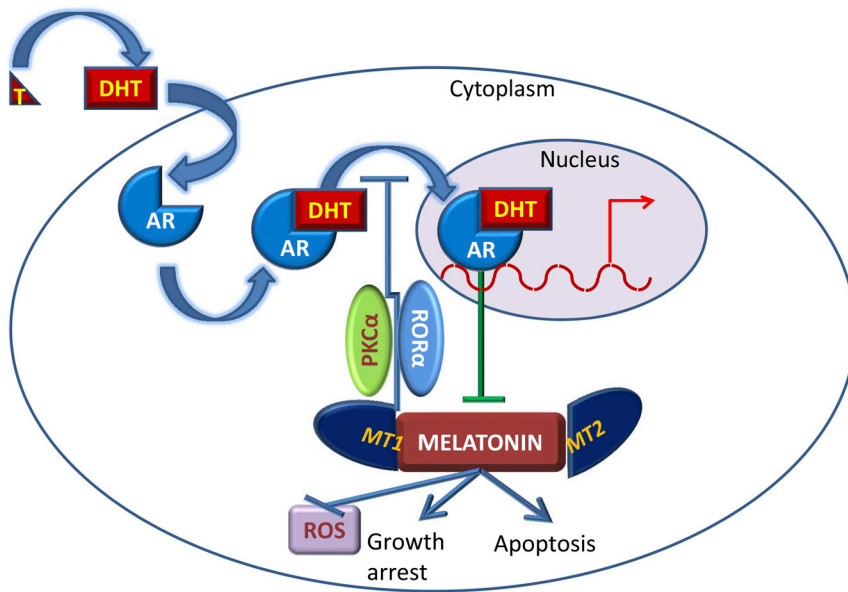
Melatonin regulation of prostate carcinogenesis

Recent studies showed that patients with high melatonin levels or a high melatonin/cortisol ratio were less likely to develop CaP or experience CaP progression (Tai *et al.* 2016). Comparison of serum levels of melatonin in elderly men with benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN) and localized CaP, as well as in normal adult men without CaP, demonstrated that melatonin induced significant circadian rhythms in normal men and patients with BPH (enlarged prostate) and PIN (considered to be a precursor for prostate cancer) but not in patients with CaP (Bartsch *et al.* 1985). In support of these reports, a rodent model affirmed that melatonin suppresses prostate tumorigenesis (Toma *et al.* 1987).

However, further analysis found that, while afternoon melatonin administration induced tumour remission in castrated rodents, continuous melatonin administration did not have any significant effect on CaP tumours (Buzzell 1988), suggesting that melatonin is only effective

when administered in synchronization with the patient's natural circadian cycle. Further, the study also emphasized that melatonin is more effective with low AR activity. In a study of 186 patients with previously untreated metastases from colorectal cancer, 93 patients were assigned chronotherapy with oxaliplatin, fluorouracil and folinic acid and 93 were assigned constant-rate infusion via multichannel programmable ambulatory pumps (Levi *et al.* 1997). An objective response was obtained in 47 (51%) of the chronotherapy group, and in 27 (29%) of the constant-rate group. Chronotherapy reduced five-fold the rate of severe mucosal toxicity and halved that of functional impairment from peripheral sensitive neuropathy (Levi *et al.* 1997). *In vitro* studies demonstrated higher growth inhibitory efficacy of melatonin not only in the absence of androgens (Siu *et al.* 2002) but also in the presence of oestrogens which are known to suppress androgen production (Lupowitz & Zisapel 1999). Thus, there is possible interaction between the melatonin-dependent pathway and the AR axis that likely antagonize each other.

The growth-suppressive effects of melatonin seems to be dependent on the GPCRs MT1/Mel1A and MT2/Mel1B receptors (Moretti *et al.* 2000, Xi *et al.* 2000, 2001); whereas in the absence of these receptors, melatonin does not appear to have a tumour-suppressive role. The fact that melatonin's effects on tumour suppression were only observed when melatonin was used in a circadian manner (afternoon administration), but not when it was continuous, suggest that continuous melatonin administration causes degradation of the melatonin receptors likely via a feedback mechanism, as is often seen in many GPCRs. Significantly, MT1 and MT2 are highly expressed in AR null cells (Gilad *et al.* 1999, Marelli *et al.* 2000, Sainz *et al.* 2005), and in 22Rv1 cells expressing high levels of alternately spliced AR variants (and low levels of full-length AR) (Tam *et al.* 2007), but not in cells expressing high AR levels (Moretti *et al.* 2000). These results

**Figure 5**

Negative feedback loop between melatonin and the androgen receptor. Testosterone (T) is converted to dihydrotestosterone (DHT), which in the prostate, binds to the androgen receptor (AR) in the cytoplasm of prostate-derived cells, including prostate cancer. DHT binding activates the AR, which then translocates to the nucleus and binds to androgen response elements (ARE) in the target genes, thereby stimulating their transcription. AR transcriptional activity represses the expression of the melatonin receptors MT1 and MT2, thereby preventing their binding to melatonin. This prevents melatonin's ability to induce growth arrest and apoptosis, and to prevent oxidative stress. In turn, melatonin and its receptors inhibit AR translocation to the nucleus, not only by preventing testosterone conversion to DHT but also by mechanisms involving PKC α or ROR α .

suggest that AR activity suppresses the expression of the melatonin receptors. Therefore, melatonin is perhaps more effective in CaP models of castration where AR activity is low, allowing MT1/2 expression, which can then bind melatonin (Fig. 5).

In turn, it was reported that melatonin inhibits AR localization in the nucleus, where it is active, thereby suppressing AR activity (Lupowitz *et al.* 2001, Rimler *et al.* 2001, 2002a) and preventing AR binding to its target gene (Rimler *et al.* 2002b). Other studies suggest that melatonin prevents testosterone conversion to DHT, a strong AR ligand (Philo & Berkowitz 1988). Melatonin's effect on the AR has been suggested to be mediated by the inhibition of the RZR/ROR receptors (Karasek & Pawlikowski 1999), while inhibition of AR nuclear translocation by melatonin was likely via a PKC α -dependent mechanism (Sampson *et al.* 2006). Thus, melatonin and the AR pathway inhibit each other (Fig. 5), likely to gain control of the downstream targets leading to cell cycle progression and apoptosis. Whether the tumour progresses or not will depend on which pathway gains control in the end.

Melatonin's effects on growth arrest are mediated by PKC α (Tam *et al.* 2008), and by aldo-ketoreductase (AKR) and prostaglandin F-synthase (PGFS)-dependent pathways (Byrns *et al.* 2008), whereas its effects on cell death required elevation of p21 (Park *et al.* 2009), p27^{Kip1} (Tam & Shiu 2011), activation of TNF α - and NF-kB-dependent pathways (Sainz *et al.* 2008, Rodriguez-Garcia *et al.* 2013, Shiu *et al.* 2013) and JNK and p38MAPK activation (Joo & Yoo 2009). Melatonin inhibition of HIF1 α signalling and reactive oxygen species generation has also been established (Park *et al.* 2009, Cho *et al.* 2011,

Paroni *et al.* 2014). Several microRNAs (miRNAs) including miR3195 and miR374b were significantly upregulated while other miRNAs were downregulated in melatonin-treated cells under hypoxia compared with untreated control (Sohn *et al.* 2015). In addition to hypoxia and androgen deprivation, melatonin also seems to sensitize CaP cell lines to cell death under hypoglycaemic conditions (Kabasakal *et al.* 2011, Gobbo *et al.* 2015).

As melatonin was effective in tumour suppression in castrated rodents, a Phase II clinical trial was initiated to test the efficacy of melatonin in conjunction with a LHRH agonist vs the LHRH agonist alone (Lissoni *et al.* 1997). Men treated with the combination demonstrated a decrease in PSA serum levels greater than 50% in 57% patients, a normalization of platelet number in patients with persistent thrombocytopenia before study, and a survival longer than 1 year in 64% patients (Lissoni *et al.* 1997). Therefore, it appears that melatonin may actually prolong the effects of LHRH agonists in men with advanced CaP. In support of the involvement of the melatonin receptor in the suppression of carcinogenesis by melatonin, a case report of a patient whose prostate tissue expressed the melatonin receptors demonstrated efficacy of melatonin in suppressing tumour progression (Shiu *et al.* 2003).

Effect of the circadian clock in hormone and chemotherapy

While melatonin is the direct effector of the circadian clock, our growing understanding of the circadian rhythm has produced various strategies to increase

the effectiveness of cancer treatment. Hot flashes are a common side effect of ADT using LHRH agonists in men with advanced CaP. A recent study demonstrated that there were significant 24-h circadian rhythms of hot flashes following ADT administration (Hanisch & Gehrman 2011) where the peak of the rhythms occurred in early afternoon. As altered circadian rhythms have been identified in untreated CaP patients, the authors conclude that the acrophases of hot flashes and elevated activity levels may represent a normalization of circadian rhythms following ADT (Hanisch & Gehrman 2011).

Recently, a retrospective study testing disease control and treatment-related toxicity in patients undergoing high-dose radiotherapy (HDRT) (median 78 Gy) for CaP evaluated those receiving daytime treatment (before 17:00 h) ($n=267$) vs evening treatment (after 17:00 h) group ($n=142$) (Hsu *et al.* 2016). This study found that evening HDRT was significantly associated with worse freedom from \geq grade 2 late GI complications (hazard ratio=2.96; $P<0.001$), especially in patients older than 70 years. Moreover, biochemical failure-free survival was worse in the evening group than the daytime group (72% vs 85%, hazard ratio=1.95, $P=0.05$) (Hsu *et al.* 2016). No known circadian-based trials of hormone and chemotherapeutic agents that are effective in CRPC patients have been reported as yet. Hormone-refractory metastatic CaP has been treated with circadian-timed fluorodeoxyuridine (FUDR) chemotherapy; however, without objective response (Rajagopalan *et al.* 1998). The lack of objective response, of course, could result from the lack of efficacy of FUDR in CaP patients.

As chronomodulation was not implemented in many CaP trials of chemotherapeutic agents, circadian-timed delivery of chemotherapeutic agents in other cancers will be examined. In 1985, 31 patients with advanced ovarian cancer received eight monthly courses of doxorubicin (adriamycin) that were followed 12 h later by cisplatin, with doxorubicin randomly administered at either 06:00 h or 18:00 h (Hrushesky 1985). Those receiving doxorubicin in the evening and cisplatin in the morning required reductions in dosage and delays in treatment (indicative of greater toxicity), compared with those receiving doxorubicin in the morning and cisplatin in the evening (Hrushesky 1985). A similar study concluded that the combination (doxorubicin+cisplatin) is active against advanced ovarian cancer, but that its toxicities can be significantly decreased by dosing doxorubicin in the early morning and cisplatin in the late afternoon (Levi *et al.* 1990).

Thereafter, this schedule (doxorubicin in the early morning and cisplatin in the late afternoon) has been used in studies on endometrial and bladder cancer patients, with considerable success (reviewed in Kobayashi *et al.* (2002)). Similar success was achieved in patients with metastatic renal cell carcinoma receiving fluorodeoxyuridine (FUDR) when delivered by a circadian-modified infusion schedule, in which 68% of the daily dose was administered between 15:00 h and 21:00 h, compared with continuous infusion (Hrushesky *et al.* 1990). As mentioned earlier, a study of chronomodulated infusion (administered to coincide with relevant circadian rhythms) of oxaliplatin, fluorouracil and folinic acid compared with a constant-rate infusion method demonstrated that chronotherapy was significantly less toxic and more effective than constant-rate infusion in patients with metastatic colorectal cancer (Levi *et al.* 1997). While no studies on the use of chronomodulation in hormone and chemotherapy in CaP patients have been reported, the above studies in ovarian, endometrial, bladder, renal and colorectal cancer patients suggest that the use of chronomodulation in CaP patients may yield considerable benefit as well.

Conclusion

This review concisely describes current knowledge of the mechanisms by which the circadian clock interacts with AR-signalling pathway. While in normal individuals, the clock genes, especially *Per1/per2* and *Bmal1* were expressed in a circadian rhythmic manner, this rhythmicity was lost in CaP patients, while the neurohormone melatonin, which can regulate *Bmal1* levels, restored circadian function. We provide evidence that melatonin reduced cytotoxicity in individuals receiving chemo- and hormonal therapy, and sensitized patients to LHRH agonists. A recent study suggests that chronomodulation of radiation therapy benefit CaP patients receiving radiotherapy in the morning compared with those receiving the treatment in the evening. Given the success of chronomodulation in the treatment of other cancers with chemotherapy, the studies references here hypothesize an improvement in outcome and a reduction in toxicity if CaP patients were to be tested for chronodependence in chemo- and hormonal therapy.

Declaration of interest

The authors declare no conflict of interest. The work reported here does not represent the views or opinions of the Department of Veteran Affairs or the United States Government.

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