Melatonin: an inhibitor of breast cancer

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Abstract

The present review discusses recent work on melatonin-mediated circadian regulation, the metabolic and molecular signaling mechanisms that are involved in human breast cancer growth, and the associated consequences of circadian disruption by exposure to light at night (LEN). The anti-cancer actions of the circadian melatonin signal in human breast cancer cell lines and xenografts heavily involve MT_1 receptor-mediated mechanisms. In estrogen receptor alpha (ERα)-positive human breast cancer, melatonin suppresses ERα mRNA expression and ERα transcriptional activity via the MT_1 receptor. Melatonin also regulates the transactivation of other members of the nuclear receptor superfamily, estrogen-metabolizing enzymes, and the expression of core clock and clock-related genes. Furthermore, melatonin also suppresses tumor aerobic metabolism (the Warburg effect) and, subsequently, cell-signaling pathways critical to cell proliferation, cell survival, metastasis, and drug resistance. Melatonin demonstrates both cytostatic and cytotoxic activity in breast cancer cells that appears to be cell type-specific. Melatonin also possesses anti-invasive/anti-metastatic actions that involve multiple pathways, including inhibition of p38 MAPK and repression of epithelial–mesenchymal transition (EMT). Studies have demonstrated that melatonin promotes genomic stability by inhibiting the expression of LINE-1 retrotransposons. Finally, research in animal and human models has indicated that LEN-induced disruption of the circadian nocturnal melatonin signal promotes the growth, metabolism, and signaling of human breast cancer and drives breast tumors to endocrine and chemotherapeutic resistance. These data provide the strongest understanding and support of the mechanisms that underpin the epidemiologic demonstration of elevated breast cancer risk in night-shift workers and other individuals who are increasingly exposed to LEN.

Introduction

N-acetyl-5-methoxytrypamine (melatonin) is an indolic hormone that was identified by Lerner *et al*. (1960) as a factor from the mammalian pineal gland that lightens melanocytes. Melatonin production is regulated by photoperiod insofar as its synthesis and secretion are repressed by light but induced at night in response to darkness (Brainard *et al*. 2001). Consequently, a diurnal pattern of melatonin synthesis that rises during the

night but diminishes throughout the day is seen in humans. In addition to the pineal gland, there is evidence for melatonin synthesis in extra-pineal organs, including the retina, gastrointestinal tract, skin, bone marrow, and lymphocytes (Acuña-Castroviejo et al. 2014). Furthermore, the 'master biological clock' located in the suprachiasmatic nucleus (SCN) of the hypothalamus takes this photoperiodic information and modulates the circadian synthesis and secretion of melatonin (Berson et al. 2002, Hastings et al. 2003). As will be discussed later in the present report, melatonin is not only an output of the central circadian clock, but it is also an important modulator/regulator of the central circadian clock and of peripheral oscillators in tissues and organs, including the breast (Stehle et al. 2003).

Most of the normal physiologic and metabolic processes are temporally organized in response to photoperiods and are thus predictably modulated throughout the day and night, which allows the organism to anticipate and adapt to environmental changes. Similarly, the multitude of processes that govern cancer initiation, promotion, and progression, including invasion and metastasis, are temporally modulated by the host's circadian rhythmic outputs from the central circadian pacemaker in the SCN (Stehle et al. 2003, Truong et al. 2014). At the same time, the same molecular clock genes and their protein products that are seen in the master clock are expressed in both normal and cancerous peripheral tissues, and they are termed peripheral oscillators. These peripheral oscillators are coordinately expressed and regulated in a circadian manner (You et al. 2005). Numerous laboratories are currently focused on defining the importance of these clock/oscillator genes and proteins and their involvement in cellular processes, including cell cycle regulation, cell proliferation, cell survival, apoptosis, DNA damage/repair, and tumor suppression/promotion (Kohsaka & Bass 2007, Troung et al. 2014). It is now evident that the central circadian system and even peripheral oscillators play important roles in the regulation of intermediary metabolism and cancer (Blask et al. 2005, Slominski et al. 2012, Kelleher et al. 2014). Melatonin, as a consequence of its ability to be regulated as an output of the central circadian clock as well as an input to both the central clock and peripheral oscillators, also plays an important role in the regulation of intermediary metabolism and cancer (Hastings et al. 2003).

The level of melatonin in the blood is regulated by its synthesis in the pineal gland in response to dark and light as well as by its peripheral degradation, primarily in the liver (Kelleher et al. 2014). Three major pathways are involved in melatonin degradation: i) hepatic biotransformation, which generates 6-hydroxy-melatonin and secretion in the urine after conjugation with sulfate or glucoronide, ii) alternative indolic pathways, which produce 5-methoxyindole acetic acid, or 5-methoxy-tryptophol, and iii) a kynuric pathway that produces N1-acetyl-N2-formyl-5-methoxykynuramine.

A plethora of biological actions have been reported for melatonin; however, the one that is best described is its chronobiotic action as a neurohormone and entrainer to the light/dark cycle and as a synchronizer of peripheral clocks to the SCN (Slominski et al. 2012). Elevated nighttime levels of melatonin in the blood inform the body's cells and organs that it is nighttime (Slominski et al. 2012) and help organize target organs and organ systems into appropriate homeostatic metabolic rhythms. Alterations in either day length or the timing/phasing of light exposure can desynchronize SCN activity and consequently the production of melatonin by the pineal gland, a phenomenon that is referred to as circadian disruption.

Over the past two decades, significant research efforts have been made to define the role of melatonin in the etiology of cancer. In the past few years, the disruption of melatonin's circadian profile by exposure to light at night has been described as playing an important role in the development, promotion, and progression of breast cancer (Reiter 1991, Claustrat et al. 2005, Jasser et al. 2006, Reiter et al 2007, Stevens et al. 2007, Hill et al. 2011). Although light exposure at night (LEN)-induced circadian disruption can potentially impact many aspects of both the central circadian clock and peripheral oscillators, including expression of the clock genes period 1 (Per1) and period 2 (Per2), which are known tumor suppressors, in tissues and organs such as the breast (You et al. 2005), data also suggest that LEN-induced circadian disruption also dramatically inhibits the amplitude and phase of the nocturnal melatonin circadian signal in the blood. Given melatonin's role as an oncostatic/anti-cancer agent and its tightly regulated synthesis by the light/dark cycle, it is believed that the impact of LEN-induced circadian disruption on breast cancer may be heavily mediated by the disruption of the nocturnal melatonin signal (Reiter et al. 2007, Hill et al. 2011).

Although melatonin production can occur in numerous tissues (e.g., retina and gut), it is primarily synthesized in the pineal gland from the amino acid tryptophan in response to the onset of darkness (Huether 1993). As an indolamine, melatonin is highly

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lipophilic and rapidly diffuses into the blood stream for delivery to distant organs and the cerebrospinal fluid to bathe the hypothalamus and CNS. The fact that melatonin's biological activity is mediated via two major mechanisms – G-protein coupled receptor (GPCR)-mediated activity and non-receptor–mediated antioxidant activity – is borne out in the literature (Dubocovich *et al.* 2003, 2010).

The objective of the present article is to review the current literature, including our own studies, on the role played by the nocturnal melatonin signal in the inhibition of breast cancer promotion, drug resistance, and metastasis via its regulation of tumor metabolism, signal transduction, gene expression (Dauchy *et al.* 2014), genomic instability and Line 1 expression (Belancio *et al.* 2015), reactive oxygen species (ROS) (García *et al.* 1988), and peripheral oscillators (Xiang *et al.* 2012) using *in vitro* and *in vivo* models of human breast cancer. We also review recent studies that focus on the impact of circadian disruption of the melatonin nocturnal signal by LEN and dim LEN (dLEN) on breast cancer progression, promotion, and drug resistance.

Melatonin receptors in breast cancer

Many of the anti-cancer effects of melatonin are mediated primarily through binding to the two membrane-associated GPCRs. The two GPCRs found in humans and mammals are the MT_1 melatonin receptor (formerly the Mel1a), which is encoded by the *MTNR1A* gene, and the MT_2 melatonin receptor (formerly the Mel1B), which is encoded by the *MTNR1B* gene (Brydon *et al.* 1999, Dubocovich *et al.* 2010). Both MT_1 and MT_2 receptors are expressed in various organs, but they show different yet overlapping expression profiles and overlap in their variable coupling G protein and signal transduction pathways. Numerous reports have demonstrated that melatonin, via binding and activation of the MT_1 and MT_2 GPCRs, modulates the activity of a variety of G proteins, including $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_q$, and $G\alpha_{11}$ (Kiefer *et al.* 2002, Lai *et al.* 2008). The MT_1 receptor is coupled to the inhibition of cAMP via pertussis toxin-sensitive inhibitory $G\alpha_i$ proteins (Dinet & Korf 2007, Lai *et al.* 2008). Activation of the MT_1 receptor promotes the inhibition of forskolin-stimulated cAMP formation, the suppression of protein kinase A (PKA) activity, and the phosphorylation of the CREB (Kiefer *et al.* 2002, Dinet & Korf. 2007). Melatonin, via activation of the MT_1 receptor, has also been reported to modulate ion channels in cells (Steffens *et al.* 2003, Schuster 2007).

The MT_2 receptor also couples to G proteins to inhibit forskolin-stimulated cAMP production, the modulation of cGMP formation, and an increase in protein kinase C (PKC) activity (Witt-Enderby *et al.* 2003). The complexity of melatonin signaling through melatonin receptors is compounded by the fact that MT_1 and MT_2 receptors can form homo- or hetero-dimers to modify receptor function and activity (Ayoub *et al.* 2002). Levoye *et al.* (2006) have also identified the GPR50 as a melatonin-related receptor with 45% identity to the $MT_{1/2}$ melatonin receptors. The GRP50 is unable to bind melatonin, but it can dimerize with MT_1 and MT_2 receptors to suppress the affinity of MT_1, but not MT_2 for melatonin.

The activation of the MT_1 receptor appears to mediate much of melatonin's oncostatic actions in ERα-positive MCF-7 human breast cancer cells. Employing MCF-7 human breast cancer cells, we reported that melatonin-activated MT_1 receptors couple to $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_q$, and $G\alpha_{11}$ proteins (Lai *et al.* 2008). Overexpression of the MT_1 receptor can potentiate the anti-proliferative effects of melatonin on breast cancer cells both *in vivo* and *in vitro*, but these effects are reversed by nonselective MT_1 and MT_2 melatonin receptor antagonists (Collins *et al.* 2003, Yuan *et al.* 2002). Furthermore, using confocal microscopy, we demonstrated that the MT_1 receptor localizes to the cell membrane in breast tumor cells, with some co-localizing with caveolin-1 in membrane-associated signaling lipid raft platforms (Lai *et al.* 2009). Analysis of MT_1 receptor expression in a small sample of 50 breast tumor biopsy specimens revealed a positive correlation between the MT_1 receptor and ERα expression. Jablonska *et al.* (2013) also demonstrated that expression of the MT_1 receptor in breast tumors correlates with ERα expression and is an independent prognostic marker in ERα-positive breast tumors for overall survival and event-free survival. Recently, Oprea-Iles *et al.* (2013) reported a higher incidence of MT_1-negative tumors in African American women (48%) as compared to Caucasian women (11%) in a cohort of 167 triple-negative breast cancer (TNBC) patients. Furthermore, in TNBC, MT_1 positivity was associated with a lower stage and smaller tumor size at the time of diagnosis, whereas MT_1 negativity in TNBC was significantly associated with a higher risk of disease progression, shorter progression-free survival, and disease-related death regardless of race. To date, a number of laboratories have demonstrated MT_1 expression in human breast tumor biopsies in a variety of breast tumor types, ranging from ERα-positive (luminal A) to TNBC (basal-like). All studies to date have correlated MT_1 expression with an improved prognosis

MT$_1$/MT$_2$ receptor independent actions of melatonin

Melatonin can bind and activate cell membrane–associated MT$_1$/MT$_2$ GPCRs (You et al. 2005, Kohsaka & Bass 2007, Truong et al. 2014). The lipophilic nature of melatonin also allows it to transverse the cell, nuclear, and even mitochondrial membranes to bind to cytosolic, nuclear, and mitochondrial proteins, which elicits a variety of non-receptor mediated effects in breast cancer. Numerous studies have shown that melatonin binds the Ca^{2+}-regulatory protein calmodulin (CaM), which leads to decreased sensitivity of adenylate cyclase (AC) in binding to CaM (Dai et al. 2002, Schuster et al. 2005). Repressed AC activity is associated with reduced cAMP levels within cells, which can lead to altered PKA, CREB, and p300 co-regulator expression/activation as well as to the attenuation of phospho-activation and transactivation of various transcription factors and nuclear receptors (NRs), including ERα (Kiefer et al. 2002, Del Rio et al. 2004, Sánchez-Barceló et al. 2005).

It was originally reported by Becker Andre et al. (1994) that melatonin binds as a ligand to the retinoic acid-related orphan receptors alpha (RORα), which are members of the NR/steroid receptor superfamily. That report, however, was withdrawn (Erratum 1997), when other laboratories working on RORα were unable to reproduce melatonin's binding to these receptors. Unfortunately, the fact that melatonin is not a ligand for the RORα receptor has not been well accepted by all groups studying melatonin, and the literature is rife with discussions of melatonin as a ligand for RORα. As is discussed later in the present report, melatonin can in fact modulate RORα transcriptional activity via activation of its MT$_1$ receptor.

Initial reports by Poeggeler et al. (1993) that identified melatonin as a potent free radical scavenger have been confirmed by many other groups that further demonstrated that melatonin impacts quinone reductases to reduce oxidative damage by ROS in various tissues, including breast tumor cells. These reports also confirm that this effect of melatonin is not mediated through MT$_1$ or MT$_2$ receptors. Furthermore, Blask et al. (1997) showed that administration of melatonin to MCF-7 and ZR-75-1 breast cancer cells in vitro induced the expression of the potent antioxidants glutathione and glutathione-S-transferase, which also promoted the inhibition of

tumor metabolism by leading to the suppression of cell proliferation. Other non-receptor-mediated effects of melatonin include its immune system modulation (Lissoni et al. 1991, Pawlikowski et al. 2002, Carrillo-Vico et al. 2003), its tumor surveillance (Cos & Sanchez-Barcelo 2000), and its ability to decrease telomerase activity (Leon-Blanco et al. 2003).

Anti-proliferative actions of melatonin in breast cancer

Numerous studies have shown that melatonin exerts oncostatic effects on a variety of malignancies (Hill et al. 2011), but its effects on breast cancer have been studied most extensively. Both clinical data and animal studies have provided evidence that melatonin reduces the incidence of experimentally induced cancers (Tamarkin et al. 1981, Blask et al. 1991, Teplitzky et al. 2001) and significantly inhibits the growth of some human breast tumors (Hill & Blask 1988, Hill et al. 1992, Blask et al. 2011, Mao et al. 2014). In general, it has been found that melatonin exerts both cytostatic anti-proliferative effects and cytotoxic apoptotic effects in breast cancer cells via a variety of mechanisms (Blask 2009, Mediavilla et al. 2010). We reported in 1988 that ERα-positive MCF-7 breast cancer cells were growth-inhibited by physiologic concentrations (1 nM) of melatonin (Hill & Blask 1988). Subsequent studies have validated that melatonin suppresses the proliferation of both ERα-positive and ERα-negative human breast tumor cell lines as well as various animal models of mammary cancer (Hill et al. 1992, 2011, Mao et al. 2014). Most studies support the suggestion that the majority of melatonin's anti-proliferative actions are mediated via activation of the MT$_1$ receptor, because MCF-7 cells that have been transfected with the MT$_1$ receptor (Collins et al. 2003, Yuan et al. 2002) show significantly enhanced anti-proliferative activity in response to melatonin. A variety of receptor-mediated mechanisms have been described for melatonin's anti-proliferative actions, including its repression of ERα transcriptional activity in ERα-positive breast tumor cells (Kiefer et al. 2002, Ram et al. 2002), its inhibitory actions on Ca^{2+} signaling and CaM expression (Dai et al. 2002), and its induction of p53 expression (Proietti et al. 2013) as well as its target gene p21 (Mediavilla et al. 1999). Work by Santoro et al. (2013) demonstrated that the blockage of MT$_1$/MT$_2$ receptors in MCF-7 cells impairs p53-mediated prevention of DNA damage. Other mechanisms associated with the anti-proliferative actions of melatonin include cell cycle with

the arrest of breast tumor cells in the G1 phase of the cell cycle (Cos *et al.* 1991), the repression of estrogen synthesis in the gonads (Blask *et al.* 2011) or the tumor by the repression of aromatase (Martínez-Campa *et al.* 2009), and finally the suppression of tumor metabolism (the Warburg effect) via the repression of the tumor uptake of free fatty acids (FAs), particularly linoleic acid (LA) and its conversion to 13-hydroxyoctadecadienoic acid (13-HODE) (Reiter 1991, Blask *et al.* 2014).

Melatonin modulation of the estrogen/ERα-signaling pathway

Early reports that melatonin could suppress the synthesis of gonadal steroids such as estrogen (Woo *et al.* 2001) were the first indication of crosstalk between melatonin and the estrogen signaling pathway. Subsequent studies, including our own (Hill & Blask 1988, Hill *et al.* 2011), which showed that melatonin inhibited the estrogen-mediated proliferation of human breast cancer cells in culture, suggested that melatonin influences estrogenic actions on breast and mammary tissue by three key mechanisms: i) suppression of the gonadal synthesis of steroids (including estrogens) to decrease their circulating levels; ii) interaction with the ERα as a selective ER modulator (SERM) to alter estrogen binding, DNA-binding, and transcriptional activity; and iii) repression of the activity of other enzymes, such as aromatase, sulfatase, and aldo-keto reductases (AKRs), which are involved in the synthesis of estrogens from cholesterol or other steroids (González *et al.* 2007, Dauchy *et al.* 2014).

Initially, melatonin was shown to be a regulator of reproduction in seasonally breeding animals under natural photoperiods (Reiter 1980) through its regulation of the hypothalamic–pituitary axis and gonadal activity (Barrett & Bolborea 2012). This regulation of the hypothalamic–pituitary–gonadal axis by melatonin resulted in the suppression of ovarian estrogen synthesis (Bondi *et al.* 2014). Although humans are not seasonal breeders and the impact of melatonin on human reproductive physiology is not completely clear (Tamura *et al.* 2008), melatonin does exert some modulatory action on steroidogenesis in human granulosa-luteal cells (Woo *et al.* 2001). Because estrogen plays a pivotal role in breast cancer etiology, these data suggested that melatonin might be an important inhibitor of breast cancer.

Our early studies demonstrated that physiologic concentrations of melatonin (1 nM) were able to suppress the proliferation of ERα-positive human breast cancer cell lines *in vitro* (Hill & Blask 1988). Subsequently, we and others have found that melatonin inhibits the proliferation of some ERα-negative breast cancer cell lines and that differences in melatonin sensitivity are evident between different cell lines (Hill *et al.* 1992, 2011). Employing ERα-positive MCF-7 breast cancer cells, Molis *et al.* (1994) reported on the melatonin-mediated suppression of ERα mRNA. Kiefer *et al.* (2002) and Ram *et al.* (2002) were the first to show that melatonin is a potent repressor of estrogen-induced ERα transcriptional activity that inhibits the expression of numerous estrogen-induced mitogenic and anti-apoptotic genes, including *Bcl2*, while inducing growth-inhibitory and pro-apoptotic genes, including *TGFα* and *Bax*. Kiefer *et al.* (2002) and Lai *et al.* (2008) subsequently demonstrated that melatonin activation of $G\alpha_{i2}$ proteins to decrease cAMP/PKA levels and phosphorylation of the ERα serine 236 (s236), a PKA sensitive site, mediates melatonin's inhibition of ERα transcriptional activity. The involvement of CaM in melatonin's repression of ERα transcriptional activity, as described by Del Rio *et al.* (2004), is consistent with the reported modulation of the Ca^{2+}/CaM pathway by PKA (Barrett & Bolborea 2012). Subsequently, Dai *et al.* (2002) have confirmed that melatonin modulates Ca^{2+} and CaM expression and signaling in MCF-7 breast cancer cells.

We employed chromatin immunoprecipitation analysis of the cyclin D1 promoter to show that melatonin blocks 17-β estradiol, the recruitment of CaM, and p300 to the cyclin D1 promoter (Hill *et al.* 2011). In the absence of estrogen, this effect is considerably blunted; it effects a delay in, a decrease in, and, to a lesser degree, the recruitment of p300 to the cyclin D1 promoter. Over a 90 min time course, melatonin, in the absence of estrogen, induces an oscillation in CaM recruitment to the cyclin D1 promoter that is anti-phase with the recruitment that is seen in the presence of estrogen. Surprisingly, if estrogen peaks in the early morning, a daily rhythm is evident in the blood (Bao *et al.* 2003, Royston *et al.* 2014). Because plasma melatonin levels typically peak between midnight and 0200 h and then decline as estrogen levels begin to rise, melatonin may be able to differentially influence CaM and p300 recruitment to the cyclin D1 promoter and other promoters in order to regulate gene transcription during the night (Hill *et al.* 2011).

The mechanism(s) involved in melatonin's modulation of ERα transcriptional activity are still not completely resolved, but they appear to involve changes in the phosphorylation of receptor and/or coactivator (e.g., CaM, steroid receptor coactivator 1 (SRC-1), cAMP binding protein (CBP)/p300, etc.) expression and phosphorylation. A recent report by Dauchy *et al.* (2014) demonstrated that dLEN, by its suppression of the

nocturnal melatonin circadian signal, induced the phosphorylation of the ERα at s167 and s118 via the induction of ERK1/2, protein kinase B (AKT), and c-SRC kinase. Notably, supplementation with exogenous melatonin during dLEN significantly repressed ERα phosphorylation at these sites. These data strongly suggest that melatonin may have an important influence on gene expression in human breast cancer cells, and that influence is mediated by the actions of the MT$_1$ receptor on specific NRs (e.g., ERα) and their signal transduction pathways.

Melatonin modulates the expression and/or transactivation of other NRs in human breast cancer cells

In addition to its suppression of ERα transactivation, melatonin also modulates the expression and/or transcriptional activity of other members of the steroid hormone/NR superfamily via its MT$_1$ receptor. For example, melatonin has been shown to repress the ligand-induced transactivation of the glucocorticoid receptor (GR) and RORα in breast cancer cells (Dai et al. 2001, Kiefer et al. 2005). Conversely, melatonin can augment/potentiate the transactivation of other NRs, including the retinoic acid receptor alpha (RARα) and the retinoic acid X receptor alpha (RXRα) (Kiefer et al. 2005, Hill et al. 2011). We initially reported (Hill et al. 2011), and Proiette et al. (2011) confirmed, that melatonin potentiates the transactivation of the vitamin D receptor (VDR) and the peroxisome proliferation activating receptor gamma (PPARγ) in breast cancer cells. However, not all NRs are impacted by melatonin, seeing as no effects of melatonin have been observed on the ERβ in human breast cancer or in HEK293 embryonic kidney cells (Lai et al. 2008). The mechanisms involved in melatonin's modulatory effects on NRs, particularly the ERα, have not been completely delineated, but they appear to involve direct changes in receptor and/or coactivator (CaM, SRC1, CBP/p300, etc.) expression and phosphorylation. Our recent report that dLEN-mediated circadian melatonin disruption induces – and melatonin administration during dLEN ablates or represses – the expression and/or phospho-activation of a number of kinases (ERK1/2, ATK, PKA, SRC, FAK, etc.) that are well known to regulate the phospho-activation of NRs and other transcription factors (Ap-1, Elk-1, NF-kB, STAT3, etc.) (Dauchy et al. 2014), clearly demonstrates the importance of melatonin in regulating gene expression in human breast cancer.

The potential clinical significance of melatonin's regulation of breast cancer NRs has been highlighted in several studies. For example, Eck et al. (1998) demonstrated that the pretreatment of MCF-7 cells with melatonin following the administration of trans-retinoic acid, which is a ligand for both RARα and RXRα, was able to promote apoptosis in MCF-7 breast cancer cells via activation of the RARα coincident with the repression of transforming growth factor beta 1 (TGFβ1) and Bcl2 (an anti-apoptotic protein) and the increased expression of the pro-apoptotic protein Bax. Follow-up studies by Teplitzky et al. (2001) and Melancon et al. (2005) found that the combination of melatonin and 9-cis retinoic acid was able to repress the development of N-nitroso-N-methylurea (NMU)–induced mammary tumors in rats by more than 90% and also to induce the regression of 78% of established NMU-mammary tumors, with 54% undergoing complete regression. The interaction between the melatonin and vitamin D/VDR pathways was demonstrated in a report by Proietti et al. (2011), which showed that melatonin could promote VDR transcriptional activity and drive MCF-7 breast tumor cells to apoptosis in vitro.

Melatonin modulation of gene expression in breast cancer

The effects of melatonin on breast cancer are quite diverse, and they range from antioxidant, immunomodulatory, and enzyme regulatory to the regulation of various kinases and transcription factors. Via activation of its MT$_1$ receptor in breast cancer, melatonin has been shown to inhibit the expression and/or phospho-activation of numerous kinases (AKT, ERK1/2, PKA, PKC, c-SRC, GSK3β, etc.), transcription factors (ERα, RORα, RAR, RXR, VDR, PPARγ, Ap-1, Elk-1, CREB, NF-kB, and STAT3), and ERα co-regulators, such as CaM, CBP/p300, and SRC-1, which are known to drive breast cancer promotion and/or progression (Gordge et al. 1996, Hill et al. 2011, Dauchy et al. 2014).

To further explore the mechanisms by which melatonin suppresses breast cancer cell growth, various laboratories, including our own, have chosen to employ a systems approach for identifying the expression profiles of genes regulated by melatonin in MCF-7 cells in vitro. Employing genomic profiling, Lee et al. (2011) evaluated microRNA (miRNA) and gene expression in MCF-7 breast tumor cells that had been treated with 1 and 100 nM concentrations of melatonin for 24 h and found significant differences in both miRNA and gene expression in response to the two different doses. In cells treated with 1 nM melatonin, five miRNAs were either up- or down-regulated as compared to 18 miRNAs in cells treated with 100 nM melatonin; only

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miR-1207-3p overlapped between the two groups. At the mRNA level, the treatment of cells with 1 nM melatonin regulated the expression of twice as many genes as the higher concentration (100 nM) of melatonin did. These effects were equally weighted between gene induction and suppression, with the majority of genes being functionally related to signal transduction, transcription, cell proliferation, and cell transport. This same group (Lee *et al.* 2013) also evaluated the effects of melatonin on gene methylation and found that a number of genes were aberrantly methylated and that their expression decreased in response to melatonin, including early growth responsive gene 3 (*Egr3*) and *POU4F2/Brn3b*, both of which are associated with increased invasive and proliferative tumor cell capacity. Conversely, melatonin (1 nM) demethylated and increased the expression of the tumor and metastasis suppressor glypican-3 (GPC-3).

A genomic profiling analysis by Liu *et al.* (2013) that examined the effect of the carcinogen methyl methanesulfonate on melatonin (1 nM)-pretreated MCF-7 cells showed altered expression of DNA damage response pathway genes. Further pathway-based bioinformatics analyses revealed that the top functional networks included DNA replication, recombination, repair, and cancer. Among these genes are several that have known roles in the regulation of DNA repair activity, including *CEP152*, a regulator of genomic integrity and cellular response to DNA damage that acts through the ATR-mediated checkpoint signaling pathway (Kalay *et al.* 2011). *N4BP2L2* mRNA was also induced following carcinogen exposure and was further elevated with melatonin pretreatment. *N4BP2L2* encodes a phosphonoformate immune-associated protein that is phosphorylated by ATM or ATR upon DNA damage.

To identify candidate genes that respond directly or indirectly to melatonin via activation of its MT_1 receptor, we used a limited (8000–9000 genes) cDNA microarray analysis to examine the gene expression profiles of MCF-7 human breast carcinoma cells that were transiently transfected with the MT_1 receptor and treated with 1 nM melatonin. Close to 300 genes were found to have significantly altered ($\geq$1.4-fold) levels of expression. Of these, melatonin was found to suppress the expression of more than 210 genes and to induce the expression of more than 80 genes. A summary list and values of these cancer-related genes and their function(s) can be found in Table 1.

Key examples include cell-to-cell adhesion and communication molecules, such as fibronectin (FN1) and desmoplakin, which were down-regulated 2.6- and 2.3-fold respectively; the cytokine bone morphogenic protein

7 (BMP7) was down-regulated 2.5-fold, and the growth factors amphiregulin (AREG) and insulin-like growth factor 1 receptor (IGF1R) were down-regulated 2.0- and 1.4-fold respectively. The Ca^{2+} homeostasis-associated S100 calcium-binding protein (S100P) was also down-regulated 2.6-fold, whereas nucleobindin 2 (NUCB2) was up-regulated 2.8-fold in response to melatonin activation of the MT_1 receptor.

Genes associated with chromatin remodeling and transcriptional regulation, including SRC-1 and the CBP/p300-interacting transactivator (CITED2), which are well-established co-regulators of the NR family and other transcription factors, were down-regulated 1.4- and 1.7-fold respectively, whereas calreticulin (CALR), an NR-interacting protein, was up-regulated 1.6-fold. The transcription factor endothelial PAS domain protein 1 (EPAS1), which is also termed hypoxia inducible factor 2 alpha (HIF2α) and is associated with increased angiogenesis, was down-regulated 2.3-fold. A variety of proteins associated with cell proliferation were down-regulated, including Ki-67 (-1.8-fold). However, the cell cycle inhibitor p21 was up-regulated 1.7-fold.

Metastasis-related genes, including IGF1-binding protein-5 (IGFBP-5), FN1, and ephrin A 1 (EFNA1) were down-regulated 3.3-, 2.6-, and 1.6-fold respectively. However, the tissue inhibitor of metalloproteinase 3 (TIMP3) was induced 1.9-fold. Interestingly, TIMP3 has been shown to promote breast tumor sensitivity to tamoxifen (TAM) and, as we recently reported (Dauchy *et al.* 2014), the *in vivo* repression of the circadian melatonin signal by dLEN drives breast tumors to intrinsic resistance to TAM. Angiogenesis is an essential step in tumor formation and plays a critical role in tumor metastasis. A number of angiogenesis-associated genes were down-regulated by melatonin, including EPAS1, the N-Myc downstream regulated gene (NDRG1), and EFNA1, which were decreased 2.3-, 2.1-, and 1.6-fold respectively.

Melatonin mediated apoptosis in breast cancer

Melatonin's anti-cancer actions in breast cancer can be classified as cytostatic or cytotoxic. Although considerable evidence shows that both physiologic and pharmacologic concentrations can inhibit breast tumor proliferation, cytotoxicity has also been reported in response to melatonin in breast cancer in a cell- and tumor-specific manner, particularly when pharmacologic concentrations of melatonin are employed (Grant *et al.* 2009, Hill *et al.* 2011, Proietti *et al.* 2013). It should be noted that in other specific types of cancer, the cytotoxic/apoptotic actions of

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Table 1 Representative list of differentially expressed genes by melatonin in MCF-7 cells

Gene symbol	Gene name	Fold change[a]	Function	Accession number
Cell-to-cell adhesion and communication				
FN1	Fibronectin 1	−2.6	Involves cell adhesion, cell motility, and wound healing; possible role in metastasis	AW385690
DPI, DPII	Desmoplakin (DPI, DPII)	−2.3	Intercellular junction; adhesion protein	M77830
Cytokine and cytokine receptors				
BMP7	Bone morphogenetic protein 7 (osteogenic protein 1)	−2.5	Member of the transforming growth factor β superfamily; induces epithelial cell growth	BE395650
Growth factor and receptors				
AREG	Amphiregulin (Schwannoma-derived growth factor)	−2.0	Member of the epidermal growth factor family; growth inhibition and stimulations	NM_001657
IGF1R	IGF1 receptor	−1.4	Binds IGF; plays a critical role in transformation events	X04434
Signal transduction-related				
S100P	S100 calcium-binding protein P	−2.6	Calcium-binding protein; mediates Ca^{2+} signals; overexpressed in breast cancer	BE536069
CALR	Calreticulin	1.6	Ca^{2+} binding protein; modulator of nuclear receptor function; possible role in angiogenesis	M84739
NUCB2	Nucleobindin 2	2.8	Ca^{2+} binding protein; possible role in calcium homeostasis	AF052644
Chromatin remodeler and coactivator				
CITED2	Cbp/p300-interacting transactivator	−1.7	Coactivator of transcription factors	BF110899
SRC1	Nuclear receptor coactivator 1	−1.4	Transcriptional coactivator for steroid and nuclear hormone receptors	U59302
Transcription factor				
EPAS1	Endothelial PAS domain protein 1	−2.3	Transcriptional factor; induces VEGF expression; possible role in angiogenesis	AW377189
Cell proliferation-related				
Ki-67	Antigen identified by MAB Ki-67	−1.8	Possible role in cell proliferation	X65550
p21	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	1.7	Cyclin-dependent kinase inhibitor; blocks cell cycle progression	U09579
Development and differentiation-related				
NDRG1	N-myc downstream regulated	−2.1	Member of the N-myc down-regulated gene family; possible role in growth arrest and cell differentiation; induced by hypoxia	X92845
Metastasis-related				
IGFBP5	IGF-binding protein-5	−3.3	Growth inhibitor and proapoptotic agent in breast cancer; possible role in metastasis	AA374325
FN1	Fibronectin 1	−2.6	Involves cell adhesion, cell motility, and wound healing; possible role in metastasis	AW385690
EphA1	EphA1	−1.6	Ephrin receptor subfamily of the protein–tyrosine kinase family; possible role in metastasis	Z27409
TIMP3	Tissue inhibitor of metalloproteinase 3	1.9	Natural inhibitors of the matrix metal-loproteinases; inhibits VEGF binding to VEGFR2 and angiogenesis	AI095372
Angiogenesis				
EPAS1	Endothelial PAS domain protein 1	−2.3	Transcriptional factor; induces VEGF expression; possible role in angiogenesis	AW377189

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Table 1 Continued

Gene symbol	Gene name	Fold change[a]	Function	Accession number
NDRG1	N-myc downstream regulated	−2.1	Member of the N-myc down-regulated gene family; induced by hypoxia	X92845
FN1	Fibronectin 1	−2.6	Involves cell adhesion, cell motility, and wound healing; possible role in metastasis	AW385690
EphA1	EphA1	−1.6	Ephrin receptor subfamily of the protein–tyrosine kinase family; possible role in metastasis	Z27409
CALR	Calreticulin	1.6	Ca^{2+} binding protein; modulator of nuclear receptor function; possible role in angiogenesis	M84739
TIMP3	Tissue inhibitor of metalloproteinase 3	1.9	Natural inhibitor of the matrix metalloproteinases; inhibits VEGF binding to VEGFR2 and angiogenesis	AI095372

[a]Positive number of fold change represents the up-regulation of gene expression by melatonin. Negative number of fold change represents the down-regulation of gene expression by melatonin.

melatonin are observed more frequently than they are in breast cancer. Mediavilla et al. (1999) and other laboratories have found that physiologic concentrations of melatonin reduce the in vitro proliferation of breast cancer cells by elongating cell cycle length by controlling the p53/p21 pathway, independent of promoting apoptosis. We have, however, documented a significant rise in apoptosis in MCF-7 cells in vitro when melatonin was administered together with retinoids (Eck et al. 1998). Some indirect proof of melatonin-induced apoptotic activity has been seen in in vivo studies in rat mammary tumors, which showed a significant increase in caspase-3 activity and DNA fragmentation in tumor samples following melatonin administration (Adb El-Aziz et al. 2005). However, a report by Cucina et al. (2009) found that under appropriate conditions, two distinct apoptotic processes could be triggered by melatonin in MCF-7 cells, including an early response that was independent of TGFβ1 and caspase activity and a later apoptotic response that was both TGFβ1- and caspases-dependent, with caspases-7 being involved as the terminal effector.

More recent research by Proietti et al. (2014) has confirmed the work of Cucina by showing that within 3 h of treatment with 1 nM melatonin, a dramatic decrease in murine double minute 2 (MDM2), a regulator of p53 ubiquitination, was observed. Down-regulation of MDM2 allowed elevated expression and acetylation of p53, which increased p21 levels, led to decreased cell cycle progression, and promoted p53-mediated apoptosis. Furthermore, those authors reported that melatonin decreased the expression of the survival protein silent mating type information regulation 1 homolog (Sirt1) via modulation of the MDM2/murine double minute X (MDMX)-p53

pathway. Both flow cytometry and DNA fragmentation analyses documented a two-phase apoptotic response to melatonin (early at 24 h, late at 96 h). Early apoptosis appears to be caspase-independent, whereas the later response appears to involve TGFβ1, caspase-7, caspase-9, PARP cleavage, and a down-regulation of the Bcl-2:Bax ratio. These melatonin-mediated apoptotic responses are even more complex and appear to involve the release of p53 and p73, with p53 being activated in the early response and p73 mediating the caspase-dependent late response.

A number of studies have shown that both in vitro and in vivo models of breast cancer are less responsive to the apoptotic effects of melatonin when it is used as a single agent. However, melatonin appears to amplify the cytotoxic effects induced by other hormones or by conventional drugs (Eck et al. 1998, Carrillo-Vico et al. 2003, Dauchy et al. 2014). Although we do not yet have a complete understanding regarding the mechanisms by which melatonin exerts its full anti-cancer effects, when it is used in pharmacologic concentrations, melatonin activates responses that involve the intrinsic and/or extrinsic apoptotic pathway in cancer cells, namely, through an increase in the p53:MDM2p ratio and through down-regulation of Sirt1.

Melatonin effects on tumor metabolic activity

Efficient biosynthesis of the cellular and molecular building blocks required for tumor growth is fueled by a process that involves the robust uptake of circulating glucose and its conversion to lactate by cancer cells via glycolytic metabolism in the presence of ample oxygen. This type of glucose metabolism is termed aerobic glycolysis (or the

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Warburg effect), and it represents the bioenergetic process that is preferred by cancer cells over oxidative phosphorylation in order to accommodate rapidly expanding tumor biomass (Warburg 1925, DeBerardinis et al. 2008, Vander Heiden et al. 2009, Locasale & Cantley 2010). AKT, HIF-1α, and c-MYC (Elstrom et al. 2004, Gordan et al. 2007) are important signal transduction and transcriptional networks that drive the Warburg effect to reroute bioenergetics in cancer cells to generate the molecular intermediates that are required to sustain continual cancer cell proliferation (DeBerardinis et al. 2008, Vander Heiden et al. 2009, Locasale & Cantley 2010). In addition to glucose metabolism via the Warburg effect, the cellular uptake of LA, an essential omega-6 FA that is the most prevalent polyunsaturated FA in the Western diet, is also critical for cancer cell proliferation and tumor growth (Sauer & Dauchy 1992, Sauer et al. 1997, 1999). Cancer cells take up LA via a cAMP-dependent transport mechanism and via activation of the enzyme 15-lipoxygenase-1 (Blask et al. 1999, 2005, Sauer et al. 1999, Dauchy et al. 2004), the activity of which is up-regulated by activation of epidermal growth factor (EGF) and IGF1Rs (Glasgow & Eling 1994, Glasgow et al. 1997), which metabolize LA to the mitogenic metabolite 13-HODE. 13-HODE has been shown to exert a positive feedback effect on EGF and IGF1R growth and survival signaling pathways in a variety of tumors, including human cancer xenografts (Sauer et al. 1999, Dauchy et al. 2004, Blask et al. 2005) and to enhance the downstream phospho-activation of AKT and ERK1/2, which leads to the amplification of cell proliferation and survival responses (Hsi et al. 2002, Blask et al. 2011).

The host–cancer balance is maintained by the circadian organization of these daily bioenergetic, metabolic, signaling, and proliferative activities into coordinated rhythms that help ultimately slow the expansion of tumor biomass. Both host and tumor rhythms are driven by the circadian nocturnal melatonin signal, which, by virtue of its intrinsic chronobiotic and oncostatic properties, provides a light/dark cycle-entrained temporal framework for tumor growth (Blask et al. 2011). For example, in human breast cancer xenografts grown in athymic female nude rats, the greatly elevated tumor uptake of LA and metabolism to 13-HODE and aerobic glycolysis that occurs during the daytime is involved in promoting a corresponding increase in cell proliferative/survival activities in tumors, whereas during the nighttime, these processes become quiescent, as is shown by the much lower cell proliferative rates and the increased cell loss. Therefore, the relatively slow net tumor growth rate that occurs in

the presence of an intact nocturnal circadian melatonin signal reflects an overall balance in tumor circadian dynamics. This balance is characterized by an up-regulation of daytime metabolism, signaling, proliferation, and cell survival, which is offset by a highly significant down-regulation of these activities during the nighttime (Blask et al. 2014).

Under the conditions of exposure to low intensity light at night and circadian disruption that only suppresses nocturnal melatonin production, both the Warburg effect and the uptake of LA and its metabolism to 13-HODE in breast cancer xenografts become completely arrhythmic and operate at constitutively high levels throughout the entire day (all 24 h). This form of circadian disruption not only provokes a melatonin-deficient state but also compromises normal rhythms of blood glucose, insulin, and IGF1, which leads to hyperglycemia and hyperinsulinemia in addition to persistently high IGF1 blood levels (Wu et al. 2011). Thus, a chronic lack of circadian organization in proliferative activity and metabolic signal processing results in a 24 h/day hypermetabolic and hyperproliferative state that culminates in markedly accelerated overall tumor growth rates. Additionally, key tumor signal transduction and transcriptional factors, such as cAMP, AKT, and the HIF-1α regulation of LA metabolism and the Warburg effect in tumors, demonstrate melatonin-driven circadian oscillations that are dramatically disrupted by dLEN. This indicates their unique role in regulating metabolic fluxes in coordination with the classical allosteric feedback mechanisms that control intermediary metabolism in human breast cancer (Blask et al. 2011).

Melatonin regulation of genomic instability

A well-established connection between shift work-associated circadian disruption and increased cancer risk in humans and animal models strongly supports the cellular inability to maintain genome stability in response to extended LEN. This assumption is based on the causative association between genomic instability and cancer. As described in the next section, LEN triggers changes in gene expression and modifications of protein activity that are relevant to the cellular ability to respond to and repair DNA damage. Genomic instability could originate from DNA damage that results from internal and external sources. As mentioned earlier in the present report, melatonin is a powerful antioxidant that can reduce ROS production and accumulation by improving mitochondrial function and by stimulating antioxidative enzymes.

Another source of genomic instability in mammalian cells are transposable elements, which are represented by non-long terminal repeat retro-elements in the human genome. Using a 'copy-and-paste' mechanism of amplification, long interspersed element 1 (L1) has grown to more than 500 000 copies, which are distributed throughout the human genome (Lander et al. 2001). About 80–100 of these loci remain functional (Brouha et al. 2002, 2003). The expression of some of these fixed active L1s, as well as a number of polymorphic loci, contribute an estimated 0.04–0.07 de novo inserts per normal cell (neuron) (Evrony et al. 2012). A full-length, functional L1 locus generates an mRNA and two proteins, ORF1p and ORF2p. The L1 proteins bind to the L1 mRNA to form a retro-transpositionally competent ribonucleoprotein (RNP) complex, but they have distinct roles in retrotransposition. The ORF1p serves as a structural protein with nucleic acid chaperon activity (Belancio et al. 2010). The ORF2 protein has an endonuclease, which cuts the host DNA, and a reverse transcriptase, which synthesizes L1 cDNA in the nucleus (Belancio et al. 2010). The L1 integration process is most likely completed with the aid of poorly defined cellular factors. In addition to retrotransposition, L1 can damage genomic DNA via the generation of double-strand breaks (DSBs) (Belancio et al. 2010), the structure and mutagenic potential of which remain unknown.

The Belancio laboratory has recently reported that melatonin, via the MT_1 receptor-mediated action, suppresses the expression of the endogenous retrotransposon L1 in a tissue-isolated model of human cancer (Deharo et al. 2014). This finding suggests that the LEN-induced suppression of nocturnal melatonin synthesis activates L1 expression. Our tissue culture based experiments demonstrated that MT_1 expression decreases L1 mobilization about tenfold in HeLa cells by down-regulating L1 ORF1 protein levels (Deharo et al. 2014). These data suggest that LEN promotes L1 expression and damage in tumors and possibly normal tissues. This previously unanticipated connection between L1 activity and environmental light exposure supports the possibility that L1-induced damage may contribute to the LEN-associated cancer risk.

Effects of LEN on melatonin and breast cancer

Based on early studies that demonstrated melatonin's anti-cancer actions in breast cancer, Stevens (1987) hypothesized that the suppression of nighttime pineal melatonin production in response to light at night might explain the rise in breast cancer rates that have accompanied industrialization and electrification in the USA and other Westernized countries. LEN is a well-recognized environmental disruptor of the central circadian timing system, which is located in the SCN (Reiter 1991, Claustrat et al. 2005, Stevens et al. 2007, Straif et al. 2007). The nighttime production of melatonin by the pineal gland represents a highly reliable output signal of the circadian clock, whose suppression by LEN is intensity-, duration-, and wavelength-dependent (Revell & Skene 2007, Rüger et al. 2013). Given this, numerous studies have shown that the circadian melatonin signal regulates metabolic and cell signaling activities to inhibit breast cancer initiation, promotion, and progression (Grant et al. 2009, Hill et al. 2011, Proietti et al. 2013). Following early epidemiologic studies by Davis et al. (2001) and Schernhammer et al. (2001) that used the Harvard Nurses Cohort, the World Health Organization has designated night-shift work that involves LEN-induced circadian/melatonin disruption as a probable carcinogen (class 2a) and a risk factor for the development of breast cancer (Straif et al. 2007).

A paradigm-shifting study by Blask et al. (2005) demonstrated that when tissue-isolated breast tumor xenografts in female nude rats were perfused with blood collected from women that was exposed to dim light (0.2 lux) at night (melatonin-poor), LA uptake, LA conversion to 13-HODE, cAMP levels, and the expression of the MAPK downstream effectors MEK/ERK were increased. Conversely, these same parameters were greatly reduced when tumor xenografts were perfused with nighttime blood taken from women during dark night (melatonin-rich). The inhibitory effects of circadian melatonin during dark night was shown to be receptor-mediated, because MT_1/MT_2 receptor antagonists were able to block these effects. Wu et al. (2011) reported increases in tumor growth rates and enhanced ERK1/2, AKT, and AKT-stimulatory 3-phosphoinositide-dependent kinase-1 (PDK1) activity in breast tumor xenografts in response to light exposure during the night or day by employing the novel tissue-isolated MCF-7 human breast cancer xenograft model in circadian/melatonin-intact female nude rats. Furthermore, they reported that these changes were blocked by melatonin (Wu et al. 2011). Also utilizing the tissue-isolated human breast tumor xenograft model in female nude rats, Mao et al. (2012) observed that the phosphorylation of GSK3β, an enzyme that is critical in cell metabolism and proliferation/survival, exhibited a circadian rhythm in tumor xenografts. Exposure to light at night by suppressing nocturnal pineal melatonin synthesis induced AKT phospho-activation at serine 473, which promoted its inhibitory phosphorylation at serine 9 of GSK3β and blocked GSK3β activation and ubiquitination activity.

As alluded to earlier in the present report, a recent study by Blask *et al.* (2014) that used tissue-isolated human breast tumor xenografts grown in female nude rats showed that tumor xenograft LA uptake, metabolism, and proliferation and survival signaling pathways in tumors are dynamically coordinated within the circadian time structure of the 24 h light:24 h darkness cycle by nocturnal pineal melatonin production, which is driven by the SCN. That work demonstrated that dLEN and its associated suppression of nocturnal circadian melatonin alter the host–cancer balance in numerous cancer-promoting signaling pathways, thereby driving hyperglycemia and hyperinsulinemia in the rat and hyper/runaway aerobic glycolysis (the Warburg effect) and proliferation in the tumor.

Our most recent work examined the effects of dLEN and melatonin on the development of TAM resistance (TAM-R) in breast cancer (Dauchy *et al.* 2014). Although the majority of this work is discussed in the section entitled 'Melatonin: a regulator of resistance to endocrine and drug therapy,' this study clearly demonstrates that dLEN, via its repression of the nocturnal circadian melatonin signal, promotes tumor aerobic glycolysis (the Warburg effect) and the expression and/or phospho-activation of the key signaling pathways and nodes involved in tumor proliferation and survival that drive resistance in breast cancer cells to endocrine therapies and chemotherapies. These signaling pathways that are induced by dLEN include the PI3K/AKT pathway, the EGFR/HER2 and downstream RAS/MAPK/ERK pathways, the p21 activating kinase 1 (PAK1), and PI3K/AKT/pyruvate dehydrogenase kinase 1 (PDK1)/mTOR/ p90 ribosomal S6 kinase (RSK) family members, all of which can drive cancer cells to proliferation, survival, drug resistance, and metastasis (Lee *et al.* 1992, McCubrey *et al.* 2007, Li *et al.* 2008, Romeo *et al.* 2012, Sims *et al.* 2013, Roskoski 2014).

Other signaling pathways that are elevated or activated in response to dLEN-induced circadian/melatonin disruption include c-SRC, FAK, cAMP, PKA, CREB, STAT3, NF-kB, and PKCα and δ (Lazennec *et al.* 2001, Gonzalez-Angulo *et al.* 2007, Díaz-Bessone *et al.* 2011, Zhang *et al.* 2011, Anbalagan *et al.* 2012). In tissue-isolated tumors that are grown under a lighting schedule of 12 h light:12 h darkness (LD 12:12) with nocturnal circadian melatonin elevated during dark night or under an LD 12:12 dLEN schedule but supplemented with melatonin in the nighttime drinking water, melatonin (endogenous or exogenous) was able to block or dramatically suppress the expression and/or phosphor-activation of each of these signaling pathways, which is turn suppressed tumor cell

proliferation and resistance to endocrine therapies and chemotherapies (Dauchy *et al.* 2014).

Circadian synchronization is controlled, in part, by ambient light that decreases melatonin synthesis and secretion, which occurs in the pineal gland but is coordinated by the SCN of the hypothalamus. Peripheral cell autonomous circadian clocks that are termed 'peripheral oscillators' and are controlled by the master clock in the SCN exist for every cell of the body, including the breast. These peripheral oscillators are comprised of the same genes as the master clock (Sellix 2013). The clock genes in a peripheral oscillator can subsequently regulate the clock-controlled genes that are involved in the cell cycle, including c-Myc, Wee1, cyclin D, and p21 (Wood *et al.* 2009). Light may directly affect tumor growth via the key oscillator (clock) genes PER1 and PER2, which in turn regulate cell cycle and apoptosis-regulated genes (Moriya *et al.* 2007, Gery & Koeffler 2009, Wood *et al.* 2009). For example, Gery *et al.* (2007) reported that PER2 plays a role in ERα ubiquitination, whereas Xiang *et al.* (2012) demonstrated that re-expression of PER2 in MCF-7 breast tumor cells induces p53 mRNA expression. PER2 has been reported to be a tumor suppressor gene whose expression inhibits the formation of a variety of tumors, including breast, prostate, lung, and lymphoma (Gery & Koeffler 2009). Thus, a loss of PER2 could well be involved in the initiation or progression of breast cancer. The repression of PER2 gene expression by methylation of the PER2 promoter or phosphorylation by casein kinase 1ε might be involved in the initiation or progression of breast cancer (Gery *et al.* 2007). Interestingly, Xiang *et al.* (2012) showed that PER2 mRNA expression is non-rhythmic in MCF-7 breast cancer cells *in vitro* and that serum shock of MCF-7 cells induced the rhythmic expression of most core clock genes except for PER2, a phenomenon that was notably restored by the administration of melatonin after serum shock.

Earlier in the present study, melatonin was reported to modulate the methylation of some genes. For example, Stevenson & Prendergast (2013) demonstrated in seasonally breeding male and female Siberian hamsters that DNA methylation of the proximal promoter for the type 3 diodinase (dio3) gene in the hamster hypothalamus is reversible and critical for photoperiod time measurement. Furthermore, they showed that short-day photoperiods with winter-like levels of melatonin inhibited hypothalamic DNA methyltransferase expression, reduced dio3 promoter DNA methylation, and up-regulated dio3 expression and gonadal regression. In a genome-wide profiling study of breast cancer cell lines, Lee *et al.* (2013)

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demonstrated that the administration of 1 nM melatonin to cells was able to alter DNA methylation patterns and to decrease the expression of known oncogenic genes, including *EGR3* and *POU4F2/Brn3b*, while simultaneously up-regulating the expression of the tumor suppressor gene, *GPC3*. Thus, the circadian melatonin signal can be dramatically altered by LEN and may affect overall DNA methylation and clock gene expression, including PER2, to promote tumor progression. Conversely, the presence of nighttime melatonin may inhibit tumor progression via the epigenetic regulation of gene expression.

A recent study by Dai *et al.* (2011), using a univariate logistic regression analysis, demonstrated that polymorphisms of CLOCK and CRY1 genes were associated with breast cancer risk. Furthermore, we reported (Dai *et al.* 2001, Dong *et al.* 2010) that melatonin represses the transcriptional activity of RORα1, a member of the NR/steroid hormone receptor superfamily, a core circadian clock gene, and a transcriptional inducer of the core clock gene *BMAL1*. In MCF-7 breast cancer and MCF-10A human breast epithelial cells, melatonin administration significantly repressed RORα1 transactivation by inhibiting its induction of *BMAL1* gene expression. Thus, melatonin, via its MT_1 receptor, directly suppresses elements of the peripheral oscillator in breast epithelial and cancer cells (Xiang *et al.* 2012).

Melatonin: a regulator of epithelial–mesenchymal transition and metastasis

An early study by Cos *et al.* (1998) noted that the *in vitro* invasive capacity of ERα-positive MCF-7 breast tumor cells was suppressed by melatonin via the regulation of E-cadherin and β-integrin. Unfortunately, the luminal A (ERα-positive/PR-positive) MCF-7 human breast tumor cell line is considered by most to be poorly metastatic (Yang & Kim 2014). However, using MCF-7 human breast tumor cell clones that overexpressed either the ErbB2/Her2-neu oncogene or the cytokine receptor CXCR4 and MCF-7 cells that serially passaged through nude mice until they developed a metastatic phenotype (MFCF7/6 cells) (Bracke *et al.* 1991), Mao *et al.* (2010) demonstrated that melatonin indeed possesses anti-invasive/anti-metastatic action-suppressing cell invasion by 60–85% in trans-well/matrigel insert assays. In that study, Mao demonstrated that the anti-invasive actions of melatonin were at least partially mediated by the inhibition of p38 MAPK and matrix metalloproteinases (MMP) 2 and 9, which are involved in the degradation of the basement membrane and metastatic cell extravasation.

The cytoskeleton is an important component of the cellular architecture, and it is composed of an intricate network of fibers, microtubules, microfilaments, and intermediate filaments and their associated proteins (Roberts 1974). The cytoskeleton shows dynamic changes, and together with related adhesion proteins, it modulates much of the metabolic and signal transduction machinery of the cell (Ingber 2003). Melatonin, via activation of its MT_1/MT_2 receptors, has been reported to significantly impact microtubule organization, and thus cytoskeleton organization in breast cancer cells. Benítez-King *et al.* (1990) demonstrated a complex interaction between melatonin and the cytoskeleton in MCF-7 cells. Their studies showed that melatonin induces the formation of focal adhesions in MCF-7 cells and alters the arrangement of microfilaments and stress fibers to form thicker bundles that are assembled with phospho-vinculin to promote adhesion contacts (Ortíz-López 2009).

PKC, via the activation and induction of Rho-associated kinase (ROCK), induces stress fiber thickening and decreases focal adhesion events known to promote breast tumor cell migration and invasion (Soto-Vega *et al.* 2004, Ramirez-Rodriguez *et al.* 2007). Melatonin has been reported to inhibit stress fiber formation and thickening via the suppression of PKC (Soto-Vega *et al.* 2004, Ramirez-Rodriguez *et al.* 2007, Yuan *et al.* 2008). In a new study that is currently in press (Xiang *et al.* 2015), we demonstrate that in MCF-7 tissue-isolated tumor xenografts grown in female nude rats that were housed in a photoperiod of 12 h light:12 dLEN, PKCα levels were elevated but were dramatically inhibited in tumors that were supplemented with nighttime melatonin during dLEN (data not shown). Although these tumor xenografts were not analyzed for stress fiber formation, the inhibition of PKCα by melatonin combined with the reports described earlier in the present section suggest that melatonin may inhibit the invasive/metastatic capacity of breast cancer cells by inhibiting PKCα-induced stress fiber formation.

The developmental process of epithelial–mesenchymal transition, in which epithelial cells acquire a mesenchymal phenotype and become migratory (Tomaskovic-Crook *et al.* 2009), has also been seen in cancer cells as they acquire invasive and metastatic phenotypes (Jechlinger *et al.* 2003, Rubin 2014). EMT in cancer is characterized by cellular changes, including a loss of cell adhesion proteins, cytoskeleton reorganization, and the acquisition of mesenchymal cell spindle-like morphology with increased motility and invasiveness. A key hallmark of EMT is the reduced expression of

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E-cadherin, a structural component of adherent junctions that is critical for epithelial cell polarity and adhesion (Hollestelle *et al*. 2013, Chen *et al*. 2014).

Also linked to the progression of EMT is the Wnt/β-catenin pathway, wherein β-catenin is a core component of the adherent junctions because of its binding to E-cadherin (ten Berge *et al*. 2008). Upon the dissociation of β-catenin from E-cadherin, it is able to translocate to the nucleus and interact with the T cell factor/lymphocyte enhancer factor (TCF/LEF), a key transcription factor that promotes the expression of Wnt target genes, including Snail, Slug, and c-MYC, which are transcriptional repressors of E-cadherin, as well as vimentin, an intermediate filament protein and well-known marker of mesenchymal cells (Gilles *et al*. 2003, Zhou *et al*. 2004, Rubin 2014). The destruction complex of glycogen synthase kinase 3 beta (GSK3β), axin, adenomatous polyposis coli, and casein kinase 1 promotes the ubiquitination of excess cytoplasmic β-catenin (Gilles *et al*. 2003, Zhou *et al*. 2004). When it is activated, GSKKβ phosphorylates β-catenin, thereby promoting its ubiquitination/degradation. The inhibitory phosphorylation by AKT or WNT that occurs at s9 of GSK3β stabilizes β-catenin allows its translocation to the nucleus and dimerization with TCF/LEF to promote Wnt target gene expression (Zhou *et al*. 2004). Interestingly, similar to β-catenin, GSK3β, via phosphorylation-mediated mechanisms, can drive the ubiquitination of Snail, Slug, and c-MYC (Zhou *et al*. 2004). Thus, GSK3β, because of its ability to regulate both Snail and β-catenin, is a clinically important target in the EMT process.

Employing the tissue-isolated human breast xenograft tumor nude rat model, Mao *et al*. (2012) demonstrated that GSK3β exhibits a circadian rhythm of phosphorylation. LEN suppresses nocturnal circadian melatonin and disrupts the circadian rhythm of GSK3β phosphorylation. In the presence of melatonin, GSK3β was activated in breast tumor xenografts via melatonin's blockade of AKT's inhibitory phosphorylation of GSK3β, which allowed GSK3β to induce β-catenin ubiquitination and inhibit EMT. Thus, chronic disruption of the circadian melatonin profile by LEN, which inhibits GSK3β activity and promotes EMT, appears to be an important contributor to metastatic spread in breast cancer patients.

Melatonin: a regulator of resistance to endocrine and drug therapy

Resistance to endocrine therapy and chemotherapy are major impediments to the successful treatment of breast cancer (Ravdin *et al*. 1992, Sabnis & Brodie 2010). Preclinical and clinical evidence link resistance to anti-estrogen and chemotherapeutic drugs in breast cancer cells with the overexpression and/or activation of various pro-oncogenic tyrosine kinases. Approximately 60–75% of breast cancers express ERα and PR that are markers and determinants for the use of endocrine therapies, including selective ERα modulators, such as TAM (Ravdin *et al*. 1992, Sabnis & Brodie 2010). Presently, anywhere from 30 to 50% of patients with ERα-positive breast tumors display intrinsic resistance to TAM, whereas most patients that are initially responsive will eventually develop acquired resistance to it (Sabnis & Brodie 2010). The anthracycline doxorubicin (Dox) is one of the most frequently used chemotherapeutic agents for patients whose breast tumors are endocrine resistant or metastatic (Lewis-Wambi & Jordan 2006). However, as with endocrine therapies, many cancers are intrinsically resistant to conventional chemotherapeutic agents, and other patients that initially respond well acquire resistance during treatment (Gariboldi *et al*. 2003).

Resistance to endocrine or drug therapies may occur through a variety of reported mechanisms (deGraffenried *et al*. 2004, Vallabhaneni *et al*. 2011, Burris 2013, Austreid *et al*. 2014). Cells can become resistant through the activation of several key signaling pathways, including the EGFR/HER2/MAPK/ERK and PI3K/AKT pathways, among others (Roskoski 2014). Numerous studies have reported that the expression and/or phospho-activation of a variety of kinases and transcription factors are elevated in human breast cancer cell lines and clinical breast tumor biopsies with both intrinsic and acquired resistance to endocrine therapies and chemotherapies. Furthermore, intrinsic or acquired resistance to endocrine therapies or chemotherapeutic drugs has also been linked to the up-regulation of ABC transporter efflux molecules (Gottesman *et al*. 2002), such as ABCB1 (MDR-1/P-gp), ABCC1 (MRP1), and ABCG2 (or breast cancer resistance protein, BCRP), as well as to the up-regulation of drug metabolizing enzymes (Kassner *et al*. 2008, Novotna *et al*. 2008), all of which lead to diminished levels of the active drug within the cancer cells. Finally, mounting evidence supports the concept that dysregulated cellular metabolism (aerobic glycolysis, the Warburg effect) is linked to drug resistance in cancer therapy via the up-regulation of tumor glucose and lactate and via increased cell signaling (Elstrom *et al*. 2004, Fantin *et al*. 2006, Robey & Nay 2009, Hua *et al*. 2014).

Several *in vitro* studies have provided evidence that melatonin may enhance the efficacy of TAM

(Wilson et al. 1992, Lissoni et al. 1995, Lissoni et al. 1999) and Dox (Fan et al. 2010). In our recent studies that examined the effects of the dLEN-mediated disruption of the circadian melatonin signal on breast cancer, we reported that in tissue-isolated MCF-7 breast tumor xenografts from female nude rats housed in a 12 h light:12 h dLEN environment, key tumor-promoting kinases (ERK1/2, AKT, SRC, and FAK) and transcription factors (ERα, CREB, and STAT3) were highly expressed and/or phospho-activated. All of these signaling nodes have been found to be elevated in TAM-R breast cancer. Additionally, tumor xenografts from rats housed in dLEN showed constitutive aerobic glycolysis (the Warburg effect) with increased tumor glucose uptake and lactic acid production. These tumor xenografts from rats exposed to dLEN showed complete TAM-R when treated with 4-hydroxy-tamoxifen (4OH-TAM). Conversely, tumor xenografts from rats housed in LD 12:12 with a dark night (elevated endogenous melatonin) and those that were housed in 12 h light:12 h dLEN but were supplemented with melatonin during dLEN showed abolished or greatly diminished expression and/or phospho-activation of these tumor-promoting kinases and transcription factors, dramatic suppression of the Warburg effect, and greatly enhanced sensitivity to and synergism with 4OH-TAM, such that tumor xenografts rapidly and significantly regressed.

Circadian melatonin disruption by dLEN in breast tumor xenografts suggest that dLEN promotes but melatonin inhibits the phosphorylation of ERα at key regulatory sites, including S118 and S167 (Dauchy et al. 2014). These ERα sites are reported to be phosphorylated in TAM-R tumors and to convert the response of the ERα to TAM from antagonist to agonist. As noted earlier in the present report, we (Lai et al. 2008) and others (Del Rio et al. 2004) have shown that melatonin can repress estrogen-induced ERα transactivation in vitro and have found significant repression of ERα phosphorylation at S118 and S167 by melatonin (Dauchy et al. 2014). Our most recent studies confirm this effect in vivo and show that melatonin, via the repression of key kinase signaling pathways, alters ERα phospho-activation and thus the responsivity of ERα to TAM by ensuring that TAM acts as an ERα antagonist. In unpublished studies, we have found that exposure to dLEN increases the expression of the ABC transporter ABCG2 (BCRP), a known efflux pump for 4OH-TAM and Endoxifen (Selever et al. 2011). Our data suggest that melatonin, in addition to regulating tumor metabolism and cell signaling, can also sensitize the response of breast tumors to TAM by regulating its interaction with ERα and by repressing the efflux of TAM from breast tumor cells.

In work that was recently presented at the American Association of Cancer Research meeting on Cancer Prevention (Xiang et al. 2015), we reported that MCF-7 tissue-isolated breast tumor xenografts grown in female nude rats housed in a dLEN environment showed complete intrinsic resistance to the anthrecycline Dox. This resistance, as seen with TAM-R, was associated with elevated aerobic glycolysis (the Warburg effect) and enhanced expression and/or phospho-activation of key signaling pathways involved in tumor proliferation, survival, and progression. Conversely, tumor xenografts in hosts exposed to dLEN but supplemented with nighttime melatonin showed inhibition of the Warburg effect and abolished/diminished expression and/or phospho-activation of key kinases and transcription factors involved in Dox-R and tumor progression. These tumor xenografts were highly sensitive to the synergistic actions of melatonin and Dox, as evidenced by their rapid regression (data not shown). These studies demonstrate that the disruption of the circadian melatonin signal by dLEN is sufficient to regulate tumor metabolism and signaling to drive tumors to complete intrinsic resistance to specific endocrine and drug therapies.

Conclusion

The study of melatonin as an anti-cancer hormone has spanned nearly five decades since the early observation by Lapin & Ebels (1976) that extracts from the pineal gland repress the growth of different tumors in rats and mice. Numerous studies have documented the oncostatic properties of this indolamine in human breast tumor cell lines in vitro and in animal models that have included human breast tumor xenografts in athymic nude mice and nude rats, carcinogen-induced mammary tumors in rats, and genetically engineered models of breast cancer in mice. Tremendous knowledge regarding the actions of melatonin on breast cancer has been gained through the concerted efforts of investigators involved in melatonin, cancer, genetic, and epidemiological research. The actions of melatonin in breast cancer appear to be mediated via both growth-inhibitory (cytostatic) and pro-apoptotic (cytotoxic) effects and are clearly complex, insofar as they are mediated through a number of mechanisms and involve a multitude of molecular pathways (Fig. 1). The majority of studies indicate major involvement of the MT_1 receptor for mediating both the cytostatic and
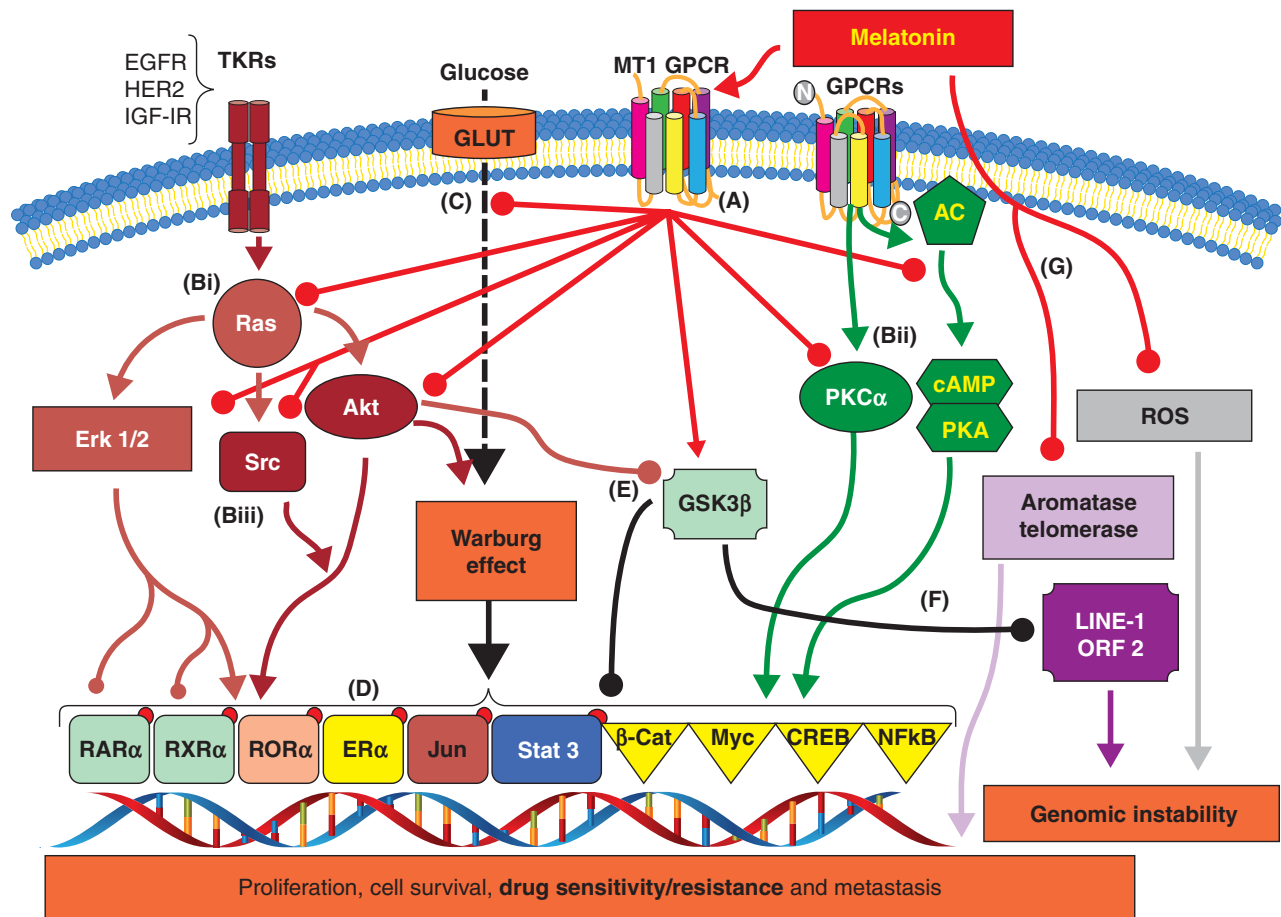
Figure 1

Mechanisms involved in melatonin's inhibition of breast cancer. Melatonin's anticancer actions are mediated through either the membrane bound MT_1 or MT_2 GPCRs or receptor-independent mechanisms. (A) By binding to its MT_1 receptor, melatonin can activate Gαi proteins to promote dissociation into Gαi-GTP and Gαi/βγ. Activation of the Gαi proteins can repress multiple signaling pathways, including the TRK and GPCR pathways, which activate signaling cascades such as MAPK/ERK1/2 (Bi), PI3K/AKT, PKC (Bii), and cAMP/PKA, as well as non-receptor kinases pathways, including c-SRC (Biii) and FAK. Each of these kinase-driven signaling pathways are known to play important roles in promoting breast cancer cell proliferation, cell survival, metastasis, and drug resistance. (C) The conversion of cellular metabolism from oxidative phosphorylation in breast epithelial cells to aerobic metabolism (the Warburg effect) in breast tumors is also involved in activating key signaling pathways (particularly the AKT and ERK pathways) to drive cell proliferation, cell survival, metastasis, and drug-resistance. These TKR and GPCR pathways subsequently regulate gene expression by activating downstream transcription factors (D) including NRs such as ERα and RORα (a clock gene) and basic helix–loop–helix transcription factors such as STAT3, MYC, β-catenin, NF-kB, and CREB. These kinase pathways also alter ERα phosphorylation by converting its response to TAM from antagonist to agonist, and via they inhibit the activity of other NRs including RARα and RXRα, via phosphorylation events in order to bock their differentiative and anti-proliferative actions on breast tumor growth. (E) Melatonin can also repress the AKT inhibition of GSK3β, which allows GSK3β to phosphorylate β-catenin, Snail, and even c-MYC and to promote their ubiquitination. Furthermore, via the activation of GSK3β (F) melatonin may be able to repress LINE-1 activity and DNA damage. Because of its highly lipophilic nature, melatonin can diffuse through cell, nuclear, and mitochondrial membranes and receptor-independent mechanisms (G) to inhibit aromatase expression and it can repress genomic instability through its suppression of telomerase activity and through its antioxidant activity on ROS.

pro-apoptotic actions of melatonin. Yet although melatonin's action as a potent endogenous scavenger of ROS is clearly important to genomic stability and tumor initiation, the extent of its role in breast tumor promotion and progression remains to be determined. In general, melatonin's cytostatic actions appear to be heavily mediated via its interaction with the estrogen/ERα signaling pathway, although new ERα-independent pathways have been identified in a number of recent studies. However, the cytotoxic actions of melatonin are mediated via a number of pathways, including p53, p73, TGFβ1, NRs such as RARα, RXRα, and VDR, and even clock genes such as PER2.

A number of new and important avenues regarding melatonin's actions in breast cancer have recently emerged, and they hold the potential to better understand

melatonin's anti-cancer properties and its potential future as a therapeutic agent for breast cancer. First, work by Blask et al. (2014) has demonstrated that the circadian melatonin signal inhibits aerobic glycolysis (the Warburg effect) in breast tumors by blocking tumor uptake of LA and glucose. Future studies need to define the mechanisms that are involved and the downstream consequences of these metabolic changes on cell signaling and tumor progression. Second, our recent reports, combined with studies by Cos et al. (1998), highlight the exciting, but poorly understood, anti-metastatic actions of melatonin in breast cancer. New in vivo studies are needed in circadian-complete (melatonin-producing) animal models of breast cancer to define the mechanisms and pathways that are involved in melatonin's anti-metastatic action. Third, given the circadian nature of melatonin synthesis and release and its regulation by photoperiod and exposure to light at night, new models need to be developed so that we can begin to fully understand how LEN impacts breast cancer initiation, promotion, and progression. These studies will be essential for identifying the interactions and independent effects that are mediated by melatonin via the central clock and peripheral oscillators in the breast and in breast cancer. Fourth, exciting new studies by the Belancio laboratory (Deharo et al. 2014) that demonstrate the circadian/melatonin regulation of LINE-1 elements and genomic instability in breast cancer are only the beginning of an exciting new story about melatonin's role in regulating genomic stability/instability and the initiation of breast cancer. Finally, additional research is required to clarify the consequences of dLEN-induced circadian melatonin disruption on endocrine and chemotherapy resistance in breast cancer. Follow-up studies are needed to define whether melatonin, given its potent role as a circadian-regulated kinase and transcription factor inhibitor in cancer, in combination with these therapeutic agents constitutes a new, more efficacious therapy for breast cancer than do multiple-kinase inhibitors in combination with chemotherapeutic agents. The broad effect of melatonin on breast cancer, including its inhibition of tumor metabolism, signaling, and genomic instability, its activity as a scavenger of ROS, its synergism with other cancer therapeutic agents, its lack of toxicity, and its wide availability and minimal cost, should make its movement into clinical trials a high priority.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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