Review

Clock genes and the role of melatonin in cancer cells: an overview

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ABSTRACT

Circadian rhythms control most biological processes in every organism and their disruption or an aberrant function in the expression of clock genes are associated with a number of cancers including some hormone-dependent and independent cancers. The processes involved in carcinogenesis and tumor progression are complex, but understanding the daily profiles of the core clock genes and their clock-controlled genes is essential to evaluate specifically the molecular program of the cancer phenotype; this may be helpful in providing a more realistic strategy for both diagnosis and treatment during the course of the disease. Because melatonin production and secretion oscillates rhythmically through the light:dark cycle and is related to the circadian machinery genes (*Clock*, *Bmal1*, *Periods*, and *Cryptochromes*), its regulatory role on clock genes in cancer cells may bring additional evidence regarding the mechanism(s) by which melatonin is involved. Mechanistically, melatonin acts via proteasome inhibition and sirtuins to indirectly modulate clock genes in cancer; however, melatonin seems to be capable of directly altering the expression of clock genes to affect cancer development. Depending on cancer cell type, melatonin might up or downregulate specific clock genes to control cell cycle, survival, repair mechanisms, etc. In parallel, melatonin exerts pro-apoptotic, anti-proliferative and pro-oxidative effects, metabolic shifting, reduction in neovasculogenesis and inflammation, and restores chemosensitivity of cancer cells. Finally, melatonin improves the life quality of patients. This review focuses on the main functions of melatonin on clock genes, and reviews, from a clinical and experimental standpoint, how melatonin regulates the expression of clock genes in some prevalent cancer types such as breast, prostate, liver, and colon cancers, leukemia and melanoma. We further emphasized possible signaling mechanisms whereby melatonin interferes with clockwork genes and circadian-controlled genes within cancer cells.

Keywords: clock genes; cancer cells; melatonin; *Clock; Bmal1; Per; Cry*.

1. INTRODUCTION

1.1. Connecting the interactions of the circadian clock and melatonin.

All living organisms on earth are constantly receiving external inputs, most of them obeying a cyclical pattern. As a consequence, cells respond to these stimuli in a well-organized biological rhythm (1). The functional aspects of living organisms (cell metabolism, core temperature, hormone synthesis and secretion, feeding time, sleep-wake cycle, and many others) are dictated by the circadian clock elements. The circadian clock is composed of a set of genes with specific and rhythmic oscillations driven by the central circadian pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus; peripheral oscillators are found in nearly all cells in the body (2). Although light is considered the most evident entrainment signal, other important changes such as temperature variations, body activity, meal times and social cues represent good synchronizers of our peripheral clocks (3). In addition to SCN, there are other oscillators in mammalian nervous system including retina, paraventricular nucleus, pituitary, and pineal gland, being this latter responsive to inputs of norepinephrine released by postganglionic sympathetic fibers (4). These clock elements operate as individual cellular oscillators, thereby requiring maximum coordination and internal coupling in the respective tissues to guarantee synchrony; however, through multiple events these coupling mechanisms can be lost or weakened by reduced rhythm amplitudes such as those observed in aging and in some pathologies (4).

Circadian clocks are encoded by a network of interlocking feedback loops composed of transcriptional activators and repressors; these autoregulatory mechanisms lead to the fluctuating expression of clock genes and proteins throughout the day (Figure 1). The main transcription-translational oscillator components contributing to the control of the molecular clock are the circadian locomotor output cycles kaput (CLOCK) which contains histone acetyltransferase activity and the brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1); these two components form a heterodimer CLOCK/BMAL1 complex (5). These transcription factors induce the expression of clock-controlled genes (CCG) by binding to E-box components on its promoter at CACGTG. This complex activates the transcription of other genes including period (*Per* 1, 2 and 3) and cryptochrome (*Cry* 1 and 2) at the beginning of the day while its activity is decreased at night. After forming a complex, PER and CRY proteins act as a repressor of the *Clock* and *Bmal1* thereby closing the feedback loop (6). Another regulatory loop for *Bmal1* levels is under the control of the activation of RAR-related orphan receptor alpha (RORα) and repression of REV-ERBα nuclear transcription factor (6). While the transcription factor D-box binding protein (DBP) binds to D-boxes [TTA(T/C)GTAA], the nuclear receptors, RORα and REV-ERBα, bind to the *Rev-Erb/ROR*-binding element, thereby regulating their targets to differentially oscillate the circadian transcripts in all tissues (7). REV-ERBα is controlled by the nuclear receptor corepressor 1 (NCoR1) which recruits HDAC3 to deacetylate histone while repressing target genes, such as *Bmal1* (8). This nuclear receptor system also includes the role of peroxisome proliferator-activated receptor (PPARα) and is strongly associated with the regulation of important metabolic pathways, thereby documenting a crosslink between metabolism, nuclear receptors, and circadian clock; disruption or deletion of specific clock genes may result in severe metabolic diseases (9).

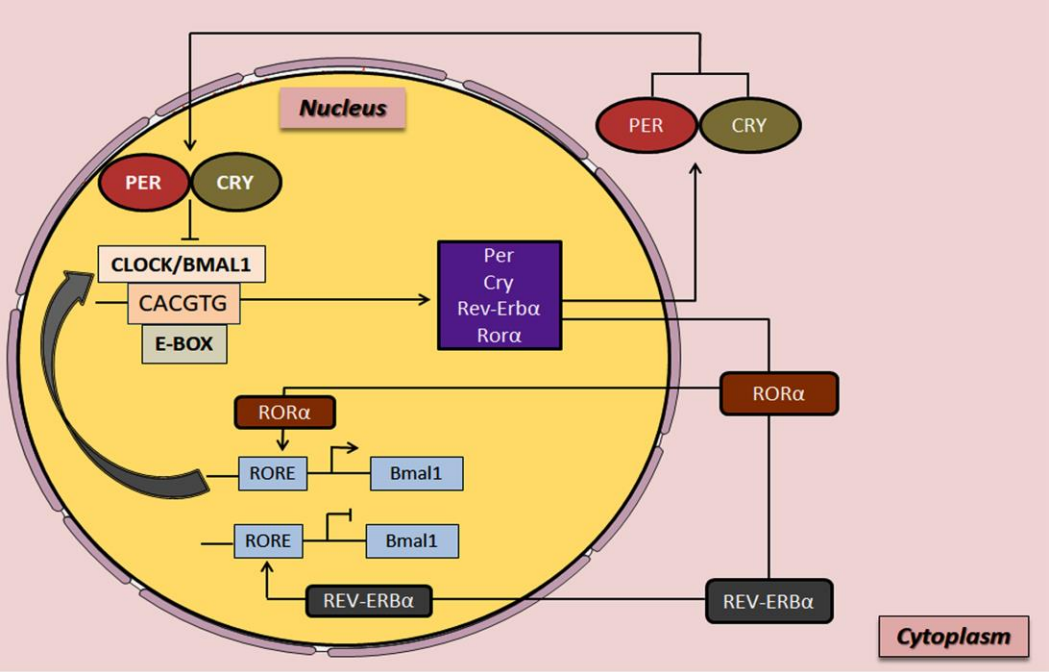

Fig. 1. Network of feedback loops components of the circadian machinery.

CLOCK and BMAL1 heterodimer activate the expression of Pers (Per1, 2, and 3), Crys (Cry1 and 2), Ror, and Rev-Erb. PER and CRY proteins enter the nucleus to repress the CLOCK/BMAL1 complex. While the REV-ERBα receptor inhibits Bmal1 expression, the nuclear receptor RORα positively regulates Bmal1 expression which in turn allows the circadian clock to progress forward.

CCG are fundamental genes involved in the organic processes including cellular dynamics, DNA damage response and metabolism. In addition to their involvement in transcriptome and epigenome, the circadian regulation of the phosphoproteome has emerged with approximately 25% of these phosphorylation sites found to timely oscillate whereas 10% of overall nuclear proteins follow a circadian accumulation pattern (10, 11). Disruption in CCG functions might severely affect normal cellular events such as the cell cycle, apoptosis, metabolism and energy, DNA repair, epithelial-to-mesenchymal (ETM) transition, and others (12). Depending on how deep, simple or complex these changes are, critical implications in a range of molecules could be as determinant in the development of cancer. Regarding circadian clock function, the recognition of potential molecules and its role in the tumorigenesis are important for treatment purposes, where the timing of drug administration and its effectiveness associated with the patient response can be optimized based on the differences in normal and cancer cells (12).

Melatonin is a hormone produced by the pineal gland and other tissues and has a well-conserved circadian rhythm in the blood of approximately 24 hr oscillation. The melatonin levels are elevated at night (80-120 pg/mL) but drop considerably during the day (2-20 pg/mL) (13). A number of studies have shown a strong correlation between reduced melatonin levels with increased risk of developing cancers including breast, prostate, colon, lymphoma, liver, and others (14-16). As a critical endogenous messenger, melatonin may regulate and adjust the biological clock (17, 18). Notably, melatonin has a circadian pattern of production and secretion (19) that is controlled by the master circadian clock in the SCN of the hypothalamus (Figure 2).

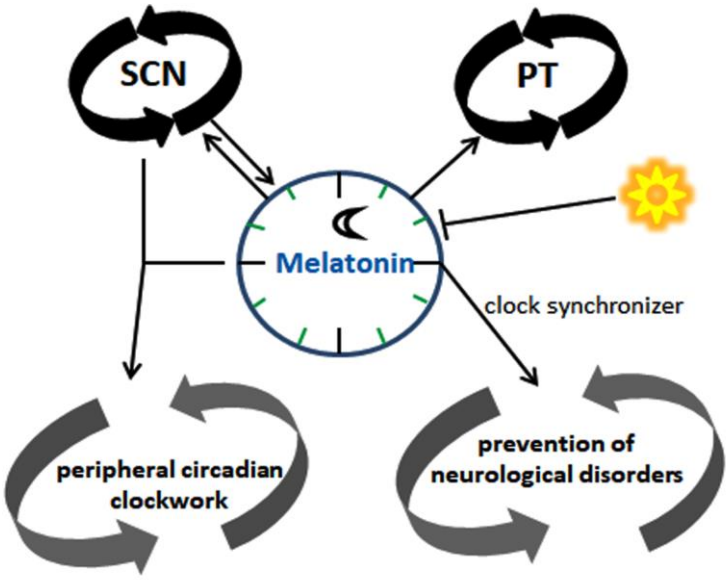

Fig. 2. Schematic illustration of the role melatonin in the clock system.

Suprachiasmatic nucleus (SCN) controls melatonin secretion in the absence of light and melatonin promotes chronobiotic activities associated with clock regulation in the SCN and pars tuberalis (PT). In peripheral tissues, SCN-dependent clock genes are also influenced by melatonin; in the case of damaged cells, melatonin seems to restore their circadian rhythmicity. Acting as a rhythm synchronizer, melatonin has been shown to regulate clock genes centrally and low melatonin levels may result in some neurological disorders.

Experimental evidence of the maintenance of clockwork machinery within the pineal gland indicates that the rhythmic expression of *Bmal1*, *Per2*, *Cry2*, and *Rev-Erbα* only occurs when norepinephrine (NE) synchronically stimulates the gland; this is accompanied by a promotion of melatonin synthesis (20). Melatonin is produced by various enzymes that are stimulated by dark (21, 22) and its mechanisms of action are multiple and include: (i) signaling via melatonin receptors (MT1 and MT2) to inhibit the linoleic acid uptake and 13-HODE formation, (ii) induction of a detoxifying enzyme termed QR2, (iii) enhance calmodulin degradation, (iv) function as scavenger of reactive oxygen and nitrogen species, (v) binds to nuclear receptors (RZR/RORα and RZRβ) to transactivate target genes, and others (16, 23).

Melatonin influences all cells to precisely provide circadian and seasonal information on the timing and photoperiods (24). Animal models (e.g., C57BL, C3H, and MT1/MT2 double knockout C3H/HeN mice) have been developed to investigate the melatonin signaling on the SCN and pars tuberalis (PT) of the pituitary gland (25). Melatonin receptors are expressed in the SCN and exhibit chronobiotic activities associated with clock regulation. Because MT1 is demonstrated to be involved in phase-shifting circadian rhythms, the development of selective MT1 agonists or antagonists may be beneficial for the treatment of circadian pathologies (26). Circadian rhythm of clock gene expression and locomotor activity is identical in melatonin-deficient or -proficient mice, and in mutant mice lacking melatonin receptors, thereby proving that the rhythmicity generated in the SCN is independent of melatonin. In this perspective, melatonin has only a significant action for entrainment of the SCN when the retinohypothalamic

tract is intact (25). In the SCN of mice, disruption of MT1 receptor did not abolish the effect of melatonin on the phase shifting of the neuronal firing pattern (27); these data suggest that the phase-shifting effect of melatonin is not strictly dependent on its receptors. A past study with mice without functional MT1 or MT2 receptors showed that MT1 mediates the interaction of melatonin with the neuropeptide PACAP in the SCN, while MT2 is only activated in the absence of MT1; this support a functional redundancy of the receptors subtypes in the SCN (28). Melatonin regulates circadian rhythms in the PT through several mechanisms. According to Wagner et al. (29), melatonin signals for synchronizing an endogenous oscillator to the photoperiod in PT. Studies with melatonin and PT showed that melatonin can control *Cry1* and *Per1* expression in a time-dependent manner (30, 31). Also in the PT, the transcription of *Cry1* and *Cry2* rise when melatonin is elevated at night in Soay rams (32) Absence of melatonin is associated with profound changes in the responsiveness of PT and activation of melatonin receptors seem to acutely inhibit the cyclic AMP signaling and the forskolin-induced CREB phosphorylation (33); melatonin is thought to sensitizes the adenylate cyclase signaling in a period-dependent manner. The rhythmic regulation of *Per1*/PER1is dependent on melatonin, and deletion of the MT1, but not MT2 gene, caused a reduction in the expression of *Cry1*, *Bmal1*, and *Clock* genes (34). Although these researchers concluded that melatonin acts to reset the circadian rhythms in the PT, no mention is made of the fact that melatonin works on ubiquitin-proteasome system.

Recent findings have documented the proteasome activity to be responsible for the phase shifting of the circadian clock-related proteins; in this scenario, the modulatory action of melatonin on the stability of these proteins is undisputable (35). In 2007, Gatfield and Schibler (36) provided evidence for the role of the ubiquitin-proteasome system in the precise maintenance of circadian timing; proteins need to be destroyed by the proteasome to maintain clock "precision". There are specific ubiquitin ligases involved in the degradation of period proteins, cryptochromes, BMAL1, and REV-ERBα (37). Kodadek et al. (38) proposed that proteasomal timing of deubiquitinases turns off the activity of transactivators; the single deubiquitinase, USP2, is suggested to be associated with period, cryptochrome, and BMAL1 proteins (37). There is increasing evidence that melatonin may interfere with the activity of ubiquitin-proteasome system in a variety of tissues including SCN (24, 35, 39) to provide selective stability for proteins and circadian rhythmicity. Based on the model proposed by Vriend and Reiter (35), elevation in melatonin levels is associated with proteasome inhibition and, if that is the case, melatonin works to adjust transcriptional cycles in the nucleus of the cells. As the increase in melatonin during the dark phase is related to the high availability of BMAL1 levels, proteasomal inhibition by melatonin may control the levels of CRY, PER, and REV-ERBα which in turn regulate *Bmal1* transcription.

1.2. Melatonin and clock genes: general molecular relationships.

Pineal melatonin exhibits a rhythmic pattern of secretion, since it is produced mostly at night in this organ. During the day, melanopsin-secreting retinal ganglion cells perceive light and suppress melatonin production in the pineal (40). The comprehensive review by Bonmati-Carrion et al. (40) show that the daily circadian effects of dark period protects the synthesis and release of melatonin and ensures human health and life quality. Melatonin, via feedback mechanisms, controls neuronal firing by the SCN cells and alters clock genes (35), but its critical involvement with the expression of circadian clock components is still uncertain in terms of

mechanisms (Figure 2). Contrary to photic cues, melatonin does not change immediately the expression of clock genes in the rat SCN. For example, Poirel et al. (41) injected melatonin (1 mg/kg) into the SCN at the end of the subjective day, but only observed its effects on the second subjective night after injection where the expression of *Per1, Per3*, *Bmal1*, and *Avp* were phase-shifted. They concluded that melatonin acting on the molecular loop was post-translational rather than a transcriptional mechanism. Another similar study by Mattam and Jagota (42) administered 30 µg/kg of melatonin 1 h before the beginning of the dark phase (Zeitgeber time (ZT)-11) in rats of three different ages for 11 days. Although gene expression varied with the age of the animals, the levels of *Per1, Per2, Cry1, Cry2* and *Bmal1* mRNAs were elevated by melatonin irrespective of the light-dark phase and all age groups studied, thereby indicating a direct transcriptional role of melatonin on these clock genes. By acting as a proteasome inhibitor, melatonin interferes with molecular loops which finally results in increase of BMAL1 and, consequently, transcription of *Per, Cry* and *Bmal1* genes. The chronobiotic effects of melatonin were also tested through the transcriptional regulation of nuclear orphan receptors mRNA (*Ror* and *Rev-Erbα*). While *Rorα* was unaffected by melatonin, levels of *Rorβ* were prevented from declining during the first hours. Expression of *Rev-Erbα* was phase-advanced in the first subjective night following melatonin injection whereas *Bmal1* was phase-shifted only on the second night (43). The authors believe that the nuclear orphan receptor genes might be the link between melatonin and the molecular core of circadian clock circuitry.

An early study using the adrenal cortex of mice showed that PER1, CRY2 and BMAL1 proteins were significantly lower in melatonin-deficient animals (C57BL) than in melatonin-producing mice (C3H) (44). Also, using melatonin-proficient Clock (Delta19) mutant mice, Kennaway et al. (45) showed that melatonin is rhythmically produced with peak production 2 h later than controls and spontaneously dropped around 1 h before the time of lights on; in continuous darkness, melatonin rhythms were maintained in accordance with the period of mutant (45). This particular mutation is due to the transversion of A to T which causes the elimination of 51 amino acids of the CLOCK protein. While a light pulse reduced the expected 2 h delay in melatonin peak during darkness in the mutants, the melatonin peak was advanced by approximately 1 h in wild-type mice; although CLOCK (Delta19) mutation produces normal rhythmicity with an endogenous period of 26-27 h, the rhythms can be changed by light exposure. Recently, the effects of melatonin administration were evaluated in hypertensive TGR (mRen2)27 rats and the indole caused a phase-dependent action on clock genes: *Per2* was reduced in the dark and increased during the light phase whereas *Bmal1* was reduced in the light and increased during the dark phase (46). The authors suggest that melatonin may synchronize *Per* and *Bmal1* in the heart when administered at night. In vitro studies were also conducted to document the effects of melatonin on clock genes. To demonstrate the involvement of MT1 receptor and melatonin in the regulation of neuronal clock gene expression, cells from striatum were isolated and cultured and melatonin (1 nM) was added at pre-determined periods. Melatonin binding to MT1 affected the expression of *Per1, Clock, Bmal1*, and *Npas2*, decreasing *Per1* and *Clock* with no change in *Bmal1* expression. When the MT1 receptor was knocked out, the melatonin-induced changes were reversed (47).

There are a number of studies relating to the synthesis and secretion of melatonin with expression of clock genes in human diseases. In idiopathic REM sleep behavior disorder (RBD), patients lacked circadian rhythmicity of *Per2* and *Bmal1* while *Per1* remained stable and *Per3* dropped. The RBD patients had the acrophase of melatonin spread over 11 h with melatonin levels being delayed by 2 h and sleep phases were delayed by ~ 1 h with no phase shift occurring

with the clock genes (48). Patients with depression and anxiety that were treated with Ramelteon (RMT), a melatonin receptor agonist, showed clinical improvements in depression scores at 8th week and in overall sleep quality; alterations in the mRNA level of the clock genes (*Clock, Per1, Per2, Cry1, Cry2, Nr1d1, Nr2d2, Dec1* and *Timeless*) were significantly associated with RMT (49). In Alzheimer's disease (AD)-like neurodegenerative condition, the profile of melatonin in patients was found to be dampened with atypical waveforms; in these mild AD patients, clock genes *Per1* and *Bmal1* were rhythmically expressed with high amplitudes and no phase change, which suggest a moderate alteration in functional state of the circadian rhythms compared with healthy control patients (53). Optimal and periodical levels of melatonin might be one of the key events in preventing the onset of neurological disorders and neurodegeneration (Figure 2).

Experimental evidence has indicated that melatonin adjusts the pattern of clock gene expression. In an animal model of seasonal affective disorder (SAD), melatonin changed depression-like and anxiety-like behaviors and daily melatonin administration 2 h before lights off increased the amplitude of the expression rhythms of *Per1, Per2, Bmal1* and *Clock* in the SCN without changing phase shifts (51). Hardeland (52) pointed out that melatoninergic agonists (TIK-301, piromelatine, GG-012, AH-001, AH-017, agomelatine, ramelteon, GR 196429, MA-2, tasimelteon, UCM765, and UCM924) are capable of entraining circadian rhythms, if chronobiological phase is followed. In this scenario, variants of circadian genes that cause rhythm deviation such as those found in sleep disorders, bipolar disorder and seasonal affective disorders were corrected by its spontaneous oscillation.

As melatonin is considered an output signal of the central master clock, different hormones and molecules (e.g. glucocorticoids, insulin, stressor agents, and others) may have influence on its synthesis in the pineal gland in addition to altering the expression of clock genes during the dark phase (53). In brief, new studies on melatonin secretion and supplementation based on different times and specific tissues would be important in facilitating an understanding of the variations in clock genes expression.

2. CLOCK GENES AND MELATONIN IN CANCER: OVERVIEW OF THE MAIN CLOCK MOLECULES INVOLVED IN CARCINOGENESIS

Circadian clock genes play crucial roles in normal physiology, and robust epidemiological evidence and medical studies have demonstrated the association of disrupted clock genes with cancer (54-57). An important recent systematic study by Liu et al. (58) evaluated the dysregulated clock genes at different molecular levels in 20 types of cancer. Of note, clock genes are dysregulated at the expression level and their downregulation in tumor tissues was correlated with hypermethylation rather than with mutations or copy number alterations. The authors developed a circadian clock index to represent the expression of clock genes and observed that their changes are often associated with viral infections, different signaling pathways, i.e., MAPK, JAK-STAT, protein export, mismatch repair, nucleotide excision repair, cell cycle, and overall survival.

Misalignment or desynchronization is often associated with flattened rhythms that may perturb the dynamics of the circadian cycle while enhancing the susceptibility for many diseases including cancer. Circadian rhythm disruption is associated with hormone-related cancers that are frequent in shift workers. This is supported by epidemiological studies which observed that reduction in melatonin levels by light-at-night (LAN) may be associated with increased risk for breast, endometrial, prostate, non-Hodgkin lymphoma, and colorectal cancers (14, 15, 59, 60).

The molecular events whereby melatonin counteracts cancer progression are vast and include changes in LA uptake/13-HODE formation, cAMP levels, Warburg effect, oxidative and hormonal status, cellular dynamics, like many others (61). Through its MT1 and MT2 receptors, melatonin mediates its oncostatic effects in various cancer models. Depending on the cellular activity, the receptors can form homo- or heteromeric complexes to promote a diversity of signaling capacity (62); this is of significant value to improve our understanding of the pharmacological effects of melatonin on cancer cells and what is the involvement of circadian rhythm regulation.

Figure 3 summarizes how clock genes often oscillate in cancer cells and the role of melatonin as a clock synchronizer. Here, we emphasized only those cancers in which melatonin is proven to have clock effects.

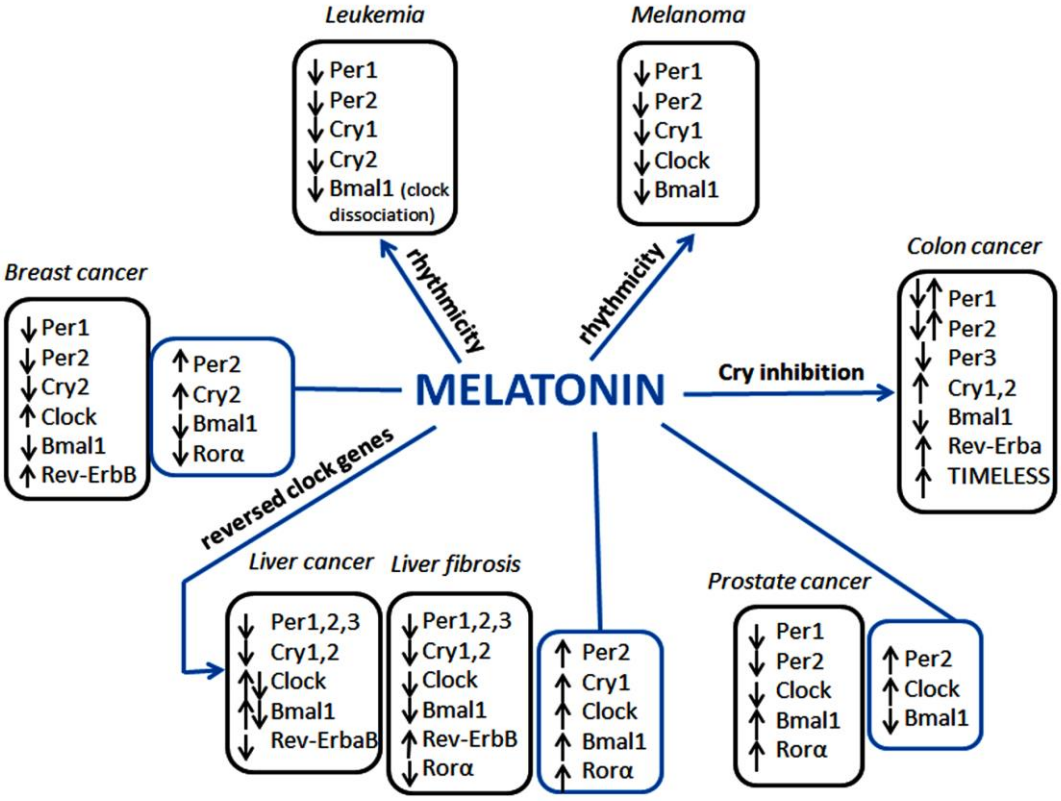

Fig. 3. Effects of melatonin on the clock genes.

Altered expression of clock genes is a common feature in cancer cells and these genes show a distinct pattern of oscillation. Down- or up-regulation of the clock genes which are described to appear in leukemia, melanoma, and breast, liver, prostate, and colon cancer are present in the black boxes. Through its influence on clock machinery, melatonin alters or resynchronizes a number of these genes similar to that observed in normal cell (blue lines and blue boxes). Up arrow: overexpression; down arrow: underexpression; Per: period; Cry: cryptochrome; Clock: circadian locomotor output cycle kaput; Bmal1: brain and muscle Arnt-like protein-1; Rev-Erb: nuclear receptor; Rorα: RAR-related orphan receptor alpha; TIMELESS: protein involved in the circadian clock.

CCG basically controls most of the molecular functions in cancer cells, playing a central role as oncogenes or tumor-suppressor genes (e.g. Clock and Bmal1). Disruption of circadian timekeeping is positively associated with cell proliferation, migration, invasion, and metabolism of cancer cells (63, 64). The first and obvious prediction that shifting working women are at higher risk of breast cancer (BC) than day-working women was observed in the mid-1980s by Stevens (65) and was associated with suppression of melatonin by the pineal gland. Later, disruption in circadian rhythms by LAN (e.g., night shift work), chronic jet-lag or altered sleep-wake cycles was correlated with increased risk of various hormone-dependent cancers. The International Agency for Research on Cancer has classified the "shift-work involving circadian disruption" as being probably carcinogenic (Group 2A) to humans (66).

Regarding to circadian clock, *Per* genes control circadian rhythms and may exert tumor suppressive function. Because methylations in the *Per1* and *Per2* promoters are associated with BC (67), primary tumors have decreased expression of *Per1* and *Per2* compared to normal tissues (68). Otherwise, overexpression of *Per2* abolishes cell proliferation in cultured cells and in an animal model (69, 70). On one hand, mutations in *Per2* showed a shorter circadian period and a great susceptibility to develop other malignant cancers induced by radiation (71, 72). Conversely, expression of *Per1* and *Per2* failed to orchestrate circadian rhythms in C3HFej/HeB mice with mammary cancer (73) while overexpression of the clock gene *Per2* in pancreatic carcinoma cells was associated with tumor growth inhibition and a pro-apoptotic effect in addition to potentiating the synergistic actions of cisplatin in these cells (74). In BC, estrogen receptor-α (ERα) signaling is related to the disruption of *Per2* as they shown rhythmic oscillations. Thus, 17β-estradiol (E2) binds to ERα and regulates the transcription of *Per2* (75, 76). Suppression of *Per2* results in ERα stabilization while overexpression of *Per2* inhibits BC cell growth and induces apoptosis (77, 78). The Clock/Bmal1 system is also important for BC. As *Bmal1* acts as tumor suppressor, its expression is often downregulated in BC cells (79), and in contrast, REV-ERBβ, which is the repressor of *Bmal1*, appears to be upregulated and may allow cancer cells to develop chemoresistance (80). Moreover, deletion of *Bmal1* gene is capable of sensitizing both non-tumorigenic MCF-10A cells and transformed breast epithelial MDA-MB-231 cells to cisplatin and doxorubicin-induced apoptosis while enhancing the invasive capacity of MDA-MB-231 cells. This effect was observed to be dependent or independent of circadian cycle and reflects an opposing carcinogenic effect. In addition, *Clock* is a tumor driver and its knockdown is associated with reduced BC proliferation together with downregulation of other genes, such as Ccl5, Bdkrb2, and Sp100 (81, 82). In conclusion, *Clock* methylation significantly reduces the risk of BC. A whole-genome expression study involving *Clock* silencing showed that Clock plays a prominent role in regulating BC-related signaling pathways (81).

A recent review that evaluated 15 epidemiological studies summarized the crosstalk between circadian gene polymorphisms and BC and indicated that *Bmal1*, *Bmal2*, *Clock*, *Npas2*, *Cry1*, *Cry2*, *Per1*, *Per3* and *Timeless* are potential candidates of BC risk (83). Melatonin exhibits a highly consistent action on clock genes in BC. In MCF-7 and MCF-10A human BC, melatonin blunts the transactivation of RORα1, via the MT1 receptor, thereby affecting *Bmal1* expression (84). Since melatonin regulates *Bmal1* expression, and *Sirt1* is downregulated by BMAL1, administration of melatonin (10^{-8} M) significantly suppressed the expression of *Sirt1*. Comparing the effects of melatonin (1 nM) in non-tumorigenic MCF-10A cells and in tumorigenic MCF-7 cells revealed an opposite action in most of the core clock genes. While melatonin did not alter the amplitude or timing of their rhythms in MCF-10A, large increases in the amplitude of *Per2* and *Cry2* expression and modest repression of *Bmal1* and *Rorα1*were found in MCF-7. These

mRNA levels remained elevated or suppressed over the entire period of 24 h (85). Hill et al. (86) showed that melatonin binding to MT1 receptor abrogated estrogen and progesterone stimulation of mammary gland and modulates the activity of RORα, RAR, RXR, ERα, and PPARc. SIRT1 was also downregulated by melatonin; melatonin-mediated apoptotic responses are complex and involve the release of p53 and p73, both of which participating in early and late apoptosis (87).

Several cancers are often affected by deleterious external circadian inputs and, as a consequence, this results in rapid tumor progression. Polymorphic variants of the main clock genes (*Per*, *Cry*, *Clock*, *Bmal1*, *Rora* and *Timeless*) are involved with the development of endocrine-related cancers likely due to changes in hormonal regulation (88). In ovarian cancer (OC), *Per1*, *Per2*, *Cry2* and *Clock* genes are significantly reduced compared with those in normal ovaries. Because of the elevations in *Cry1* and *Bmal11* expression, they could be considered possible candidates for prognostic index in epithelial OC (89). Epigenetic silencing of *Bmal1* is common in OC, and its overexpression inhibits cell growth and enhances the chemosensitivity of OC cells to cisplatin (90). Recently, inhibition of *Clock* expression reversed the cisplatin resistance of SKOV-3 cells by affecting autophagy-related protein expression (91). An association between circadian disruption by rotating work schedule and risk of fatal OC has been reported by Carter et al. (92). In the context of circadian rhythms, serum levels of melatonin are significantly lower in women with OC than in healthy women, which can be involved with the pathogenesis of OC (93). Even though no study has demonstrated a relationship between melatonin and clock genes in OC, a number of beneficial effects using physiological doses of melatonin in the treatment of OC have been recognized and mechanistically proven (94-98). Whether the molecular changes involve clock gene machinery is the subject of future investigation.

Circadian genes are correlated with prostate cancer (PCa) occurrence, and disturbed activity of the clock work is an important additional mechanism for PCa progression and its aggressive behavior (99). A population-based case-control study with 1,515 men showed an association with PCa circadian gene variants estimated in 31 clock genes. At the gene level, a significant correlation was observed between PCa and *Npas2* and *Per1* while only *Rorα* was significant for aggressive PCa (100). The rhythmicity of androgen synthesis is damaged in PCa patients and expression of *Per1* and *Per2* is reduced while *Bmal1* is increased (101). Notably, administration of melatonin suppressed PCa progression via different mechanisms, and melatonin receptor antagonized with AR related to its suppressive effects on clock genes. Comparing androgen-dependent or -independent PCa cells with normal prostate cells, Jung-Hynes et al. (102) reported that CLOCK and PER2 protein levels were downregulated while BMAL1 protein levels were upregulated. In this experiment, overexpression of *Per2* resulted in a dramatic loss of cell growth and viability. Melatonin treatment (1 mM) reversed the condition by increasing *Per2* and *Clock* and reducing *Bmal1* in addition to resynchronizing the oscillatory circadian genes (*Dbp* and *Per2*); CLOCK and PER2 protein levels were also upregulated to a level similar to that found at the gene level. Analysis of 96 single-nucleotide polymorphisms across circadian-related genes in a PCa cohort study demonstrated no correlation between the core circadian clock and fatal PCa; conversely, a strong gene-based association with fatal PCa and lower levels of 6-sulfatoxymelatonin was found (103).

Bmal1 is repressed by the P2-HNF4α, an isoform of the hepatocyte nuclear factor 4 alpha (HNF4α), which is induced in human hepatocellular carcinoma (HCC) (104). These authors also reported the molecular mechanisms underlying the incompatibility and demonstrated that forced expression of *Bmal1* prevents in vivo tumor growth. Recently, Ma et al. (63) showed that

rearrangement of F-actin is regulated by CLOCK and BMAL1 in HepG2 and HeLa tumor cells. In these cells, RHOA (ras homolog family member A), a member of the RHO family, was upregulated by CLOCK and BMAL1. This upregulation is likely due to inhibition of CUL3-mediated ubiquitination and a reduction in the interaction between RHOA and RhoGDI, thus altering the dynamics of F-actin/G-actin turnover and promoting cancer progression. Using HepG2 cells, Polo et al. (105) showed a network profile of dysregulated genes and concluded that *Clock* gene is associated with the Hub genes via cytoskeleton associated protein 5 (CKAP5). The genes that associate the circadian system with liver cancer are reported to encode a number of disordered proteins. Chronodisruption by *Per*, *Cry*, *Clock* and *Bmal1* reductions seem to accelerate human HCC (106). To prove the divergent effects of *Per* and *Cry*, male *Cry1*$^{-/-}$ *Cry2*$^{-/-}$ mice exposed to diethylnitrosamine (DEN) were evaluated. Interestingly, DEN exposure of *Cry1*$^{-/-}$ *Cry2*$^{-/-}$ mice increased primary liver cancer nearly 5-fold higher than wild type (WT) mice and the number of cholangiocarcinomas in *Cry1*$^{-/-}$ *Cry2*$^{-/-}$ mice was 8-fold higher than WT mice (107).

An interesting study by Kettner et al. (108) showed that circadian disruption by experimentally chronic jet lag resulted in persistent deregulation of liver gene expression, culminating in altered metabolism and development of HCC. The molecules which were shown to be involved in this process were bile acid receptor FXR and xenobiotic receptor CAR. Liver fibrosis, biliary senescence, and enhanced clock genes (*Clock*, *Per1*, *Per2*, *Cry1*, *Bmal1*) are associated with reduced melatonin synthesis in cholestatic rats (109); melatonin seems to decrease the clock genes and, acting via MT1 and miR-200b, promotes protection against cholestasis-induced damage and ductular reaction. Dysregulation of the circadian clock is also observed in mice with CCl_4-induced hepatic fibrosis; in the situation, melatonin (100 or 500 μM) efficiently upregulated *Bmal1*, *Clock*, *Per2*, *Cry1*, and *Rorα* expression, providing molecular insights into its protective effects (110). In a model of DEN-induced HCC, melatonin (5 or 10 mg/kg) lowered the incidence of preneoplastic/neoplastic lesions while inhibiting the expression of various cell cycle regulatory proteins and cell survival molecules (111). These authors further reported that in DEN-induced HCC, the expressions of *Bmal1*, *Clock*, *Rorα*, and *Sirt1* are increased whereas *Cry1*, *Per1*, *Per2*, *Per3*, *Rev-Erba* and *Rev-Erbb* are decreased. In this experiment, melatonin prevented changes in the expression of clock genes and, consequently, regulated the cell cycle especially through upregulation of its MT1 receptor (112).

Activation of the circadian gene *Timeless*, which is dependent on ERK, seems to be higher in a number of proliferating cancer cells and is associated with poor prognosis (113-115). In colon cancer cells, TIMELESS depletion in association with reduced levels of Wee1 or CHK1 promote decreased metabolic capacity; this is accompanied by elevated DNA damage (via γH2AX) and cell cycle (G2/M) arrest (116). Considering other circadian genes, Krugluger et al. (117) reported that downregulation of *Per1* is strongly correlated with high-grade colon cancer and disruption of circadian rhythms was described as being involved with colorectal liver metastasis (56). Down-regulation of *Bmal1* expression has been associated with tumor growth in mice (118). *Bmal1* suppression decreased etoposide-induced apoptosis and DNA damage induced by cisplatin while reducing the number of cells in the G2/M phase in colon cancer cells (C26) and fibroblast cells (L929). Mechanistically, a lack of *Bmal1* promoted reductions in *Per1*, *Per2*, *Per3*, *Wee1*, and *p53* expressions and increased levels of cdc2 and cyclins B1, D1 and E. These alterations support a role for *Bmal1* in regulating apoptosis, cell-cycle progression and DNA changes which influence tumor development and anti-cancer treatment.

Supporting these findings, Soták et al. (119) reported that *Per1, Per2, Rev-Erba* and *Dbp* are enhanced in colorectal tumors and the rhythmic expression of *Bmal1* is abolished. More recently, Korkmaz et al. (120) noted that knockout of *Bmal1* gene using CRISPR technology has different impact on apoptosis and cell invasion, affecting molecular events in carcinogenesis. Alternatively, overexpression of the *Bmal1* has been linked to colon cancer cell inhibition and high sensitivity to oxaliplatin in in both vitro and in vivo models. Higher *Bmal1* levels were also associated with longer survival of patients compared to those with low *Bmal1* levels (121). In elderly subjects, *Cry1* and *Cry2* are overexpressed in colorectal cancer and correlates with a poor survival rate and different treatment response to 5-fluorouracil and oxaliplatin (122). Melatonin levels are negatively correlated with colon cancer, and its MT1 receptor is significantly decreased in colorectal cancer patients compared with healthy mucosa tissue (123). This malignant colonic tissue had different clock gene mRNA expression and higher MT1 levels were associated with *Cry1* expression, thereby providing evidence for a role of melatonin in human colorectal cancer.

Epigenetic changes in *Bmal1*gene are associated with hematologic malignancies, including diffuse large B-cell lymphoma and myeloid and lymphocytic leukemia (124). In these cells, *Bmal1* is silenced by promoter CpG island hypermethylation and re-introduction of *Bmal1* results in inhibition of colony growth in nude mice. The epigenetic inactivation of *Bmal1* further modifies the expression of c-Myc, catalase, and p300 in their related promoters in addition to preventing their association with CLOCK protein, thus enhancing the damage of the circadian rhythms in these malignant cells. Daily pattern expressions of clock genes (*Per1, Per2, Per3, Cry1, Cry2* and *Bmal1*) are altered in chronic myeloid leukemia and indicate an association with pathogenesis and development of the disease (125). In a murine model of acute myeloid leukemia, *Clock* and *Bmal1* are required for the growth of cells and disruption of canonical circadian pathway produces anti-leukemic effects such as enhanced myeloid differentiation, impaired proliferation, and depletion of leukemia stem cells (126). Patients with chronic lymphocytic leukemia (CLL) showed downregulation in *Bmal1, Per1, Per2* and *Wee1* levels whereas c-Myc and cyclin D1 were upregulated. These effects were more pronounced and aberrant in shift-workers than non-shift-workers (127). Considering that low levels of melatonin were found in the serum of CLL patients and were still lower in shift-workers, melatonin could be considered as a biomarker of circadian disruption in CLL. Additional research involving melatonin and clock genes in leukemia is needed to elucidated possible mechanisms associated with the etiology of this disease.

Each skin component is constituted by a complex circadian organization with distinct machinery and oscillators being present in keratinocytes and melanocytes to drive the rhythmicity in the skin (128). Melanoma, the most rapidly increasing neoplasia in the white population, has high mortality rate worldwide (129). Although the functional clock of melanocytes is disrupted in melanoma, the molecular mechanisms are still not completely elucidated. Rhythmic expression of *Clock, Timeless*, *Per1*, *Cry1*, and *Bmal1* was observed in human skin, but the relationship between melanoma and clock gene polymorphisms remains to be unraveled (130). Lengyel et al (131) analyzed the expression of *Per1, Per2, Clock* and *Cry1* in human melanoma and then uncovered a possible link between clock genes and skin cancer; both mRNA and protein levels showed a reduction of 30-60% in melanoma biopsies. Notably, *Clock* gene was upregulated in non-cancerous cells but not in melanoma cells, indicating its role in altered metabolism of tumor cells. Recently, clinical results from The Cancer Genome Atlas indicated that *Bmal1* is positively correlated with overall survival in melanoma patients; in

pretreatment samples, high *Bmal1* expression was associated with important benefits from immune checkpoint inhibitors (132). Alterations in the expression of clock genes in the human skin seem to be an early event during tumor development (133). These authors documented that disruption in melatonin levels by LAN or other light disturbances might predispose to the development of skin cancer, especially melanoma. As melatonin protects against harmful UV light, its suppression can shift circadian rhythms thus promoting melanoma. Experimental evidence with C57 mice with melanoma showed that melatonin (2 mg/kg BW/day) reduced tumor weight and metastatic dissemination and improved circadian rhythmicity. It should be emphasized that endogenous rhythms and melatonin administration cooperate when considering that the subjective night restricts melanoma development (134). Given that dermatoses are related to oxidative, photo-, and radiation-induced damage, melatonin is attractive to provide reparative mechanisms and even prevent skin cancer. Due to its low toxicity, its topical and transepidermal application represent a promising area for future investigation in dermatotherapy (135).

Sulli et al. (136) mentioned the role of REV-ERBs as being lethal in cancer and oncogene-induced senescence. After testing the activity of the pharmacological agonists of REV-ERBα and REV-ERBβ, termed SR9009 and SR9011, they found an impressive anticancer effect in glioblastoma due to alterations in a number of oncogenic drivers (HRAS, BRAF, PIK3CA and others) which even persisted in the absence of p53 and under hypoxic conditions. It is believed that these REV-ERB agonists are inhibitors of autophagy and de novo lipogenesis and have a selective action in malignant and benign tumors. More recently, melatonin potentiated the anticancer effects of the synthetic REV-ERB agonist 'SR9009' in human Hep3B cells (112).

Mechanistically, solid and numerous data exist on how a specific rhythm dictated by the circadian clock of the tumor cell is involved in initiation, cancer progression and metastasis. However, understanding the initial steps by which a normal cell becomes susceptible and is altered by the molecular circadian machinery and what are the clock-related events responsible to these cellular transformations could be helpful in preventing or treating specific cancers. Chronochemotherapy, which is the administration of antitumor agents at specific period of the day to achieve optimal results with fewer side effects, has been preconized to be based on the molecular link between the clock elements and the DNA damage response (e.g., excision repair, checkpoints and apoptosis). Recognizing the intrinsic molecular core that controls the timing of cell reactions and the cellular responses to physical or chemical stimuli may provide additional cues to develop new strategies for chemotherapeutic regimens (137).

3. MELATONIN INFLUENCES CLOCK GENES IN TUMOR CELLS VIA DIFFERENT PROCESSES

Figure 4 depicts possible mechanism(s) whereby melatonin influences clock genes and related molecules in cancer cells. There is an old long-standing and cooperative relationship between SIRT1, a histone deacetylase involved in chromatin remodeling and then in gene activation, and melatonin (138). SIRT1 activity depends on a circadian rhythm of nicotinamide phosphoribosyltransferase (NAMPT) synthesis, the limitating enzyme involved in the biosynthesis or salvage of NAD^+, which is controlled by BMAL1 (139). In terms of cellular oscillators, NAD^+-driven activity of SIRT1 modulates CLOCK/BMAL1 complex possibly by acting as an antagonist of the protein acetyltransferase CLOCK which deacetylates BMAL1. Another important function of SIRT1 is related to the enhancement of *Bmal1* and *Clock* gene

transcription by the ROR response element in the promoters (140). Although a positive regulation of SIRT1 by melatonin is considered to be protective against the aging process, melatonin reportedly downregulates SIRT1 in cancer cells (16, 138). This is further associated with the apoptotic and pro-oxidative action of melatonin in cancer cells compared to normal cells (141, 142). Because CLOCK/BMAL1 exert an influence on *Sirt1* promoter by two E-box elements (143), tumor cells with high CLOCK and BMAL1 levels strongly upregulate *Sirt1* expression to promote deacetylation of transcription factors thus inducing cell division (4). In this case, BMAL1 rhythms are more effective than CLOCK. Notably, melatonin is reported to decrease the expression of *Clock*, *Bmal1*, and *Sirt1* in tumor cells thereby having an anti-proliferative action (16, 144). Although the mechanism(s) whereby melatonin affects SIRT1 is not completely identified, it is important to consider that melatonin diminishes the oscillatory components to a condition closer to normal (4). Depending on specific state of the cells and tissues, melatonin might up or downregulate SIRT1 with implications in the regulation of oscillators to finally control cellular activity. Expression of *Bmal1* is generated through transactivation by RORα while it is repressed by REV-ERBα. Hill et al. (145) collected information and showed that RORα enhances *Bmal1* expression in MCF-10A and MCF-7 breast cancer cells, and melatonin, especially via MT1 receptor, blunts the transcriptional activation of RORα, finally blocking the induction of *Bmal1* expression and reducing SIRT1 levels. Through SIRT1 inhibition, melatonin can enhance the expression and activity of DNA repair proteins (BRCA1/2, Ku70, p53) and apoptotic factors such as FOXOA3, ERRα, and BAX. Also, via RARα signaling, melatonin may render tumor cells more sensitive to apoptosis.

Several cell cycle checkpoints are often associated with DNA repair after damage, and a part of them, are directly mediated by clock proteins (146). The cell cycle is affected by the circadian clock, and an altered rhythm of the circadian clock may predispose to oncogenic signaling and cancer development; the genes c-Myc, cyclin D, p21, and Wee1 are disrupted by the molecular clock whereas activation of PER2 leads to c-Myc overexpression and tumor growing (147). Hunt and Sassone-Corsi (148) revisited the regulatory elements involved in both cell cycle and circadian clock. In this review, PER1 is reported to antagonizes the cell cycle as well as the function of BMAL1/CLOCK in a similar oscillatory manner; while Per1 seems to be downregulated in human tumors, ectopic PER1 reduced the expression of Wee1, cyclin B1, and cdc2. BMAL1, PER, and CRY interact with these genes (147), and more importantly, the proteasome controls the levels of these cell cycle-related genes (149-153). Since we previously mentioned that melatonin acts as a proteasome inhibitor, its effects on inhibition of cyclin D1 expression in MCF-7 breast cancer cells (153) together with reductions in CDK2, CDK4, cyclin D1, E, and c-Myc in X02 cancer stem cells (154) might be largely associated and are further consistent with the regulatory interaction of cell cycle and clock proteins on the cancer growth and differentiation. Both melatonin and bortezomib, a proteasome inhibitor, have demonstrated a selective pro-apoptotic action in different cancer cells. Since bortezomib and melatonin indirectly interfere with cellular levels of p53, NF-kB, p21, Bcl-2, and BAX through ubiquitin-proteasome system, it may be that the combination of melatonin with proteasome inhibitors might reduce drug toxicity and resistance while increasing efficiency of treatments (24). In the context of human glioma cells, bortezomib and melatonin enhanced the sensitivity of tumor cells to TRAIL-induced apoptosis.

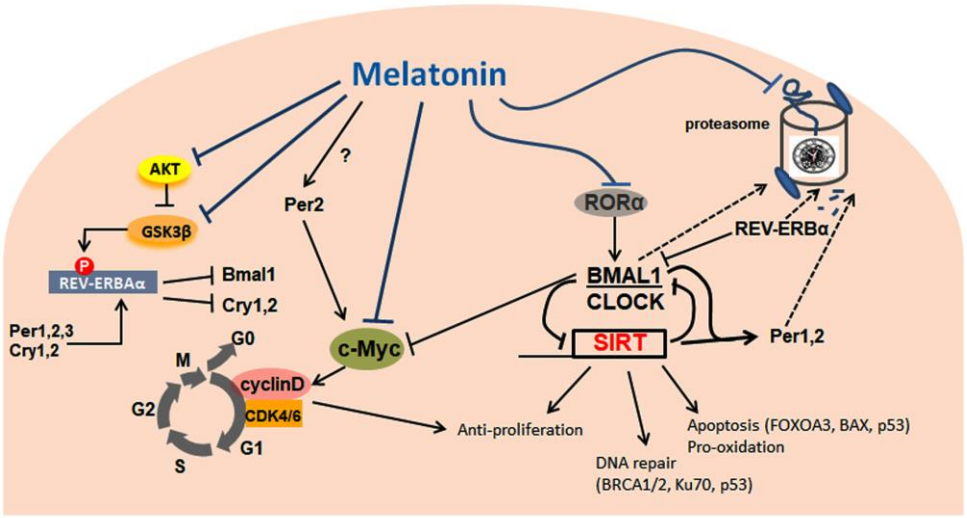

Fig. 4. Proposed mechanisms by which melatonin influences clockwork in cancer cells.

BMAL1 and CLOCK upregulate Sirt1 expression in cancer cells to promote cell division; melatonin, especially via MT1, leads to decreased expression of the Clock/Bmal/Sirt1 system by blunting the transcriptional activation of RORα, thereby having an anti-proliferative, and perhaps pro-apoptotic and pro-oxidative actions. Through proteasome inhibition, melatonin may act post-translationally to maintain the adjusted clock by stabilizing proteins depending on time of day. Cell cycle interacts with clock to mediate cancer growth and differentiation. Both CLOCK/BMAL1 heterodimers and melatonin can repress c-Myc and, therefore, cyclin D/CDKs, possibly resulting in cell cycle arrest; whether melatonin affects Per2, which is associated with c-Myc expression, remains unclear. REV-ERBAα is phosphorylated by GSK3β, a downstream target of AKT, and transcriptionally represses Bmal1 and Cry genes. As melatonin has an inhibitory effect on AKT and GSK3β phosphorylation, it could control the levels of these genes in cancer cells. Per: period; Cry: cryptochrome; Clock: circadian locomotor output cycle kaput; Bmal1: brain and muscle Arnt-like protein-1; REV-ERB: nuclear receptor; RORα: RAR-related orphan receptor alpha; SIRT: sirtuin; c-Myc: proto-oncogene c-Myc; CDK4/6: cyclin-dependent kinase 4/6; AKT: protein kinase B; GSK3β: glycogen synthase kinase 3-beta; FOXOA3: forkhead box OA3; BAX; bcl-2-like protein 4; p53: tumor suppressor; DNA: deoxyribonucleic acid; BRCA1/2: breast cancer genes 1 and 2; ku70: Ku protein required for DNA repair; P indicates protein phosphorylation; ?: uncertain mechanism.

Circadian dynamics of molecular clock rhythmically control the proliferative rate of the cells. CLOCK/BMAL1 heterodimers induce repression of *c-Myc* expression via E-box-mediated reactions in the P1 promoter (155). As activation of c-Myc promotes transition from G0 to G1 and from G1 to S and elicits p53-mediated apoptotic effects and BMAL1/CLOCK reduces cellular proliferation in the absence of DNA damage, this circadian machinery represents another cell cycle target for the control of proliferation. Consistently, melatonin has shown anti-tumor and anti-proliferative effects by reducing *c-Myc* expression at mRNA and protein levels (154). In regard to BMAL1/CLOCK system, melatonin could have a different action depending on tumor cell.

In tissue-isolated breast cancer xenograft, tumor growth rate and increased levels of ERK, AKT, and AKT stimulatory 3-phosphoinositide-dependent kinase-1 are correlated with light

exposure at night and reduced levels of melatonin (156, 157). In parallel, when REV-ERBAα is phosphorylated by the GSK3β, a downstream target of AKT, the expression of *Bmal1* and *Cry* is repressed (100). Considering that AKT inhibits GSK3β in addition to promoting cell survival, it is plausible to consider that inhibition of AKT may enhance expression of *Bmal1*. Melatonin has indeed shown a marked inhibitory effect on phosphorylation of PI3K, AKT, and GSK3β in breast cancer cells (158), which could be regulating the levels of *Bmal1*. In brief, *Bmal1* has been demonstrated to suppress cancer invasion by blocking the PI3K-AKT-MMP-2 signaling pathway (159).

4. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

It seems true that circadian output signals often feedback into the oscillators, controlling normal cell functions; the input signals determine the alignment of the molecular circadian clock and disruption of this machinery may result in cancer. Thus, it is predicted that humans can be the victims of their own habits governed by modern life style, suffering by unexpected consequences due to changes in the functional pattern of the oscillators. Depending on the damage to clock genes and the sensitivity of other cell sensors that are affected, the ultimate response will be harmful and, in most of cases, irreversible.

As melatonin is mainly released at night, it likely influences clock molecules that are increased during darkness. The contrary is also true: disruption in circadian rhythm that affects CLOCK/BMAL1 system, which contributes to arylalkylamine *N*-acetyltransferase (AA-NAT) gene expression (160), may result in deficient melatonin production. The development of melatonin-deficient models and melatonin-receptor knockout mice for testing drug activity and affinity is promising for a more comprehensive understanding of the role of melatonin and the crosstalk between cancer and clock genes activities.

Through proteasome inhibition, melatonin is shown to significantly conserve BMAL1 levels in the cells; recognizing BMAL1 as a tumor suppressor for the most of cancer cells reinforces the protective role of melatonin. Furthermore, experimental data has demonstrated that melatonin modifies ubiquitin ligases for CRY, PER, REV-ERBα and BMAL1. That being the case, melatonin may help in synchronizing clock molecules depending on time in which these proteins are active in cancer cells. In this review, we emphasize that administration of melatonin on experimental cancer models, varying from physiological up to supra-physiological or pharmacological concentrations, is capable of resynchronizing peripherally-induced rhythms which are altered in breast, prostate, liver, and skin cancer (e.g., melanoma); the adequate coordination of these clock genes may result in anti-proliferation and apoptosis of tumor cells. Whether melatonin treatment could realign circadian clockwork genes in other malignancies is the subject of future research.

Disruption in circulating melatonin levels were also associated with impaired circadian machinery in leukemia, liver fibrosis, melanoma, ovarian and colon cancer. Chronodisruption dramatically interferes with melatonin production, reducing its multifaceted actions on cell physiology and thereafter predisposing to intracellular damage (e.g., weakness of circadian clock). This can result in neoplastic transformation according to the type of molecular disorganization. Because clock genes actively work in DNA remodeling to maintain the physiological state of the cell, and recognizing the numerous benefits of melatonin in cancer cells, we include in its repertoire the potential to revert an epigenetically-imposed perturbation. Melatonin also is reduced with aging and lifetime accumulation of genetic changes associated

with decreased repair mechanisms may predispose to cancer development. Through its antioxidant and welfare properties, melatonin has been given in-depth consideration as serving as a chemopreventive agent. Investigations of the circadian cycles in the presence of melatonin may provide additional cues for improving the understanding of the molecular basis of carcinogenesis in addition to guiding optimal timing for administration of anti-cancer agents with low toxicity based on their time of administration.

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AUTHORSHIP

LGAC, LALJ, HSS, FRFS, and MSC: concept and design of the review and drafted the manuscript. RR: critical revision of the manuscript. All authors significantly contributed with compilation of the literature and approved the final version of the manuscript.

CONFLICT OF INTEREST STATEMENT

Authors declare no conflict of interest.

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