HHS Public Access

Author manuscript

Endocr Relat Cancer. Author manuscript; available in PMC 2015 December 01.

Published in final edited form as:

Endocr Relat Cancer. 2015 June ; 22(3): R183–R204. doi:10.1530/ERC-15-0030.

Melatonin: an Inhibitor of Breast Cancer

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Abstract

This review discusses recent work on melatonin-mediated circadian regulation and metabolic and molecular signaling mechanisms involved in human breast cancer growth and 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, via the MT1 receptor, suppresses ERα mRNA expression and ERα transcriptional activity. As well, melatonin regulates the transactivation of other members of the nuclear receptor super-family, estrogen metabolizing enzymes, and the expression of core clock and clock-related genes. Furthermore, melatonin also suppresses tumor aerobic metabolism (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-to-mesenchymal transition. Studies demonstrate that melatonin promotes genomic stability by inhibiting the expression of LINE-1 retrotransposons. Finally, research in animal and human models indicate that LEN induced disruption of the circadian nocturnal melatonin signal promotes the growth, metabolism, and signaling of human breast cancer to drive breast tumors to endocrine and chemotherapeutic resistance. These data provide the strongest understanding and support of the mechanisms underpinning the epidemiologic

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Declaration of interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

demonstration of elevated breast cancer risk in night shift workers and other individuals increasingly exposed to LEN.

Keywords

Melatonin; Breast Cancer; Nuclear receptors; Molecular Signaling; Circadian Disruption; Genomic instability; Drug resistance

Introduction

Melatonin (N-acetyl-5-methoxytrypamine) is an indolic hormone identified by Lerner and colleagues as a factor from the mammalian pineal gland that lightens melanocytes (Lerner et al. 1960). Melatonin production is regulated by photoperiod as its synthessis 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 rising during the night but diminished 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, 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 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 photoperiod and thus predictably modulated throughout the day and night. Thus, allowing anticipation and adaptation of the organism to environmental changes. Similarly, the multitude of processes governing cancer initiation, promotion, 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; Troung et al. 2014). At the same time, the same molecular clock genes and their protein products seen in the master clock are expressed in peripheral tissues both normal and cancerous and termed peripheral oscillators, and these peripheral oscillators are coordinately expressed and regulated in a circadian manner (You et al. 2005). Numerous laboratories are focused on defining the importance of these clock/oscillator genes and proteins are involved 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 its 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: (1) hepatic biotransformation generating 6-hydroxy-melatonin and secretion in the urine after conjugation with sulphate or glucoronide, (2) by alternative indolic pathways which produce 5-methoxyindole acetic acid or 5-methoxytryptophol, and also (3) through a kynuric pathway that produces N1-acetyl-N2-formyl-5-methoxykynuramine.

A plethora of biological actions have been reported for melatonin however best described is its chronobiotic action as a neurohormone and entrainer to the light/dark cycle, and a synchronizer of peripheral clocks to the SCN (Slominski et al. 2012). Elevated nighttime levels of melatonin in the blood inform the bodies 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 the SCN activity and consequently the production of melatonin by the pineal gland, a phenomenon referred to as circadian disruption.

Over the last two decades, significant research efforts have been afforded to defining the role of melatonin in the etiology of cancer. In the last few years, disruption of melatonin's circadian profile by exposure to light at night has been described to play 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). While 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), a known tumor suppressor, in tissues and organs such as the breast (You et al. 2005), data also suggests 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).

While melatonin production can occur in numerous tissues (e.g., retina, 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 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-mediated activity and non-receptor-mediated antioxidant activity, is born out in the literature (Dubocovich et al. 2003; Dubocovich et al. 2010).

The focus of this article is to review the current literature including our own studies on the role of the nocturnal melatonin signal in the inhibition of breast cancer promotion and drug resistnace and metastasis via the 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. 2015), and the control of peripheral

oscillators (Xiang et al. 2012) involving *in vitro* and *in vivo* models of human breast cancer. As well, we will review recent studies focusing 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 G-protein coupled receptors (GPCRs). These two GPCRs found in humans and mammals are the MT_1 melatonin receptor (formerly the Mel1a) encoded by the MTNR1A gene and the MT_2 melatonin receptor (formerly the Mel1B) 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 show different yet overlapping expression profiles and overlap in their variable coupling G proteins and signal transduction pathways. Numerous reports demonstrate that melatonin via binding and activation of the MT_1 and MT_2 G protein-coupled receptors, 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 cyclic adenosine monophosphate (cAMP) via pertussis-toxin sensitive inhibitory $G\alpha_i$ proteins (Dinet et al. 2007; Lai et al. 2008). Activation of the MT_1 receptor promotes inhibition of forskolin-stimulated cAMP formation, suppression of Protein kinase A (PKA) activity, and phosphorylation of the cAMP-responsive element-binding protein (CREB) (Kiefer et al. 2002; Dinet et al. 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, modulation of cyclic guanosine monophosphate (cGMP) formation, and increase Protein kinase C (PKC) activity (Witt-Enderby et al. 2003). The complexity of melatonin signaling through melatonin receptors is increased 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). As well, Jockers and co-workers (Levoye et al. 2006) have also identified the G protein coupled receptor 50 (GPR50) as a melatonin-related receptor with 45% identity to the $MT_{1/2}$ melatonin receptors. The GRP50 is unable to bind melatonin, but 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 mediates much of melatonin's oncostatic actions in ERα-positive MCF-7 human breast cancer cells. Employing the 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). Over expression of the MT1 receptor can potentiate the anti-proliferative effects of melatonin on breast cancer cells both *in vivo* and *in vitro*, but are reversed by non-selective MT_1 and MT_2 melatonin receptor antagonists (Yuan et al. 2002; Collins et al. 2003). 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 (AA) women (48%) as compared to Caucasian women (11%) in a cohort of 167 triple-negative breast cancers (TNBC). Furthermore, in TNBC, MT_1 positivity was associated with a lower stage and smaller tumor size at time of diagnosis, while 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 compared to those with MT_1-negative breast tumors (Lai et al. 2009; Rögelsperger et al. 2009; Jablonska et al. 2013; Oprea-Ilies et al. 2013).

MT_1/MT_2 receptor independent actions of melatonin

Melatonin can bind and activate cell membrane associated MT_1/MT_2 G protein coupled receptors (GPCRs) (You et al. 2005; Kohsaka & Bass 2007; Troung 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 to elicit a variety of non-receptor mediated effects in breast cancer. Numerous studies have shown that melatonin binds the Ca^{2+}-regulatory protein calmodulin (CaM) leading 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 that can lead to altered PKA, cAMP biding protein (CREB) and p300 coregulator expression/activation, as well as 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 bound as a ligand to the retinoic acid-related orphan receptors alpha (RORα), members of the NR/steroid receptor superfamily. This report, however, was withdrawn (Erratum 1997), as other laboratories working on RORαs 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 will be discussed later, melatonin via activation of its MT_1 receptor can in fact modulate RORα transcriptional activity.

Initial reports by Reiter and co-workers (Poeggeler et al. 1993) identifying melatonin as a potent free radical scavenger has been confirmed by many other groups further demonstrating 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 that also promoted inhibition of tumor metabolism leading to 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) and 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) with its effects on breast cancer being the most extensively studied. Clinical data as well as 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 that the majority of melatonin's anti-proliferative actions are mediated via activation of the MT_1 receptor as MCF-7 cells 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), inhibitory actions on Ca^{2+} signaling and CaM expression (Dai et al. 2002), and induction of p53 expression (Proietti et al. 2013) and its target gene p21 (Mediavilla et al. 1999). Work by Santoro et al. (2013) demonstrated that blockade 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 arrest of breast tumor cells in the G1 phase of the cell cycle (Cos et al. 1991), repression of estrogen synthesis in the gonads (Blask et al. 2011) or the tumor by repression of aromatase (Martínez-Campa et al. 2009), and finally by suppression of tumor metabolism (Warburg effect) via repression of tumor uptake of free fatty acids, particularly linoleic acid 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 cross talk between melatonin and the estrogen signaling pathway. Subsequent studies, including our own, showing that melatonin inhibited estrogen-mediated proliferation of human breast cancer cells in culture (Hill & Blask 1988; Hill et al. 2011) suggested that melatonin influences estrogenic actions on breast and mammary tissue by three key mechanisms: (1) by suppression of gonadal synthesis of steroids (including estrogens) to decrease their circulating levels; (2) by

interacting with the ERα as a selective estrogen receptor modulator (SERM) to alter estrogen binding, DNA-binding, and transcriptional activity; and (3) by repressing the activity of other enzymes such as aromatase, sulfatase, and aldo-keto reductases (AKRs) 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 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). While 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-leuteal cells (Woo et al. 2001). Since estrogen plays a pivotal role in breast cancer etiology, these data suggested 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 sensitivity to melatonin are evident between different cell lines (Hill et al. 1992; 2011). Employing ERα-positive MCF-7 breast cancer cells, Molis et al. (1994) reported that melatonin-mediated suppression of ERα mRNA. Ram et al. (Kiefer et al. 2002; Ram et al. 2002) was the first to show that melatonin was a potent repressor of estrogen-induced ERα transcriptional activity, inhibiting the expression of numerous estrogen-induced mitogenic and anti-apoptotic genes including Bcl-2, while inducing growth-inhibitory and pro-apoptotic genes including TGF-α and Bax. Lai (Lai et al. 2008) and Kiefer (Kiefer et al. 2002) 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 (ChiP) 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 effecting a delay and decreasing to a lesser degree p300 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 seen in the presence of estrogen. Surprisingly, a daily rhythm if estrogen that peaks in the early morning is evident in the blood (Bao et al. 200; Royston et al. 2014). As plasma melatonin levels typically peak between midnight and 2 am and then decline as estrogen levels begin to rise, this may suggerst that melatonin can differentially influence

CaM and p300 recruitment to the cyclin D1 promoter and other promoters 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 appear to involve changes in phosphorylation of receptor and/or coactivator (e.g. CaM, SRC-1, CBP/p300 etc.) expression and phosphorylation. In a recent report by Dauchy et al. (2014), we 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 extracellular receptor kinase 1 and 2 (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 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 nuclear receptors (NRs) in human breast cancer cells

In addition to its suppression of ERα transactivation, melatonin via it MT_1 receptor, also modulates the expression and/or transcriptional activity of other members of the steroid hormone/NR superfamily. For example, melatonin has been shown to repress the ligand-induced transactviation 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 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 in breast cancer cells that melatonin potentiates the transactivation of the vitamin D receptor (VDR) and the peroxisome proliferation activating receptor gamma (PPARγ). However, not all NRs are impacted by melatonin, as no effects of melatonin were observed on the ERβ in human breast cancer or HEK293 embryonic kidney cells (Lai et al. 2008). The mechanism(s) involved in melatonin's modulatory effects on NRs, particularly the ERα, are not completely delineasted but 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 on gene expression in human breast cancer.

The potential clinical significance of melatonin's regulation of breast cancer NRs in is highlighted in several studies. For example, Eck et al. (1998) demonstrated that pre-treatment of MCF-7 cells with melatonin following theadministration of all trans-retinoic acid, 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 repression of transforming growth factor beta 1 (TGF-β1) and Bcl2 (an anti-apoptotic protein), and increased expression of the

pro-apoptotic protein Bax. Follow-up studies by Teplitizky (Teplitzky et al. 2001) and Melancon (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%, as well as 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) showing 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, ranging from antioxidant, immuno-modulatory, and enzyme regulatory, to 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 coregulators such as calmodulin (CaM), cAMP binding protein (CBP/p300), and steroid receptor coactivator-1 (SRC-1) known to drive breast cancer promotion and/or progression (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 to identify the expression profile of genes regulated by melatonin in MCF-7 cells *in vitro*. Employing genomic profiling, Lee et al. (2011) evaluated miroRNA (miRNA) and gene expression in MCF-7 breast tumor cells treated with 1 nM 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, 5 miRNAs were either up- or down-regulated as compared to 18 miRNAs in cells treated with 100 nM melatonin, with only miR-1207-3p overlapping between the two groups. At the mRNA level, treatment of cells with 1 nM melatonin regulated the expression of twice as many genes as the higher concentration (100 nM) of melatonin. These effects were equally weighted between gene induction and suppression with the majority of genes 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 a number of genes were aberrantly methylated and their expression decreased in response to melatonin, including early growth responsive gene 3 (Egr3) and POU4F2/Brn-3b, both of which are associated with increased invasive and proliferative tumor cell capacity. Conversely, melatonin (1 nM) de-methylated and increased the expression of the tumor and metastasis suppressor glypican-3 (GPC-3).

A genomic profiling analysis by Liu et al. (2013) examining the effect of the carcinogen methyl methanesulfonate on melatonin (1 nM) pre-treated 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, DNA repair, and cancer. Among these genes are several with known roles in regulation of

DNA repair activity including up regulation of *CEP152*, a regulator of genomic integrity and cellular response to DNA damage 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 pre-treatment. N4BP2L2 encodes a phospho-noformate 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 examined the gene expression profile of MCF-7 human breast carcinoma cells transiently transfected with the MT_1 receptor and treated with 1 nM melatonin using a limited (8,000–9,000 genes) cDNA microarray analysis. Close to 300 genes were found to have significantly altered levels of expression ≥ 1.4-fold. Of these, melatonin was found to suppress the expression of more than 210 genes and induce the expression of more than 80 genes. A summary list and values of these cancer related genes and their function can be found in Table 1.

Key examples include cell-to-cell adhesion and communication molecules including fibronectin (FN1) and desmoplakin that were down regulated by 2.6- and 2.3-fold, respectively; the cytokine bone morphogenic protein 7 (BMP7) was down regulated by 2.5-fold, and the growth factors amphiregulin (AREG) and insulin-like growth factor 1 receptor (IGF1R) were down regulated by 2.0- and 1.4-fold, respectively. The Ca^{2+} homeostasis-associated S100 calcium-binding protein (S100P) was also down regulated by 2.6-fold, while nucleobindin 2 (NUCB2) was up regulated by 2.8-fold in response to melatonin activation of the MT_1 receptor.

Genes associated with chromatin remodeling and transcriptional regulation including steroid receptor coactivator one (SRC-1) and the cAMP binding protein (CBP)/p300-interacting transactivator (CITED2), well-established coregulators of the NR family and other transcription factors, were down regulated by 1.4- and 1.7-fold, respectively, while calreticulin (CALR), a NR interacting protein, was up-regulated by 1.6-fold. The transcription factor Endothelial PAS domain protein 1 (EPAS1) also termed Hypoxia inducible factor 2 alpha (HIF2α), which is associated with increased angiogenesis, was down regulated by 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 by 1.7-fold.

Metastasis related genes, including insulin-like growth factor binding protein 5 (IGFBP5), fibronectin (FN1), and ephrin A1 (EFNA1) were down regulated by 3.3-, 2.6-, and 1.6-fold, respectively. However, the tissue inhibitor of metalloproteinase three (TIMP3) was induced by 1.9-fold. Interestingly, TIMP3 has been shown to promote breast tumor sensitivity to tamoxifen 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 tamoxifen. 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, N-Myc downstream regulated (NDRG1), and EFNA1, being decreased by 2.3-, 2.1-, and 1.6-fold, respectively.

Melatonin mediated apoptosis in breast cancer

Melatonin's anticancer actions in breast cancer can be classified as cytostatic or cytotoxic. While 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 melatonin are more frequently observed than 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 via control of 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 is seen in *in vivo* studies in rat mammary tumors showing 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. in 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 involved as the terminal effector.

More recent work by Proietti et al. (2014) confirms the work of Cucina 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, leading to decreased cell cycle progression and promoted p53-mediated apoptosis. Furthermore, they 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 @ 24 h and late @ 96 h). Early apoptosis appears to be caspase-independent, while the later response appears to involve TGF-β1, 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 p53 and p73 release with p53 activated in the early response and p73 mediating the caspase-dependent late response.

A number of studies support 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 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 mechanism(s) by which melatonin exerts its full anti-cancer effects, when used at 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 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 involving 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 (also referred to as the Warburg effect), and it represents the bioenergetic process preferred by cancer cells over oxidative phosphorylation 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. 2008) are important signal transduction and transcriptional networks that drive the Warburg effect to re-route bioenergetics in cancer cells to generate molecular intermediates 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 linoleic acid (LA), an essential omega-6 fatty acid (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 by 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 insulin-like growth factor-1 (IGF-1) receptors (Glasgow & Eling et al. 1994; Glasgow et al. 1997), metabolize LA to the mitogenic metabolite 13-HODE. 13-HODE has been shown to exert a positive feedback effect on EGF and IGF-1R 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), to enhance the downstream phospho-activation of AKT and ERK1/2 leading to the amplification of cell proliferation and survival responses (Hsi et al. 2003; 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 to 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 and organization of tumor growth (Blask et al. 2011). For example, in human breast cancer xenografts grown in athymic female nude rats, greatly elevated tumor uptake of LA and metabolism to 13-HODE and aerobic glycolysis during the daytime are involved in promoting a corresponding increase in cell proliferative/survival activities in tumors, whereas during the nighttime, these processes become quiescent as shown by much lower cell proliferative rates and increased cell loss. Therefore, the relatively slow net tumor growth rate in the presence of an intact nocturnal circadian melatonin signal reflects an overall balance in tumor circadian dynamics characterized by a up-regulation of daytime metabolism, signaling, proliferation and cell survival 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 a constitutively high level throughout the entire day (all 24-hrs). This form of circadian disruption not only provokes a melatonin-deficient state, but also compromises normal rhythms of blood glucose, insulin and IGF-1 leading to hyperglycemia and hyperinsulinemia, in addition to persistently high IGF-1 blood levels (Wu et al. 2011). Thus, a chronic lack of circadian-organization in proliferative activity and metabolic signal processing results in a 24-hour per day hyper-metabolic and -proliferative state that culminates in markedly accelerated overall tumor growth rates. Additionally, key tumor signal transduction and transcriptional factors such as cAMP, AKT, and HIF-1α regulation of LA metabolism and the Warburg effect in tumors show melatonin-driven circadian oscillations that are dramatically disrupted by dLEN indicating their unique role in regulating metabolic fluxes in coordination with classical allosteric feedback mechanisms controlling 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 loss of cellular ability 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 relevant to cellular ability to respond to and repair DNA damage. Genomic instability could originate from DNA damage resulting from internal and external sources. It was previously mentioned that melatonin is a powerful antioxidant and can reduce ROS production and accumulation by improving mitochondrial function as well as by stimulating anti-oxidative enzymes.

Another source of genomic instability in mammalian cells is 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 accumulated to over 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). 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 retrotranspositionally competent ribonucleoprotein (RNP) complex and 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 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 expression of the endogenous retrotransposon L1 in a tissue-isolated model of human cancer (Deharo et al. 2014). This finding supports that LEN-induced suppression of nocturnal melatonin synthesis activates L1 expression. Our tissue culture based experiments demonstrated that MT_1 expression decreases L1 mobilization by about 10-fold in HeLa cells by down regulating L1 ORF1 protein levels (Deharo et al. 2014). These data support that LEN promotes L1 expression and damage in tumor 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 light exposure at night (LEN) on melatonin and breast cancer

Based on early studies demonstrating melatonin's anti-cancer actions in breast cancer, Stevens (Stevens 1987) hypothesized that 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 United States and other westernized countries. Light exposure at night is a well-recognized environmental disruptor of the central circadian timing system located in the SCN (Reiter 1991; Claustrat et al. 2005; Stevens et al. 2007; Straif et al. 2007). 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 that, 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 and early epidemiologic studies by Davis and Stevens (Davis et al. 2001), and Shernhammer et al. (2001) using the Harvard Nurses Cohort, the World Health Organization has designated night shift work involving LEN-induced circadian/melatonin disruption as a probable carcinogen (class 2a) and risk factor for the development of breast cancer (Straif et al. 2007).

A paradigm shifting study by Blask and co-workers (Blask et al. 2005) demonstrated that when tissue-isolated breast tumor xenografts in female nude rats were perfused with blood collected from women at night but exposed to dim light (0.2 lux) at night (melatonin-poor), LA uptake and its conversion to 13-HODE, cAMP levels, and the expression of MAPK downstream effectors MEKs/ERKs were induced. 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 effect of circadian melatonin during dark night was receptor mediated, as 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 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 promoting its inhibitory phosphorylation at serine 9 of GSK3β to block GSK3β activation and ubiquitination activity.

As alluded to above, a recent study by Blask et al. (2014) using 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 the tumor were dynamically coordinated within the circadian time structure of the 24-hour light/dark cycle by nocturnal pineal melatonin production driven by the SCN. This work demonstrated that dLEN and its associated suppression of nocturnal circadian melatonin altered the host/cancer balance in numerous cancer promoting signaling pathways driving hyperglycemia and hyper-insulinemia in the rat and hyper/runaway aerobic glycolysis (Warburg effect), and proliferation in the tumor.

Our most recent work examined the effect of dLEN and melatonin on the development of tamoxifen resistance (TAM-R) in breast cancer (Dauchy et. al 2014). Although the majority of this work will be discussed below 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 (Warburg effect) and the expression and/or phospho-activation of key signaling pathways and nodes involved in tumor proliferation and survival that drive resistance in breast cancer cells to endocrine and chemo-therapies. These signaling pathways 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 one (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 elevated or activated in response to dLEN-induced circadian/melatonin disruption include c-SRC, FAK, cAMP, PKA, CREB, STAT3, NF-kB, and protein kinase C alpha and delta (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 grown in a lighting schedule of 12h light/12 h dark (LD 12:12) with nocturnal circadian melatonin elevated during dark night, or in 12:12dLEN 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 phospho-activation of each of these signaling pathways to dramatically suppress tumor cell proliferation and resistance to endocrine and chemotherapies (Dauchy et. al 2014).

Circadian synchronization is controlled, in part, by ambient light decreasing melatonin synthesis and secretion in the pineal gland and coordinated by the SCN of the hypothalamus. Peripheral cell autonomous circadian clocks termed “peripheral oscillators,” controlled by the master clock in the SCN, exist with every cell of the body including the breast, and are comprised of the same genes as the master clock (Sellix 2013). The clock genes in a peripheral oscillator can subsequently regulate clock-controlled genes 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 & Hoeffler 2009; Wood et al. 2009). For example, Gery et al. (2007) reported that PER2 plays a role in ERα ubiquitination, while 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 & Hoeffler 2009). Thus, loss of PER2 could well be involved in the initiation or progression of breast cancer. Repression of PER2 gene expression by methylation of the PER2 promoter or phosphorylation by casein kinase 1ε (Gery et al. 2007) might be involved in the initiation or progression of breast cancer. 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 administration of melatonin after serum shock.

In an earlier section entitled “Melatonin modulation of gene expression in breast cancer,” 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 III 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 and reduced dio3 promoter DNA methylation to up regulate dio3 expression and gonadal regression. In a genome-wide profiling study of breast cancer cell lines, Lee et al. (2013) demonstrated that administration of 1 nM melatonin to cells was able to alter DNA methylation patterns and decrease the expression of known oncogenic genes including early growth response gene 3 (EGR3) and POU4F2/Brn-3b while 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 epigenetic regulation of gene expression.

A recent study by Dai et al. (2011), using a univariate logistic regression analysis, has 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 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-to-mesenchymal transition (EMT) 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 as suppressed by melatonin via 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 over-expressed either the ErbB2/Her2-neu oncogene or cytokine receptor CXCR4, or MCF-7 cells 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% to 85% in trans-well/matrigel insert assays. In this study Mao demonstrated that the anti-invasive actions of melatonin were at least partially mediated by inhibition of p38 MAPK and matrix metalloproteinases (MMP) 2 and 9, which are involved in degradation of the basement membrane and metastatic cell extravasation.

The cytoskeleton is an important component of the cellular architecture composed of 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, modulates much of the metabolic and signal transduction machinery of the cell (Ingber 2001). 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. Benitiz-King et al. (1990) demonstrated a complex interaction between melatonin and the cytoskeleton in MCF-7 cells. Their studies showed that melatonin induced the formation of focal adhesions in MCF-7 cells and altered the arrangement of microfilaments and stress fibers to form thicker bundles assembled with phospho-vinculin to promote adhesion contacts (Ortíz-López 2009).

Protein kinase C (PKC) via activation and induction of Rho-associated kinase (ROCK) induces stress fiber thickening and decrease 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 new studies currently in press (Hill et al. 2014), we demonstrate that in MCF-7 tissue-isolated tumor xenografts grown in female nude rats housed in a photoperiod of 12h light:12 dLEN PKCα levels were elevated but were dramatically inhibited in tumors 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 above reports suggest that melatonin may inhibit the invasive/metastatic capacity of breast cancer cells through inhibition of PKCα-induced stress fiber formation.

The developmental process of EMT in which epithelial cells acquire a mesenchymal phenotype and become migratory (Tomaskovic-Crook et al. 2009), is also 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 include loss of cell adhesion

proteins, cytoskeleton reorganization, acquisition of mesenchymal cell spindle-like morphology with increased motility and invasiveness. A key hallmark of EMT is the reduced expression of E-cadherin, a structural component of adherent junctions, critical for epithelial cell polarity and adhesion (Hollestelle et al. 2013; Chen et al. 2014).

Also linked to progression of EMT is the Wnt/β-catenin pathway, with β-catenin being a core component of the adherent junctions through its binding to E-cadherin (ten Berge et al. 2008). Upon dissociation of β-catenin from E-cadherin, it is able translocate to the nucleus and complex with the T cell factor/lymphocyte enhancer factor (TCF/LEF) a key transcription factor to promote the expression of Wnt target genes including Snail, Slug, and c-MYC transcriptional repressors of E-cadherin, and 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 3β (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). GSK3β when activated phosphorylates β-catenin promoting its ubiquitination/degradation. Inhibitory phosphorylation by AKT or WNT as s9 of GSK3β stabilizes β-catenin, allowing its translocation to the nuclear translocation 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, known repressor of E-cadherin (Zhou et al. 2004). Thus GSK3β, due to 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. Light exposure at night suppresses the nocturnal circadian melatonin to disrupt 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 inhibiting-phosphorylation of GSK3β, allowing GSK3β to induce β-catenin ubiquitination and inhibit EMT. Thus, chronic disruption of the circadian melatonin profile by LEN (occupational exposure to light at night or age-related sleep disturbances) and its inhibition GSK3β activity and promotion of EMT, appears to be an important contributor to the 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 over-expression 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 tamoxifen (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 while most patients that are initially responsive will eventually develop acquired resistance to TAM (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 others that initially respond 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 activation of several key-signaling pathways including the EGFR/HER2/MAPK/ERK and PI3K/AKT pathways, as well as a variety of other signaling pathways (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 and chemotherapies. Furthermore, intrinsic or acquired resistance to endocrine therapies or chemotherapeutic drugs has also been linked to up-regulation of ABC transporter efflux molecules (Gottesman et al. 2002) such as ABCB1 (MDR-1/P-gp), ABCC1 (MRP1), and ABCG2 (BCRP) as well as drug metabolizing enzymes (Kassner et al. 2008; Novotna et al. 2008), all of which lead to diminished levels of active drug within the cancer cells. Finally, mounting evidence supports the concept that dysregulated cellular metabolism (aerobic glycolysis, Warburg effect) is linked to drug resistance in cancer therapy via up regulation of tumor glucose and lactate, and 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 examining the effects of 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 12h light:12h 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 (Warburg effect) with increased tumor glucose uptake and lactic acid production. These tumor xenografts from rats exposed to dLEN, showed complete TAMR when treated with 4-hydroxy-tamoxifen (4OH-TAM). Conversely, tumor xenografts from rats housed in LD 12:12 with dark night (elevated endogenous melatonin), or in 12L:12 dLEN but supplemented with melatonin during dLEN, showed abolished or greatly diminished expression and/or phospho-activation of these tumor promoting kinases (ERK1/2, SRC, FAK) and transcription factors (CREB, ERα, STAT3), 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 show that dLEN promotes but melatonin inhibits the phosphorylation of the 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 convert the response of the ERα to TAM from antagonist to agonist. As noted earlier, we (Lai et al. 2008), and others (Del Rio et al. 2004),

have shown that melatonin can repress estrogen-induced EPα 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 repression of key kinase signaling pathways, alters ERα phospho-activation and thus ERα's responsivity to TAM to ensure TAM acts as an ERα antagonist. In unpublished studies we have found that exposure to dLEN increases the expression of the ABC transporter ABCG2 also termed breast cancer resistance protein (BCRP) a known efflux pump for 4OH-TAM and Endoxifen (Selever et al. 2011). Our data suggest that melatonin, in addition to its regulation of tumor metabolism and cell signaling, can also sensitize the response of breast tumors to TAM by regulating its interaction with the ERα and by repressing the efflux of TAM from breast tumor cells.

In work recently presented at the American Association of Cancer Research meeting on Cancer Prevention (Hill et al. 2014), we reported that MCF-7 tissue-isolated breast tumor xenografts grown in female nude rats housed in dLEN environment showed complete intrinsic resistance to the anthrecycline, Dox. This resistance, as seen with TAM-R, was associated with elevated aerobic glycolysis (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 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 5 decades since the early observation by Lapin and Ebels (Lapin & Ebels 1976) that extracts from the pineal gland repressed 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 including 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 being mediated through a number of mechanisms involving a multitude of molecular pathways. The majority of studies indicate major involvement of the MT_1 receptor for mediating both the cytostatic and pro-apoptotic actions of melatonin. Yet while, melatonin's actions as a potent endogenous scavenger of reactive oxygen species (ROS) are clearly important to genomic stability and tumor initiation, the extent of their 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, TGF-β1, NRs including RARα, RXRα, and VDR, and even clock genes such as PER2.

A number of new important avenues regarding melatonin's actions in breast cancer have recently emerged, representing great potential to better understand melatonin's anti-cancer properties and potential future as a therapeutic agent for breast cancer. First, work by Blask and Dauchy (Blask et al. 2014) have demonstrated that the circadian melatonin signal inhibits aerobic glycolysis (Warburg effect) in breast tumors, blocking tumor uptake of linoleic acid and glucose. Future studies need to define the mechanism(s) involved and the downstream consequences of these metabolic changes on cell signaling and tumor progression. Second, our recent reports combined with studies by Cos (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 mechanism(s) and pathways 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 to begin to fully understand how LEN impacts breast cancer initiation, promotion, and progression. These studies will be essential to identify the interactions and independent effects mediated by melatonin via the central clock and peripheral oscillators in the breast and breast cancer. Fourth, exciting new studies by the Belancio laboratory (Deharo et al. 2014) demonstrating 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 if 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 the multiple-kinase inhibitors in combination with chemotherapeutic agents. The broad action of melatonin on breast cancer including its inhibition of tumor metabolism, signaling, and genomic instability, its activity as a scavenger of ROS, synergism with other cancer therapeutic agents, lack of toxicity, and wide availability and minimal cost, should make its movement into clinical trials a high priority.

Acknowledgments

Financial Support: This work was supported by the following grants NIH Grant R21CA129875–04 (to DEB) and an American Association for Laboratory Animal Science GLAS grant (RTD and DEB), and Life Extension Foundation (VPB). Part of the cost of this work was defrayed with the support of the Edmond and Lily Safra Endowed Chair for Breast Cancer Research (SMH recipient) at Tulane Cancer Center.

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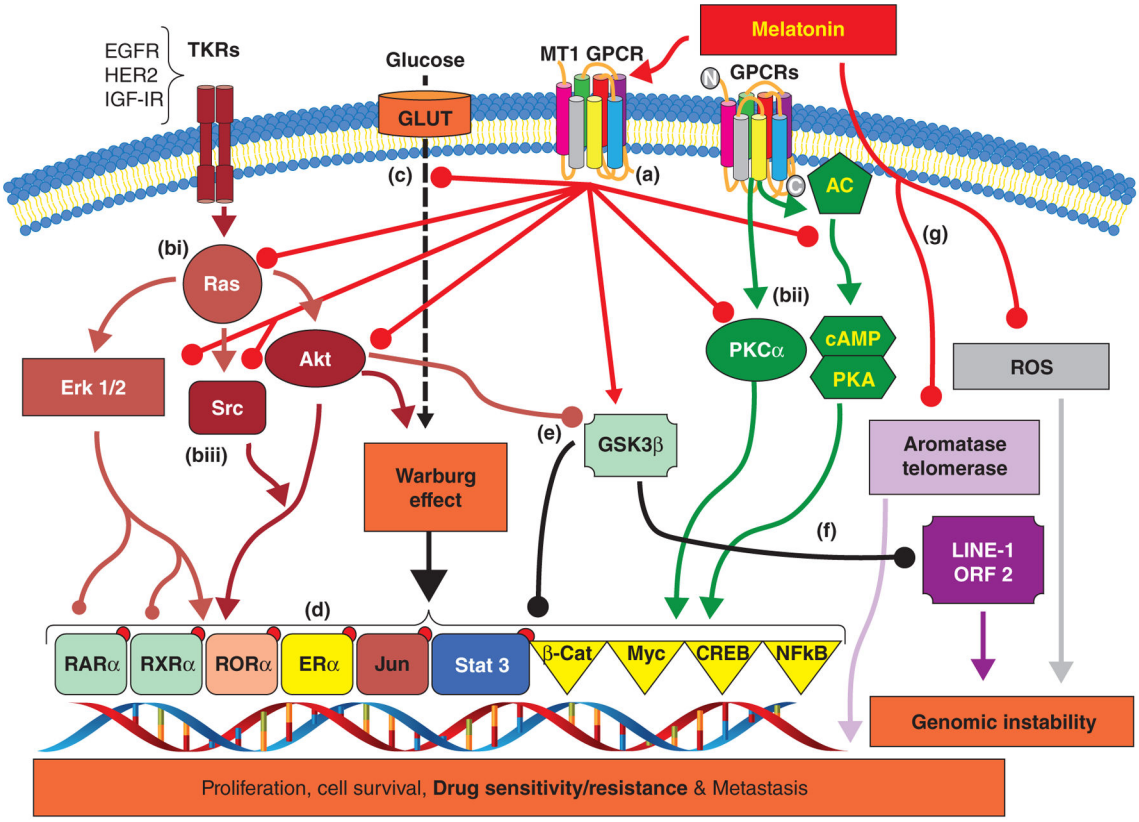

Figure 1.

Table 1

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

GENE SYMBOL	GENE NAME	FOLD CHANGE	FUNCTION	ACCESSION NUMBER
Cell-to-cell adhesion and communication				
FN1	Fibronectin 1	−2.6	Involves cell adhesion, cell motility, 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-beta superfamily, induces epithelial cell growth	BE395650
Growth factor and receptors				
AREG	Amphiregulin (schwannoma-derived growth factor)	−2.0	A member of the epidermal growth factor family, growth inhibition and stimulations	NM_001657
IGF1R	Insulin-like growth factor 1 receptor	−1.4	Binds insulin-like growth factor, plays a critical role in transformation events.	X04434
Signal transduction-related				
S100P	S100 calcium-binding protein P	−2.6	Calcium-binding protein, mediates Ca2+ signals, overexpressed in breast cancer	BE536069
CALR	Calreticulin	1.6	Ca2+ binding protein, modulator of nuclear receptor function, possible role in angiogenesis	M84739
NUCB2	Nucleobindin 2	2.8	Ca2+ binding protein, possible role in calcium homeostasis	AF052644
Chromatin remodeler and coactivator				
CITED2	Cbp/p300-interacting transactivator	minus;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 monoclonal antibody Ki-67	−1.8	Possible role in cell proliferation	X65550
p21	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	1.7	Cyclin-dependent kinase inhibitor, blocking cell cycle progression	U09579

GENE SYMBOL	GENE NAME	FOLD CHANGE	FUNCTION	ACCESSION NUMBER
Development and differentiation-related				
NDRG1	N-myc downstream regulated	−2.1	A member of the N-myc downregulated gene family, possible role in growth arrest and cell differentiation, induced by hypoxia	X92845
Metastasis-related				
IGFBP5	Insulin-like growth factor binding protein 5	−3.3	Growth inhibitor and proapoptotic agent in breast cancer, possible role in metastasis	AA374325
FN1	Fibronectin 1	−2.6	Cell adhesion, cell motility, 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 metalloproteinases, 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
NDRG1	N-myc downstream regulated	−2.1	A member of the N-myc downregulated gene family, induced by hypoxia	X92845
FN1	Fibronectin 1	−2.6	Involves cell adhesion, cell motility, 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	Ca2+ binding protein, modulator of nuclear receptor function, possible role in angiogenesis	M84739
TIMP3	Tissue inhibitor of metalloproteinase 3	1.9	Natural inhibitors of the matrix metalloproteinases, inhibits VEGF binding to VEGFR2 and angiogenesis	AI095372

* 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.