Effects of chronic melatonin treatment on cell proliferation and MAPK signaling in MCF-7 human breast cancer cells

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Abstract

The hormone melatonin may have anticancer effects in humans. In particular, previous work in animal models and cell culture links melatonin action to estrogen-dependent cell signaling pathways that control proliferation. To assess effects of melatonin alone and in combination with estrogen on the growth of MCF-7 human breast cancer cells and phosphorylation of the mitogen-activated protein kinases, ERK1/2, cells were cultured under low serum conditions and treated with physiological levels of melatonin and/or 17ß-estradiol (E2). AlamarBlue fluorescence was employed to measure total cell numbers, and ERK1/2 signaling was analyzed by PAGE/western blotting using phospho-specific antibodies. The results demonstrate that chronic treatment with 10nM melatonin over 5 days decreased the number of cells compared to untreated control cultures. When combined with 100pM E2, melatonin inhibited estradiol-induced cell growth. Though 24 hours of melatonin treatment surprisingly had no effect on ERK1/2 phosphorylation, the melatonin receptor antagonist (luzindole) alone dramatically elevated phosphorylation of ERK1/2 regardless of treatment, suggesting that melatonin receptors enforce a tonic suppressive effect on the MAPK signaling.

To my uncle Chris Green, who is fighting the good fight.

Introduction

In the last few years, increasing evidence has pointed to a connection between circadian rhythm disruption and a increased risk of cancer. In an age where electric lighting is widespread and many industrialized systems require 24-hr constant maintenance, nighttime shift-work has become commonplace. In response to multiple studies noting a modest increase in the risk of breast cancer in long-term nighttime employees as compared to daytime shift worker across various geographical regions (Straif et al., 2007), the IARC (International Agency for Research on Cancer) officially classified night shift-work involving the disruption of circadian rhythm as a probable cause of cancer (IARC, 2010).

Though multiple factors could be involved in this odd correlation between nighttime activity and cancer, research has suggested that exposure to light at night (LAN) is the largely responsible element. Exposure to light during the dark hours has been shown to lower melatonin secretion, a neurotransmitter hormone that is closely associated with circadian regulation (Vollrath, 2001). Further exploration of this phenomenon will require extensive study, both epidemiological and physiological. Here I present a body of work focusing on the anticancer effects of melatonin treatment in cultured human breast cancer cells.

Melatonin

Melatonin is an evolutionarily ancient indolealkylamine present in plants, fungi, bacteria, eukaryotic unicells, and numerous taxa of invertebrates (Hardeland and Poeggeler, 2003). Present in the human as a hormone and neurotransmitter, melatonin is produced largely in the pineal gland, where it is also stored and secreted. Characterized in 1958 by Aaron Lerner (Lerner et al., 1958) as 5-methoxy-N-acetyltryptamine (Figure 1), the biosynthesis and regulation of the hormone has since been demystified. The amino acid tryptophan is taken up from circulation into the pineal gland, where it is first

hydroxylated, then decarboxylated to form 5-hydroxytryptamine, also known as serotonin. Serotonin is N- acetylated, and this product is then O-methylated to yield the final product of melatonin. These four intracellular steps are well conserved, allowing for the production of melatonin even in lower, non-mammalian organisms.

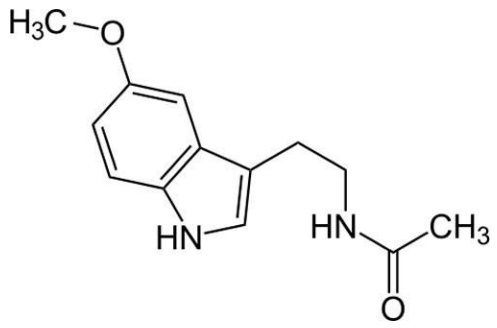

Figure 1: The molecular structure of melatonin

In order to function in an environment with such strict and constant rhythms as night and day, summer and winter, an organism must have some method of detecting and exploiting these natural patterns. Melatonin is the signal by which our bodies interpret the natural levels of light, thus allowing for the distinction between night and day, and the subsequent and necessary synchronization of the physiological system to match this rhythm. Melatonin is the darkness detector of our bodies; its production and secretion is largely inhibited by the presence of light.

Pelham et al. (1973) described the roughly sinusoidal melatonin secretion profile in human males. During daytime hours, blood plasma concentrations of melatonin were observed to be very low, averaging approximately 1.5 pg/ml. Beginning at 2000 hours, levels rose, and peaked at about 0200 hours. Typically, this peak reached about 42 pg/ml before melatonin returned to its daytime levels, at around 0900 to 1100 hours. Overall, it was estimated that approximately 20mcg of melatonin is released during a 24- hour period, eighty percent of which is formed at night (Pelham et al., 1973) (Figure 2). These regularly fluctuating levels allow the body to chemically identify the external light/dark cycle, associating high levels of melatonin with darkness, and low levels with daytime. In

order to move backwards along the causal pathway that instigates the changes in melatonin production, we must examine the central clock of the body.

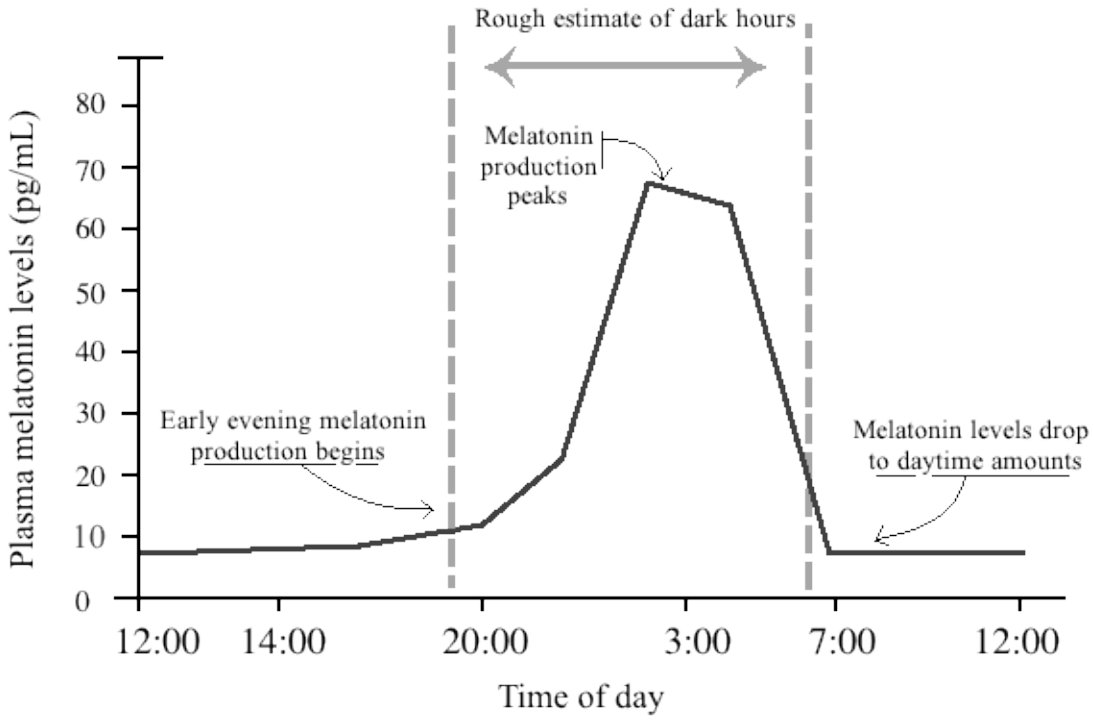

Figure 2: Blood plasma melatonin profile over a 24-hour period

Natural human secretion of melatonin in blood plasma coincides with the 24-hour light/dark cycle. Beginning in early evening, the pineal gland begins to produce melatonin. Production peaks around 2:00 am, and then tapers off to drop melatonin levels back to the low daytime quantities by morning. Figure altered from epgonline.org without permission.

In humans, the internal body clock is driven by the master pacemaker in the brain- the suprachiasmatic nucleus (SCN). Located above the optic chiasm and flanking the third ventricle at the base of the hypothalamus, this tiny organ is entrained to light/dark cycles by direct retinal innervation from the photic input pathway called the retinohypothalamic tract. Photoreceptive retinal ganglion cells use a photopigment, melanopsin, to detect even brief exposures to light at dawn and/or dusk and thereby entrain the SCN clockwork to solar time, ensuring that the approximate oscillator runs on a precise 24 hour cycle. The SCN is responsible for directing a network of systems requiring circadian rhymicity, including sleep, alertness, hormone levels, physical

activity, metabolism, and body temperature. (Maywood et al., 2007). Mini oscillators, synchronized to the SCN, are scattered throughout the peripheral tissues and assist in keeping these regular functions coordinated.

As the SCN is entrained to the light and dark phases, it in turn is responsible for driving the production and secretion of melatonin from the pineal gland. During the nighttime period, there is an increase of noradrenalin from intrapineal postganglionic sympathetic fibers that acts upon adrenoreceptors of pinealocytes, which stimulate adenylate cyclase activity and cyclic AMP (cAMP) formation. Increased cAMP levels stimulate tryptophan hydroxylase activity, converting tryptophan to serotonin. From here, serotonin is transformed to melatonin, which is then excreted into circulation and proceeds to act upon a wide variety of peripheral tissues (Drijfhout et al., 1997).

Perturbations in either the exposure to light or the detection thereof result in adaptation of the clock system. Following an artificial 8 hr phase advance of the light/dark cycle (such as with jetlag), it takes the melatonin cycle 10 days to adjust completely. (Drijfhout et al., 1997). In addition, evidence provided by the large proportion of blind people without conscious perception of light, as well as people living in the dark Antarctic winter, provides valuable information about the endogenous SCN rhythm when uninfluenced by retinal innervation. Studies on these groups of people indicate that the human endogenous circadian length runs for slightly longer than 24 hours, (Vollrath, 2001) which brings up interesting questions as to this discrepancy between the natural light cycle and the human physiological approximation.

Detection of the light/dark cycle is essential not just to maintain an appropriate sleep/wake cycle, but also to orient an organism within the seasonal rhythms. This is especially important for animals that experience breeding seasons. In 1988, a study conducted by Porter et al. was undertaken to investigate the role of melatonin and the pineal gland as intermediaries in the transfer of photic information on daily and calendar time in the control of the timing of the parr-smelt (freshwater to saltwater) transformation in the Atlantic salmon. Initial experimentation confirmed the presence of a seasonally changing light-dark rhythm of melatonin in Atlantic salmon smolts (adults), the profile of which accurately reflected the dark phase throughout the annual photoperiodic cycle. Researchers were able to set salmon about 3 weeks behind or ahead in their natural

progression towards adulthood by either pinealectomizing subjects, or implanting artificial melatonin sources. These results were interpreted to suggest that melatonin level manipulation had influenced the salmon's physiological awareness of the season, prompting an acceleration or deceleration of the maturation rate. However, the mechanism for this supposed modification remains unclear (Porter et al., 1998).

Though the melatonin cycle seems to be driven by the external shift from light to dark, evidence suggests that this correlation might not be entirely accurate. As mentioned previously, the distinctive day-to-night cycle is still present in blind or light-deprived animals, driven by the endogenous SCN circadian rhythm. Efforts to stimulate melatonin synthesis by exposure to darkness during daylight hours have been unsuccessful. However, research has shown that even short bursts of light at night are effective at suppressing melatonin formation (Klein and Weller 1972; Kanematsu et al.,1994 ; Drijfhout et al,. 1996), including pulses as short as 1 ms (Vollrath et al 1989). In addition, research suggests that the earlier the light exposure stops, the more likely the melatonin cycle is to recover (Vollrath, 2000).

As previously introduced, there has been growing awareness towards the potentially carcinogenic effect of light exposure at night (LAN), due to its effects on the circadian rhythm. The LAN theory suggests that the introduction and increasing use of electricity to light the night accounts for some of the international differences in risk of breast cancer (Stevens, 2009). This effect could be explained by lowered levels of protective melatonin, resulting from LAN-induced circadian rhythm disruption in countries with widespread artificial lighting.

In addition to melatonin suppression, LAN has also been implicated in the alteration of clock gene functioning and de-synchronization of the master clock in the suprachiasmatic nucleus from the peripheral clocks in tissue. Whether these two effects are causally connected remains to be proved, but significant evidence suggests that melatonin plays a role in regulating or supporting the function of cellular core clock genes (Xiang et al., 2012).

The Melatonin Receptors

MT1and MT2 melatonin receptors are G-protein-coupled receptors (GPCRs) that, when linked to G_i and G_q proteins, cause decreases in protein kinase A (PKA), cAMP, and cAMP response element binding protein (CREB) activation, or increases in phosphoinositide (PI) hydrolysis and protein kinase C (PKC), respectively (Bondi et al., 2007). The mechanisms underlying these actions of melatonin induced MT1 activation are currently being illuminated, and could involve multiple signaling cascades including calmodulin, estrogen receptors and/or MEK and ERK 1/2 (Witt-Enderby, 2006).

In order to study the downstream and morphological effects of activating the MT1 receptor with melatonin treatment, Bondi et al (2007) used the MT1 Chinese hamster ovary model (MT1-CHO). This model has been essential in mapping the signaling pathways underlying melatonin-induced cellular differentiation seen in other models for several reasons: the activation of MT1 receptors in MT1-CHO cells leads to a measurable response, the cells are not under the influence of the light/dark cycle, and they do not contain an endogenous pacemaker that could influence receptor expression and function. In addition, the cells can be grown in serum-free medium, reducing the complications that arise from serum levels, as discussed later. It was found that melatonin treatment induced MT1-CHO cells to hyperelongate through a MEK 1/2 and ERK1/2 - dependent mechanism that is dependent upon MT1 receptor internalization, G_i protein activation, and clathrin-mediated endocytosis. To this end, MT1 receptors were shown to form complexes with MEK 1/2 and ERK 1/2 in the presence of melatonin, and in MT1-CHO cells, 5 hours of melatonin exposure resulted in approximately two fold more activated MEK 1/2 and ERK 1/2 compared with vehicle (Bondi et al., 2007).

Luzindole as an MT1/MT2 Receptor Inhibitor

Luzindole (N-acetyl-2-benzyltryptamine) is a selective melatonin receptor antagonist. Found to be a potent and competitive melatonin receptor antagonist in rabbit retina (Dubocovitch, 1988), the compound is often used experimentally to inhibit MT1

and MT2 receptors. However, the full action of luzindole on cellular physiology has not been elucidated. Aside from directly inhibiting the MT1 and 2 receptors, luzindole has been shown to reversibly inhibit voltage-activated transient outward K+ current, in a dose-dependent manner at concentrations of 1 μM to 1 mM. This effect was still observed when luzindole was combined with an additional melatonin receptor antagonist (4 P- PDOT), suggesting that the inhibition of K+ current is not mediated through luzindole's action on the MT1 and 2 melatonin receptors (Mi-ou et al., 2003).

Cancer and Melatonin

By the mid 1970's, a link had been established between pineal function and carcinogenesis. Research by Vera Lapin in 1976 on the antineoplastic effects of sheep pineal extracts (containing melatonin) on different tumors in rodents showed a definite potential for further exploration (Lapin and Ebels, 1976). Further work showed that the incidence of experimentally-induced cancers in pinealectomized and thymectomized rats was significantly higher than in intact rats, leading researchers to suspect a link between the neuroendocrinological regulation of the pineal gland and the immunological control of cancer (Lapin, 1978; Wrba et al., 1975). Although these anticancer effects were initially linked only to general extracts taken from pineal tissue, it soon became clear that melatonin was likely the responsible element. In the 1980's and 1990's, the exploration of melatonin had erupted into a full investigation of the antiproliferative and morphological effects of melatonin as well as the link between the hormone receptors present in the cancerous tissue and the effects of melatonin. Specifically, mammary cancers responded robustly to changes in pineal function, prompting further study of this phenomenon. Shah et al found that incidences of 9,10-dimethyl-1,2-benzanthracene (DMBA) -induced mammary cancer were higher in female rats that had been "functionally pinealectomized" by exposure to light 24/7 since birth, and that administration of daily melatonin completely eliminated the effect of constant light, dropping the frequency of DMBA induced cancers from 95% to 25% (Shah et al., 1984).

Clock Genes

Defective clock genes and clock-controlled-genes (CCG) and their effect on cell cycle regulation have been implicated in cancer. Recent studies show that breast tumor cells fail to express the essential clock protein PER2, the re-expression of which incites cell cycle arrest and subsequent apoptosis in MCF-7 cancer cells, via up-regulation of the p53 pathway (Xiang et al., 2012).

Normally, a delicate and complex rhythm plays out within the suprachiasmatic nucleus (SCN) of the hypothalamus, controlling the internal clock of the body and directing the circadian rhythm. This tempo is based on the transcriptional/translational feedback loop of clock proteins Period (PER), Cryptochrome (CRY), CLOCK, and brain and muscle arnt-like protein-1 (BMAL1). This feedback loop is driven by two protein complexes, containing transcription factors CLOCK and BMAL1, that form a heterodimer which binds E-box enhancer elements in promoters of the target genes driving the transcription of Per1, Per2, Per3, Cry1, Cry2, Dec1, and Dec2 genes. Once these genes have been activated, the CRY and PER proteins are translated in the cytoplasm, where they form heterodimeric complexes that translocate to the nucleus and proceed to inhibit their own transcription by binding to the BMAL1 promoter, thereby restraining the BMAL1 gene expression (Xiang et al., 2012).

Driven by this cycle, the SCN synchronizes peripheral clocks (oscillators) placed throughout the body, allowing all normal cells in the body to be synchronized with the internal rhythms of the body, which are ultimately driven by the external rhythms of the environment.

Melatonin is part of the link between the external stimulation of the light/dark cycle that orients the internal rhythm of the SCN, and the cellular recognition of the photoperiod. Based on the evidence that the circadian clock located in the SCN regulates cell cycle and cell growth by providing circadian synchronization for cell proliferation and apoptosis (Bjarnason, et al., 2001; Oikonomou et al., 2010), Xiang et al. (2012) tested whether melatonin would behave as an actuator for enforcing normal cell growth and death upon cancer cells with dysfunctional core clock genes. They found that in MCF-7 cells, which fail to express the clock protein PER2 normally, the re-expression of

PER2 induces p53 up-regulation, cell cycle arrest, and, subsequently, apoptosis. In response to serum shock (addition of 50% horse serum to cell culture), they determined that the core clock genes of these cells could be induced to oscillate in vitro, and showed circadian rhythmicity as xenografts in hosts with intact circadian rhythms. These results are quite intriguing, providing further support to the critical nature of the circadian system in the etiology of breast cancer and implicating melatonin as a crucial link between the centrally controlled circadian clock and the proper actuation of this clock on the cellular level (Xiang et al., 2012).

17β-estradiol Action

Estrogenic steroids have been observed to incite strong proliferative responses in a variety of cell types, particularly those of female reproductive tract tissues and mammary gland tissues (Soule, 1980). MCF-7 cells are estrogen receptor alpha positive (ERα (+)), and quite responsive to estradiol (E2) stimulation. Estradiol treatment in MCF-7 cells induces a general up-regulation of positive proliferation regulators, including survival factors, cell cycle progression gene products, growth factors, and regulatory factor-receptor loops. In addition, transcriptional repressors, antiproliferative and apoptotic genes, and certain families of growth inhibitory factors are all down-regulated (Frasor et al., 2003).

The result of this transcriptional regulation is an accelerated cell cycle, as well as the re-entry of normally non-cycling G0 phase cells into active proliferation. Sensitivity to mitogenic stimulation is limited to the G1 phase of the cell cycle, transit through which is regulated by the activities of cyclin-dependent kinases such as Cdk4, Cdk6, and Cdk2.

Estradiol acts upon the cell through stimulation of the ERα receptor. Activation causes the ligand-bound receptor to dimerize and translocate to the nucleus, where it acts upon the promoter regions of target genes marked with specific estrogen response elements (EREs). Here, E2 and ER action is known to include the stimulation of gene transcription together with specific co-regulators that possess histone modifying and chromatin remodeling activities, which are able to overcome the repressive structural features of chromatin and thereby induce active transcription (Kraus and Wong, 2002). In

addition to these longer-term effects, ER activation includes the ability to invoke the membrane starting activation of specific rapid phosphorylation cascades such as Src/ERK/MAPK pathways, leading to rapid effects in the cell (Galluzzo and Marino, 2006).

Clinical Studies

In 1982, Tamarkin et al (1982) found a link between nocturnal plasma melatonin levels and the estrogen receptor concentration of breast tumors in women with stage I and II breast cancer. Though sample size was small (20 women), it was found that the nocturnal increase in melatonin was far less in women with ER positive tumors, and lowest in those women whose tumors had the highest concentration of estrogen receptors. Although the researchers were unable to conclude whether the altered melatonin profile preceded the malignancy or vice versa, this significant correlation between the peak plasma melatonin concentration and the estrogen receptor concentration of the tumor suggested a diagnostic method for determining the hormonal dependence of the tumor. (Tamarkin et. al, 1982)

In a meta- analysis performed between 1992 and 2003 of 10 randomized controlled trials of melatonin treatment in solid tumor cancer patients and its effect on survival at one year, Mills et al (2005) showed that melatonin reduced the risk of death at 1 year, and that its effects were consistent across the type of cancer and the dose of melatonin. No severe adverse events were reported, underlining the apparently innocuous nature of melatonin treatment. However, it was noted that a difficulty with this analysis was that the clinical trials in question had all been performed by the same network of investigators in Poland and Italy (Mills et al., 2005). In light of the fairly small number of clinical trials oriented around the effects of melatonin in cancer, it is clear that further independent trials are necessary to truly establish how melatonin can be used in a clinical setting.

Melatonin may also impact receptor negative tumors. In triple negative breast cancers, (ER-, PR- Her2/nu -), MT1 negative cancers showed a significantly higher hazard ratio for disease progression, disease-related death, shorter progression free

survival, and shorter overall survival. This evidence suggests that the MT1 receptor may play a role outside of its interaction with ERα (Oprea-Ilies et al., 2012).

Effects On Cell Proliferation

Outside of larger clinical studies, the effect that melatonin has on breast cancer cells *in vitro* is both compelling and puzzling. The MCF-7 cell line, being responsive to estrogens and progestins, is an excellent *in vitro* cell model to study the effects of melatonin in conjunction with estrogens and overall cell growth. Many such studies have observed a direct antiproliferative effect of melatonin treatment on *in vitro* cultured MCF-7 cells; several others have failed to do so, casting some confusion on the phenomena. However, the list of possible variations between different experiments suggests that the reported antiproliferative actions of melatonin are perhaps valid. In light of the studies concerning clock gene expression and cell cycle control, it is possible that melatonin plays a role in normalizing the excessive growth patterns of cancerous MCF-7 cells.

In response to the growing correlation between tumorigenesis and melatonin in live animal models, Hill and Blask (1988) determined that treating MCF- 7 cells with evening-time physiological serum concentrations of melatonin (10nM) inhibited proliferation as much as 60%-70%, as measured by either DNA content or cell count. This effect was found to be reversible once the melatonin was removed from the media, restoring logarithmic growth to the cells. No effect was seen in either human foreskin fibroblasts (-ER), or, curiously, the ER+ human endometrial cancer cell line RL95-2. The team did note significant morphological changes in cells treated for 4 days with melatonin, including reduced numbers of surface villi, nuclear swelling, cytoplasmic and ribosomal shedding, disruption of mitochondrial cristae, vesiculation of the smooth endoplasmic reticulum, and an increase in the number of autophagic vacuoles (Hill and Blask, 1988).

In addition to acting as an antiproliferative in MCF-7 cells, melatonin also appears to work against metastasis, reducing the invasive potential of several model cell lines. In an effort to investigate the effects of melatonin treatment on the invasiveness of

MCF-7 cells, Cos and colleagues performed a variety of *in vitro* invasion assays testing the broader oncostatic properties of melatonin. They determined that 1nM (daytime) levels of melatonin were able to counteract E2-induced invasion and cell adhesion, as well as lower the chemotactic response of MCF-7 cells. Melatonin treatment also reduced the ability of the cancer cells to adhere to the basement membrane, a consequence that proved to be more effective following 5 days of prolonged exposure of the cells to treatment. Simultaneous addition of E2 and melatonin resulted in significantly lower chemotactic response than in E2 treated cells, suggesting an inhibitory action of melatonin of the E2-mediated mechanisms of invasion (Cos et al., 1998).

Furthermore, the team also investigated the influence of melatonin on the expression of two cell-surface adhesion molecules, E-cadherin and β-1 integrin, both membrane proteins that regulate cell-cell contact and interaction with the extracellular matrix. Melatonin treatment was found to increase the β-1 integrin subunit and E-cadherin expression, and also appeared to promote the differentiation of tumor cells, an effect opposing that of estrogen. These effects were thought to be at least partially responsible for the lowered invasion status of the treated cells.

The anti-proliferative effects of melatonin were found to be reversed by the addition of E2 to the culture. Like the Hill and Blask study mentioned previously, Crespo et al (1994) found that after 4 days, melatonin treated cells exhibited morphological and morphometric features characteristic of degeneration, including mitochondrial swelling with disruption of cristae, cytoplasmic vacuolation, nuclear chromatin disaggregation and cell lysis, as well as significantly smaller cell and nuclear sizes than control groups.

While control and E2-treated cells demonstrated increased tumor-like characteristics, melatonin treated cells presented morphologically greater differentiation. Crespo et al hypothesized that melatonin acts through a cell-cycle specific mechanism by delaying the entry of MCF-7 cells into mitosis, thereby allowing the tumor cells to achieve greater differentiation. Given the known cell-cycle accelerating effects of E2, it seems likely that melatonin's inhibitory action is partially due to the counteraction of this E2-induced cell cycle acceleration (Crespo et al., 1994), or potentially through the restoration of the normal patterns of cell cycle transit.

Mao et al (2010) used matrigel invasion chambers to determine that treatment with melatonin at physiological levels significantly lowered the invasive potential of MCF-7 derived cells. The researchers designed three cell lines, two of which stably expressed either the Her2/neu receptor or the chemokine receptor CXCR4, both of which are implicated in a higher likelihood of metastasis, and their presence associated with a poor prognosis in breast cancer patients. The third line was descended from normal MCF-7 cells, but had been selected for metastatic potential. The invasive potential of these three cell types was significantly reduced after treatment with melatonin, and downstream effects of the treatment included reduced activity of two matrix-metalloprotinases (MMPs) thought to be complicit in degrading the extracellular matrix, an important step towards metastasis. This action of melatonin was demonstrated to include the repression of the P38 MAPK phosphorylation pathway, upstream of the MMPs.

The complications of serum in hormone-sensitive cell culture

A difficulty of studying hormonal effects in cultured cells concerns the necessary use of fetal bovine serum (FBS) as a supplement for cell growth and function.

FBS contains an assortment of growth factors, including various hormones and estrogen analogs. Most of the non-peptide hormones can be removed by charcoal stripping the serum, a common practice for experiments testing hormone effects. However, it is known that the non-estrogenic compound estrone sulfate is not completely removed by the charcoal treatment, and Vignon et al (1980) showed that MCF-7 cells convert estrone sulfate to estrone, an estrogenic hormone. In this scenario, estrogen sulfate enters the cells and is metabolized, yielding unconjugated estrogens including the most active form, estradiol, which are bound to the nuclear estrogen receptors. As such, it is impossible to achieve a truly hormone-free environment with standard levels of charcoal stripped FBS, despite the purifying step. Previous investigation by Toffey (2011) into the antiproliferative and antiestrogen effects of melatonin were performed under 5% cs-FBS serum levels, which prevented experimental conditions from being truly estrogen free. According to Briand and Lykkesfeldt (1984), the concentration of estrone sulfate in medium containing 0.05% FBS or 5% cs- FBS was 2 to 4 x 10~11 M,

and proved to be sufficient to translocate estrogen receptors to the nucleus of MCF-7 cells. No such translocation of estrogen receptors could be measured in cells grown in 0.05% cs-FBS, which corresponded to levels of 2×10^{-13} M estrone sulfate (Briand and Lykkesfeldt, 1984).

To further inspect the mechanisms behind the antiproliferative effect of melatonin, we proceed to examine the MAP Kinase family of signaling molecules.

ERK And MAPK Signaling Pathways

Mitogen-activated protein kinases (MAPKs) are an exclusively eukaryotic family of serine/threonine-specific protein kinases fundamentally involved in cellular response to extracellular stimuli, including heat shock, osmotic stress, mitogens, and proinflammatory cytokines. MAPKs are involved with regulation of proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis, among others processes. Networks of multiple MAPK pathways are responsible for governing cellular activities varying from gene expression, mitosis, and metabolism to motility, survival, apoptosis, and differentiation. As of the present time, five distinct groups of MAPKs have been characterized in mammals: extracellular signal-regulated kinases (ERKs) 1 and 2 (ERK1/2), c-Jun amino-terminal kinases (JNKs) 1, 2, and 3, p38 isoforms α, β, γ, and δ, ERKs 3 and 4, and ERK5 (Roux and Blenis, 2004). Of these 5 vertebrate MAPKs, the most extensively studied groups are the ERK1/2, JNKs, and p38 kinases. The wide range of functions of the MAPKs are mediated through phosphorylation of several substrates, including phospholipases, transcription factors, and cytoskeletal proteins.

Specifically relevant to the work presented here is the ERK1/2 family of MAP kinases. The mammalian ERK1/2 module is also known as the classical mitogen activated protein kinase cascade. It consists of the MAPKKKs A-Raf, B-Raf, and Raf-1, the MAPKKs MEK1 and MEK2, and the MAPKs ERK1 and ERK 2. ERK1 and ERK2 are expressed to some extent in all tissues, and share 83% amino acid identity.

ERK 1 and 2 are robustly activated by growth factors, serum, and phorbol esters, as well as to a lesser extent by ligands of the heterotrimeric G protein-coupled receptors,

cytokines, osmotic stress, as well as microtubule disorganization (Roux and Blenis, 2004).

During activation, signals are transmitted to the Raf/MEK/ERK cascade from cell surface receptors such as tyrosine kinases (RTK) and G protein-coupled receptors through different isoforms of the small GTP-binding protein Ras. Activation of membrane-associated Ras is accomplished through recruitment of a Ras-activating guanine nucleotide exchange factor called SOS (son of sevenless). Here the signal is broadened, as SOS stimulates Ras to exchange GDP for GTP by permitting it to interact with a wide range of downstream effector proteins. Of particular note among this class are the isoforms of the serine/threonine kinase Raf; together with Ras, the regulation of the two is crucial for the proper maintenance of cell proliferation. As shown by Chong et al (2003), activating mutations in these genes lead to oncogenesis. To further this connection, Ras has been shown to be mutated in 30% of all human cancers, and B-Raf is mutated in 60% of malignant melanomas (Downward, 2003). To complete the signaling cascade, the activated Raf binds to and phosphorylates the dual specificity kinases MEK1/2, which in turn phosphorylate ERK1/2.

A notable feature of the signaling cascade is its amplification properties; activation of merely 5% of Ras molecules is sufficient to induce full activation of ERK1/2 (Hallberg et al., 1994). The downstream effectors of most importance in cellular proliferation are transcription factors that regulate expression of cell cycle genes required for the transition the G1 growth phase to the S, or DNA synthesis, phase of the cell cycle.

As demonstrated with MT1-expressing Chinese hamster ovary cells, melatonin treatment has been shown to induce approximately two-fold more activated MEK 1/2 and ERK 1/2 after 5 hours of exposure, when compared to vehicle treatment. In addition, melatonin treatment caused morphological changes in MT1-CHO cells through a MEK 1/2 and ERK1/2 - dependent mechanism that was contingent on MT1 receptor internalization. In the same experiment, MT1 receptors were found to form complexes with both MEK 1/2 and ERK 1/2 in the presence of melatonin (Bondi et al., 2007). These results suggest a clear link between melatonin and ERK 1/2 activity and warrants additional exploration.

Experimental Design

A large body of evidence suggests that the hormone melatonin has anticancer effects. The purported antiproliferative effect of melatonin on tumor cells has been linked to estrogen-dependent cell signaling pathways that influence mechanisms such as cell cycle regulation and growth. In order to characterize these actions of melatonin better, I chose to use the cultured human breast cancer MCF-7 cell line to study the *in vitro* effects of melatonin with and without 17β-estradiol (E2) on cell growth, as well as the activity and expression of the MAPK family ERK. I employed MCF-7 cells cultured in hormone-free medium with low levels of serum growth factors, with or without physiological levels of melatonin and estradiol. In order to assess the effects of these treatments on cell number, I used a simple AlamarBlue fluorescence-based assay to determine cellular metabolic action, and from there extrapolate cell number counts. In addition, I explored the effects of 24 hours exposure to melatonin and/or E2 on ERK 1/2 activation, and the consequence of MT1 receptor inhibition on this signaling cascade. This was performed by SDS PAGE electrophoresis and Western blot analysis of lysate from treated cells.

Materials And Methods:

Cell Culture

Low passage number MCF-7 human breast cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing phenol red supplemented with 1X penicillin-streptomycin glutamine (PSG) (0.5mg.ml, Gibco) and 5% heat inactivated (55 C, 1hr) fetal bovine serum (FBS). Cells were grown in 10 cm plates in 5% CO2 in air at 37˚C to about 80% confluency before being rinsed twice with Hank's Balanced Salt Solution (HBSS), treated with 1X trypsin to detach cells from culture dish. Cells were suspended in DMEM:F12 1:1 (DF) medium supplemented with 1XPSG and 5% charcoal-stripped FBS (cs-FBS). Using a sterile glass bottle and stir bar, cells were then seeded into 48-well plates for proliferation experiments, on sterile glass coverslips for morphological examination, or 6 cm culture plates for ERK1/2 expression with MT-1 inhibition experiment. After the cells had adhered, the media was aspirated and replaced with 2.5% or 1% cs-FBS 24 hours before treatment.

Cell Proliferation

Total cell number was estimated using AlamarBlue (AbD Serotec, Raleigh, NC) fluorescence upon reduction by cellular dehydrogenases. The relationship between cell number and fluorescence was given by fluorescence values of known cell concentrations generated via serial dilutions of a suspension of MCF-7 cells. Following resuspension of cells as described above, a hemocytometer was used to count cells in a known volume of suspension, yielding the concentration of this stock solution. From there, serial dilutions were calculated in order to ensure that the correct number of cells were seeded. Wells of a 48-well culture plate were seeded in six replicates of 10, 20, 40, 80, and 120 thousand cells in DF 12 media with 2.5% cs-FBS. 12 hours post seeding, 1/10 volume AlamarBlue dye was added to each well, and respiring cells were incubated in an atmosphere of 5% CO_2 at 37˚C for 4 hours, followed by brief centrifugation to collect cell debris and

eliminate bubbles. 50uL of each sample containing some amount of reduced AlamarBlue dye, as well as samples of fully oxidized and fully reduced 10% media were transferred to a 96-well black microtitre plate, and fluorescence emission measurements at 590nm (530-560nm excitation) were taken using the Twinkle Microplate Fluorometer LB 970 (Berthold Technologies, Wildbad, Germany). Four replicate readings of each well were taken to ensure equipment reliability. The baseline fluorescence readings obtained from fully oxidized AlamarBlue-containing media were subtracted from the raw fluorescence data to eliminate background contribution from media. Cell number was estimated from initial cell density and log-transformed to produce a linear relationship between fluorescence and $\log_{10}$ cell number. This protocol was designed by and obtained from Margo Gilbert (Reed College, 2012).

In the proliferation experiments, 20,000 cells were seeded in wells of a 48-well plate as described above and randomly assigned to one of four treatments in 6 replicates, consisting of 24 wells per 48-well plate (1/2 plate per sample group).

Treatment media were made with DF (1X PSG), and 1% cs-FBS for each of the four treatments: (1) 0.00027% ethanol for Vehicle, (2) 10 nM melatonin (diluted in DF12 media, with equivalent 0.00027% ethanol added), (3) 100 pM E2 (diluted in ethanol), (4) 10 nM melatonin + 100 pM E2.

24 hours post-seeding, the original media was aspirated and replaced with DF (1X PSG) with 1% cs-FBS, to serum starve the cells. 24 hours later, the media was once again changed to the assigned treatment media. At each time point of 3, 5, 6.5, 7, and 9 days post-treatment, a sample group of ½ plate was removed from the incubator and assayed for cell number with AlamarBlue as described above, timed to a strict hourly schedule. Once wells were measured, the media was aspirated and left dry. The full plate was then either returned to the incubator to preserve the remaining half, or discarded if both halves had been assayed. Cells measured on days 9 and 7 were paired, as were days 6.5 and 3, and day 5 to baseline.

Over the course of the 9-day experiment, the medium was changed every two days, 24 hours before the AlamarBlue assay was performed for each timepoint. See Figure 3 for a detailed protocol schematic. Measurements were taken at 3, 5, 6.5, 7, and 9 days post treatment, as well as an initial pre-treatment time point to establish a baseline.

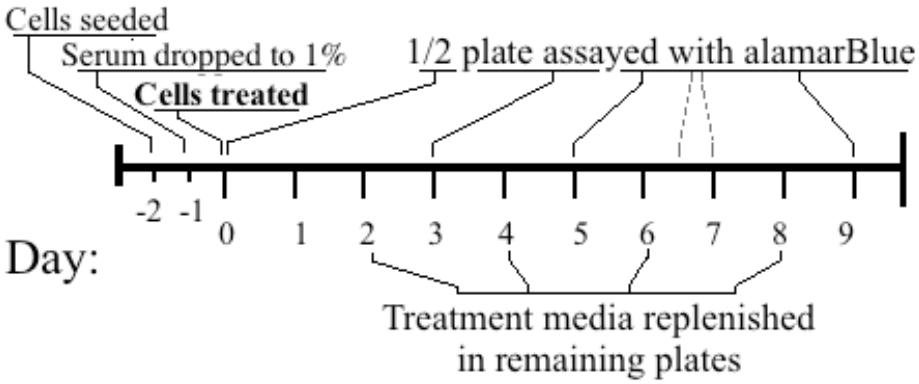

Figure 3: Timeline of proliferation experiment.

Schematic of the cell proliferation experiment, designed to measure the proliferation of MCF-7 cells when treated with estradiol and/or melatonin over the course of 9 days. Multiple 48-well plates were seeded with cells two days before treatment, and then subjected to lowered serum levels (1%) 24 hours before treatment. On days 3, 5, 6.5, 7, and 9, 24 wells representative of all treatments were assayed for cell number. The treatment media in remaining wells was replaced every two days, exactly 24 hours before measuring cell number.

Raw fluorescence data were exported to, examined, and processed in Excel (Microsoft). In order to account for unavoidable cell clumping seeding errors, the highest and lowest value of each treatment was discarded. The fluorescence readings for each treatment by day were then collected, converted to an estimation of cell number based on the standard curve linear model, then averaged. Data were analyzed using R (http://www.r-project.org/) software employing separate linear regression analyses within each timepoint in order to identify significant variance between each treatment and the vehicle. Additionally, a linear regression analysis was performed for each day between vehicle and melatonin treatments, as well as between estradiol and combined estradiol and melatonin treatments.

Western Blot of Activated ERK 1/2 with Luzindole Inhibition

Cells were seeded in hormone free DF media with 5% cs-FBS in 6 cm plates and allowed to grow until the cultures were about 80% confluent. Serum levels were then

dropped to 1% by refreshing the media. 24 hours later, quadruplet plates were treated with vehicle (0.00027% ethanol) (VEH), 100pM 17beta -estradiol (E2), 10nM melatonin (MEL), or 100 pM E2 and 10nM melatonin. Immediately following treatment, either 50uM luzindole (+LUZ) or equivalent vehicle (0.023% ethanol) was added to duplicate plates, and treated cells were allowed to incubate for 24 hours. At a later date, an epidermal growth factor (EGF) treatment was included as a technical positive control (15 minute treatment at 4 ng/ml EGF). Cell lysates were collected in ice cold HEPES/KOH lysis buffer (with 20 μl 0.2 M sodium vanadate and 1X Roche complete protease inhibitor cocktail (Roche). The monolayer of cells was collected with a rubber bladed cell scraper, and lysate was transferred to an autoclaved 1.7 ml Eppendorf tube on ice. DNA was sheared by vortexing each sample for 8 seconds. After 30 min on ice all lysates were centrifuged at 14,000 rpm eight minutes, 4˚ C. Lysate supernatants were then transferred to clean 1.7 mL Eppendorf tubes, and stored at -80˚ C. Lysate was later thawed and subjected to SDS PAGE electrophoresis on 10% polyacrylamide gels (Bio-Rad), followed by Western blot analysis. Proteins were electrophoretically transferred onto methanol activated PVDF (Bio-Rad) membrane at 20 volts, 4 hours.

Membranes were rinsed with 1X Tris buffered saline (TBS), containing 0.05% Tween-20 (TTBS) and placed in TTBS with 3% bovine serum albumin (BSA) and 0.05% sodium azide (‘blocking solution’) with constant rocking for 30 minutes at 20˚C to block non-specific protein binding sites. After blocking, membranes were rinsed twice with TTBS at 20˚C and incubated in rabbit anti-phospho ERK P 44/42 MAPKinase primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:3000 in TTBS + 0.06% BSA + 0.01% sodium azide overnight at 20˚ C with constant rocking.

Excess primary antibody was removed from the membranes by rinsing 5 times with TTBS, and the membranes were transferred to goat anti-rabbit IgG-HRP secondary antibody diluted 1:10,000 in TTBS + 0.05% BSA for 90 minutes at 20˚C, rinsed 5X in TTBS and the membranes were placed in enhanced chemiluminescence reagents (PerkinElmer, Waltham, MA) for 60 seconds with gentle hand rocking. Sites of antibody binding were visualized using FluorChemE gel imager (Cell Biosciences, Santa Clara, CA). Membranes were stripped in 2% SDS, 5% β-mercaptoethanol in 0.062 M TRIS pH 6.8 20˚C overnight, rinsed five times into TTBS and re-probed with rabbit anti-ERK 2

(Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:3000 as described above to normalize levels of activated ERK 1/2.

ImageJ software was used to quantify differences in band density among treatments, providing arbitrary relative values relating to the measured intensity of each band (Rasband, 1997-2012).

Results

The Effects of Melatonin on MCF-7 Breast Cancer Cell Proliferation

Previous work demonstrated that melatonin inhibited proliferation of breast cancer cells in culture, but the results are confounded by the presence of high concentrations of serum growth factors or the weak estrogen phenol red (Vignon, 1980). To directly test the effects of melatonin on proliferation, cells were cultured in maintenance levels of charcoal stripped fetal bovine serum (1% cs-FBS). Initial experimentation was intended to both confirm the antiproliferative effect of melatonin, and to determine the consequences of altering the (charcoal stripped) fetal bovine serum levels during cell culture.

MCF-7 cells were seeded into 48-well plates using serial dilutions of a concentrated cell suspension. 24 hours post-seeding, AlamarBlue dye was added to the media to create a 10% solution. After four hours incubation, fluorescence values were measured and plotted against $\log_{10}$ cell number as estimated from initial cell density, and fit into a linear model, producing the equation $y = 187766x - 709987$ ($R^2 = 0.99008$) (Figure 4). This model allowed for AlamarBlue fluorescence values to be used to estimate cell number in the proliferation assay.

To test for inhibitory effects of melatonin, MCF-7 cells were seeded in DMEM F12 (DF) medium containing 5% cs-FBS in three 48-well plates. 24 hours post-seeding, the media was replaced with DF supplemented with the lower concentration of 1% cs-FBS to minimize effects of serum growth factors, yet promote cell survival. 24 hours later, the media was again replaced, this time with treatment media containing either 0.00027% ethanol vehicle (VEH), 10nM melatonin (MEL), 100pM 17β-estradiol (E2), or 100 pM E2 and 10nM melatonin (E2 + MEL). All treatments contained the same amount of ethanol.

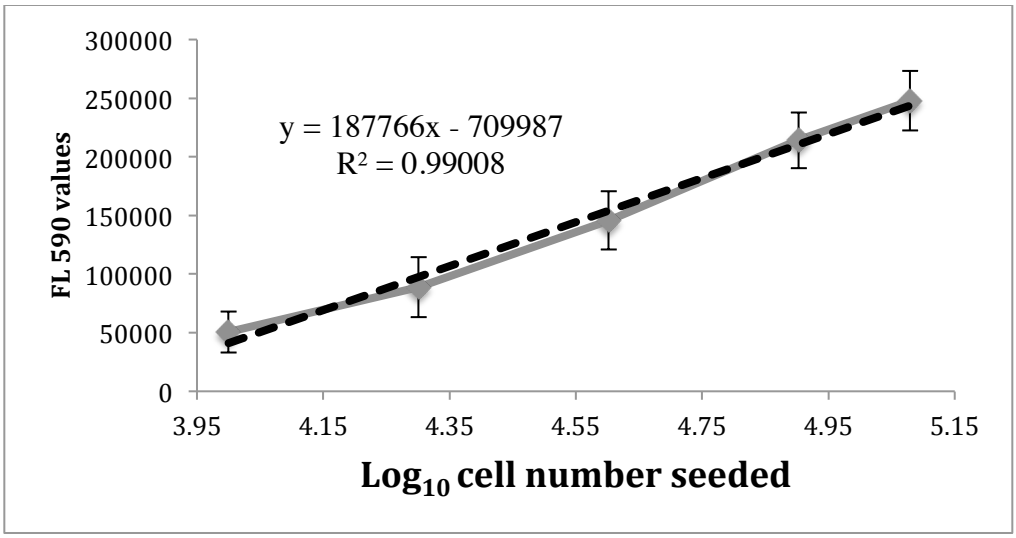

Figure 4: The linear relationship between $\log_{10}$ of cell number fluorescence at 590nm.

Linear model fit to the florescence of reduced AlamarBlue dye versus $\log_{10}$ of cell number seeded, with the equation y = 187766x - 709987, $R^2 = 0.99008$. In three separate trails, wells of a 48-well culture plate were seeded in six replicates with a serially diluted suspension of concentrated MCF-7 cells in DF media, 5% cs-FBS. 24 hours post-seeding, AlamarBlue was added to each well and incubated for 4 hours at 37°C, and dye fluorescence was measured by fluorospectrophotometry as described in Materials and Methods. Line of best fit was added using Excel software.

A detailed protocol schematic (Figure 3) is given in the Material and Methods section. Briefly, the 48-well plates were allowed to incubate at 37˚ C until fluorescence assays were performed, with treatment media replenished every 48 hours. Six sample groups of 24 wells (one half of a plate) were assayed at a pre-treatment baseline, and days 3, 5, 6.5, 7, and 9, providing estimates of cell proliferation at these time points under treatments conditions (Figure 5). While some time points had to be thrown out due to contamination and feeding irregularity, the data indicate that melatonin inhibits cell proliferation on day 5 whether or not melatonin is combined with 17β-estradiol (E2).

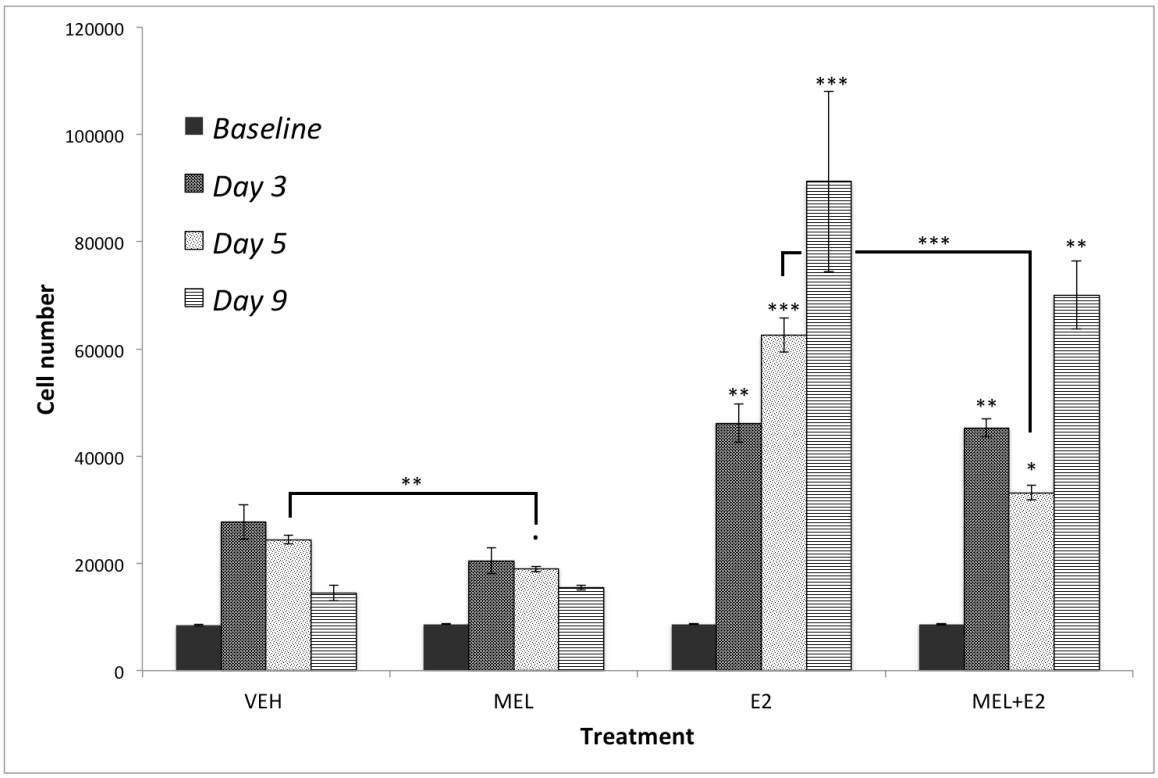

Figure 5: Effects of chronic melatonin and 17β-estradiol treatment on the total number of MCF-7 cells over 9 days.

Cells were seeded in 48-well plates in DF, 5% cs-FBS, and randomly assigned to one of four treatments in 6 replicates. Sufficient wells were seeded so that a representative sample group of 24 wells (1/2 plate) could be removed and assayed for each time point, yielding an estimation of cell number at 3, 5, and 9 days. 24 hours before treatment, serum level was dropped to 1% and maintained throughout the experiment. On the day of treatment, one 24 well sample group was removed and assayed via AlamarBlue reduction as described in Materials and Methods in order to provide a baseline reading to confirm proper seeding. Cells were treated with one of: (VEH) vehicle (0.00027% ethanol), (E2) 100pM 17β-estradiol, (MEL) 10nM melatonin, or (E2 + MEL) 100 pM E2 and 10nM melatonin. Plates were allowed to incubate at 37˚C, 5% CO2, with sample groups removed, assayed, and discarded at 3, 5, 6.5, 7, and 9 days as described above. Error necessitated the removal of days 6.5, and 7. Data from each day were subjected to linear regression analysis in R to test for variation from the vehicle control group. Statistical significance is described by significance codes placed directly over the bars for each day: P = 0 '***', 0.001 '**', 0.01 '*', 0.05 '.'.VEH and MEL treatments were analyzed for variance between the two (see linked bars), as were E2 and E2 + MEL treatments.

As expected, the addition of E2 lead to a significant increase in cell number on days 3, 5, and 9, as compared to vehicle treated cells ($p = 0.00261$, $p = 3.28e\text{-}08$, $p = 0.000301$, respectively).

A small but significant antiproliferative effect of melatonin was observed when MCF-7 cells were treated with melatonin in the absence of E2, suggesting that melatonin affects basal cell growth. This inhibitory effect was not observed after 9 days of treatment, possibly due to the effects of long-term serum deprivation ($p = 0.00329$).

When combined with E2, melatonin treatment inhibited the proliferative estrogen effect after 5 days of treatment. Cells treated with E2 + melatonin showed comparable growth after 3 days, and significantly reduced growth after 5 days ($p = 0.000423$). Though mean cell number was numerically lower after 9 days of E2 + melatonin, cell proliferation differences were not statistically significant owing to the large variation in the E2 treatment group.

Importantly, variation among baseline cell numbers was very small, indicating that all treatments initially received similar numbers of cells, and therefore subsequent differences must be accounted for by treatment effects or block effects in the experiment design.

The Effects of Melatonin on ERK Phosphorylation

The notion that melatonin directly inhibits cell signaling via activation of MT1 receptors is supported by decreased ERK1/2 phosphorylation in MCF-7 cells overexpressing MT1 receptors (Bondi et al., 2008). Overexpression frequently perturbs cell signaling in non-physiological ways, so I employed luzindole, a potent and competitive MT1/2 receptor antagonist, in order to inhibit the melatonin receptors.

Estradiol treatment of MCF-7 cells has been shown to induce rapid and transient activation of the MAPK pathway, up-regulating ERK-1 and ERK-2 activation. Based on the anti-estrogen effects of melatonin I observed in the proliferation experiments, I tested whether melatonin treatment would counteract the estrogen-influenced ERK1/2 phosphorylation. Simultaneously, I used a specific antagonist of MT-1 receptors to test whether luzindole inhibition of the MT1 receptor would act to release the MAPK

pathway, resulting in the upregulation of ERK phosphorylation. Cells were seeded in 6cm plates and grown to 80% confluency, at which point the cs-FBS level was dropped from 5% to 1%. 24 hours later, quadruplet plates were treated with one of four treatments: MEL (10nM melatonin), E2 (100pM estradiol), E2 + MEL (10nM melatonin plus 100pM estradiol), or VEH (0.00027% ethanol). Duplicate plates from each treatment group were then treated with the addition of either 50uM luzindole to inhibit the MT1 receptor, or equivalent vehicle (0.0023% ethanol). Plates were then incubated for 24 hours, after which lysate was collected as described in the Methods section and assayed for ERK 1/2 expression using SDS PAGE gel electrophoresis and Western blotting (Figure 6). Total ERK 2 expression decreased, and phosphorylation of p44 and p42 ERK isoforms noticeably increased in cells treated with luzindole regardless of any other treatments (Figure 9), (Figure 6).

All treatments slightly increased phosphorylation of ERK1/2 relative to the vehicle treatment. Visual analysis shows reasonably even levels of total ERK expression across all treatments, with the exception of E2, sample B. Examination of the blot shows a disruption in this band (Figure 7), likely the result of a bubble error during protein transfer. Slightly increased levels of phospho-ERK isoforms p44 and p42 in E2+Mel, E2, and MEL treatments as compared to VEH suggest that ERK phosphorylation is upregulated in response to 24 hours of estrogen and melatonin treatment. Semi-quantitative densitometric analysis confirms the visual pattern, as seen in Figure 8, and furthermore suggests that melatonin treatment in conjunction with E2 may have slightly reduced the phosphorylation of ERK1/2 over that of cells treated with only estradiol. This densitometric analysis is flawed, however, and cannot be considered as a significant result.

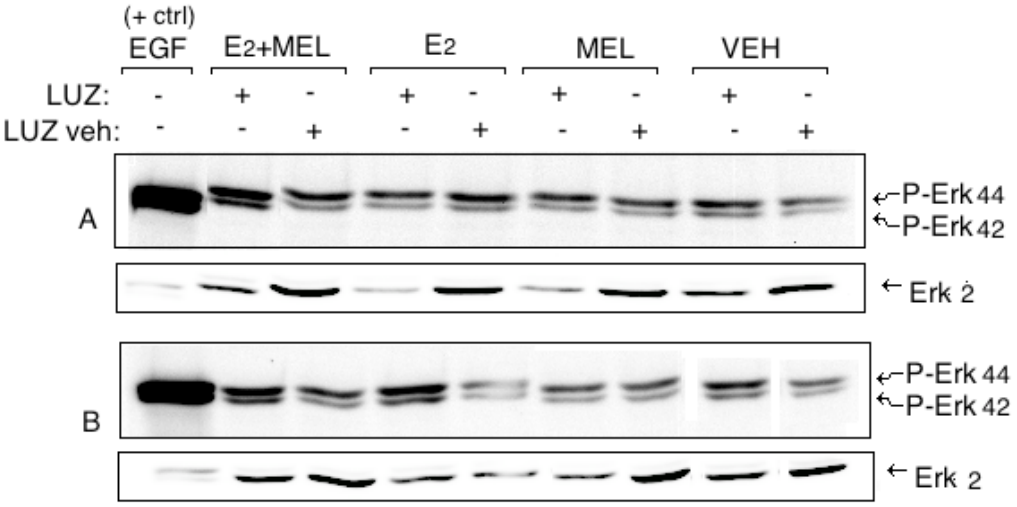

Figure 6: Western blot of ERK expression and phosphorylation in cells treated for 24 hours with melatonin and/or E2, with and without luzindole MT1 receptor inhibition.

Cells were seeded in DMEM-F12 media with 5% cs-FBS and allowed to grow to 80% confluence in 6cm plates. Once ~80% confluence was reached, serum levels were dropped to 1% by refreshing the media. 24 hours later, quadruplet plates were treated with vehicle (0.00027% ethanol) (VEH), 100pM 17beta -estradiol (E2), 10nM melatonin (MEL), or 100 pM E2 and 10nM melatonin. Immediately following treatment, either 50uM luzindole (+LUZ) or equivalent vehicle (0.023% ethanol) was added to duplicate plates (A and B), and treated cells were allowed to incubate for 24 hours. Cell lysates were collected in HEPES/KOH lysis buffer and submitted to SDS-page on 10% polyacrylamide gels, followed by Western blot analysis as outlined in Materials and Methods. An epidermal growth factor (EGF) treatment was included as a positive control (15 minute treatment at 4 ng/ml EGF). To assess the activity of ERK1/2, membranes were first stained for phosphorylated ERK1/2 (p44 and p42), then stripped overnight and reprobed for ERK 2 (N = 2).

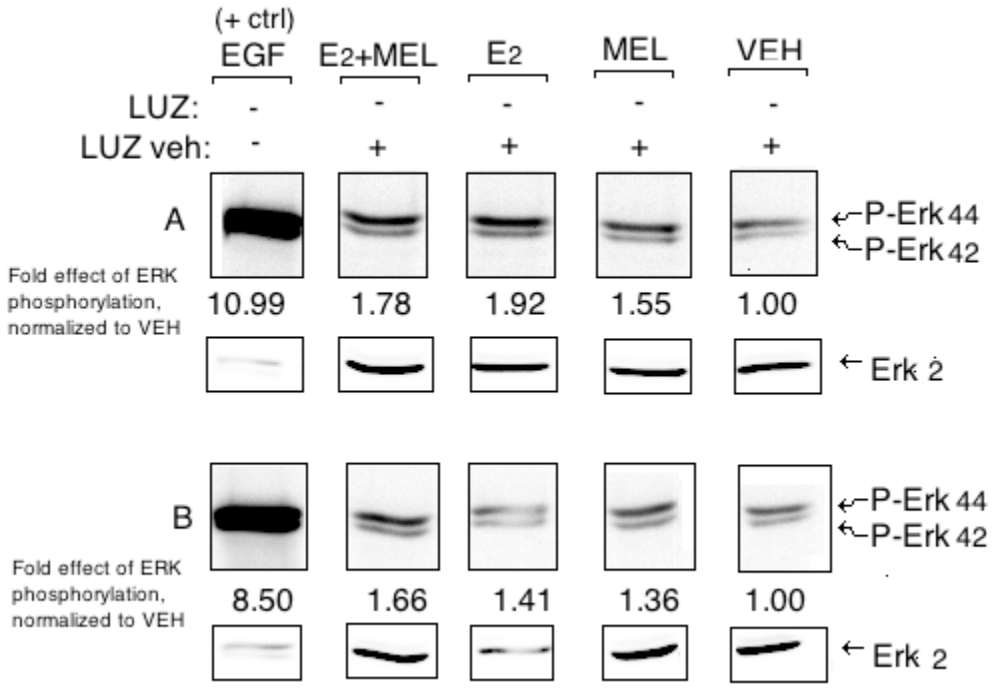

Figure 7: ERK expression and phosphorylation in cells treated for 24 hours with E2 and/or melatonin.

Isolated lanes as seen in figure 6 featuring ERK expression and phosphorylation in MCF-7 cells not treated with luzindole. The fold effect of ERK phosphorylation has been normalized to the vehicle treatment, and represents the increased ERK activation seen across four treatments. The fold difference was calculated by Western blot semi-quantitative densitometry, dividing the intensity of both p-ERK bands by the intensity of the ERK 2 band, as calculated using ImageJ colorimetric analysis (N = 2). Full details provided in the Appendix.

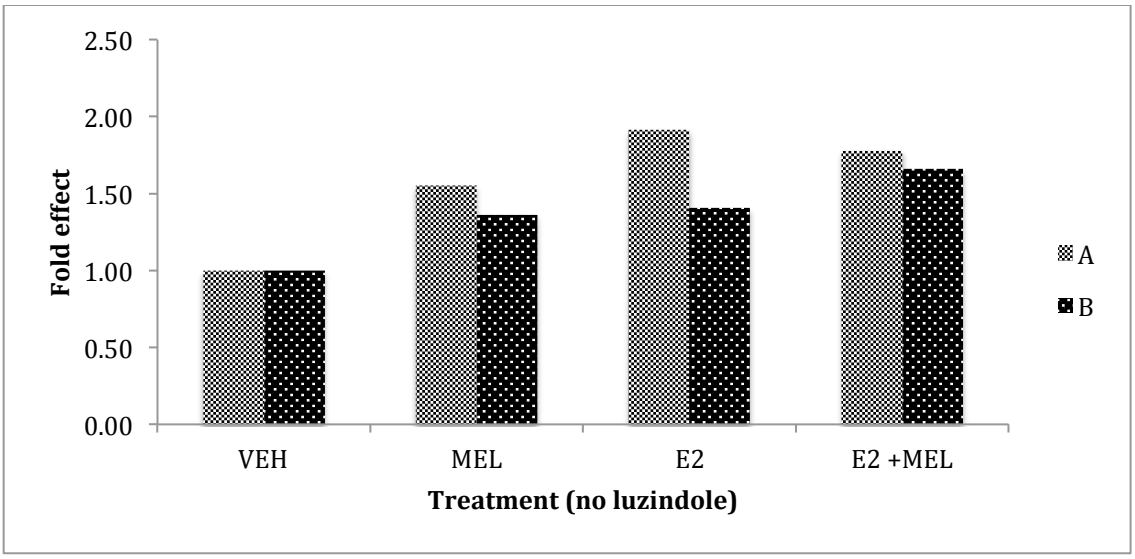

Figure 8: The fold effect of ERK phosphorylation in MCF-7 cells treated for 24 hours with estrogen and/or melatonin.

Graphical representation of the fold effect of ERK 1/2 phosphorylation (as seen in Figure 7). Fold effect quantified by ImageJ colorimetric analysis as seen in Figure 7. Fold effect is calculated as the ratio between total ERK2 expression and ERK1/2 phosphorylation. Full details provided in the Appendix.

Luzindole treatment appeared to dramatically reduce total ERK 2 expression, and increase ERK phosphorylation by about two-fold, when compared to cells without MT1 inhibition. Visual analysis of the Western blot showed decreased ERK 2 expression in MT1 inhibited cells, most notably in the E2 and MEL treatments. Densitometric analysis was used to calculate and compare the ratios of ERK expression to ERK phosphorylation between +LUZ and -LUZ inhibited cells within each treatment, revealing that luzindole increased the phosphorylation of ERK regardless of treatment (Figure 9). This evidence suggests that the inhibition of the MT1 receptor in some way releases the MAP-K pathway.

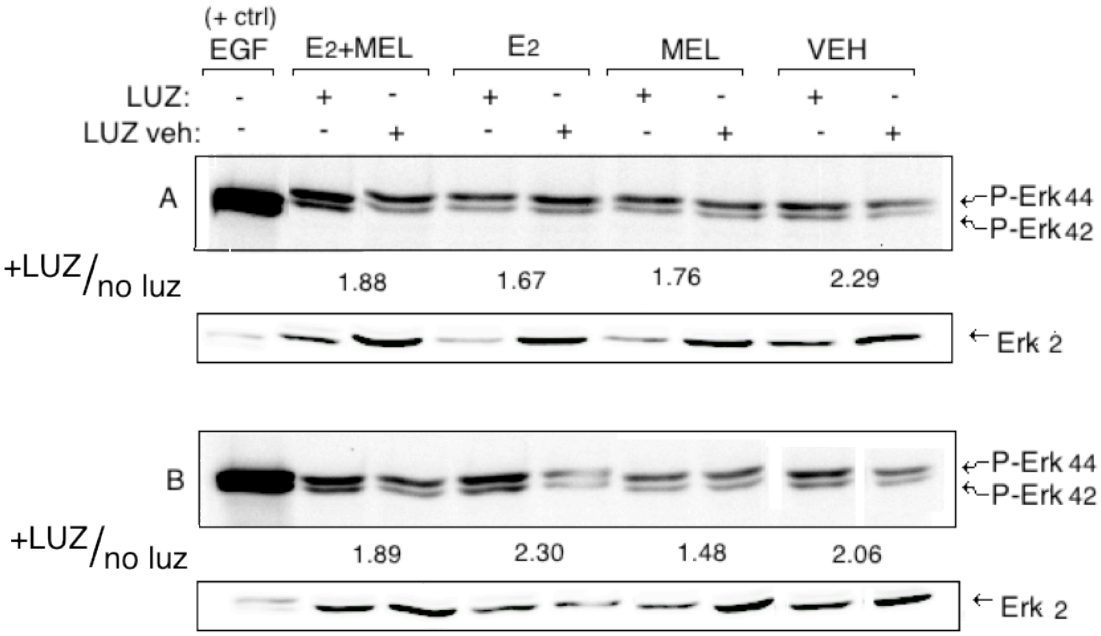

Figure 9: Increased ERK phosphorylation in MT1 inhibited (luzindole) cells.
The effects of MT1 inhibition with luzindole on ERK phosphorylation after 24 hours of treatment with E2 and/or melatonin. Image shows selected lanes from Figure 6, with the fold effect of increased ERK phosphorylation in luzindole treated cells listed below each treatment. Densitometric analysis performed with ImageJ software, and calculations performed in Excel, then graphed as seen in Figure 10 below. The fold difference was calculated by dividing the ratio of ERK2/p-ERK in LUZ+ cells by the ratio of ERK2/p-ERK in LUZ- cells (See Equation 1 in Appendix).

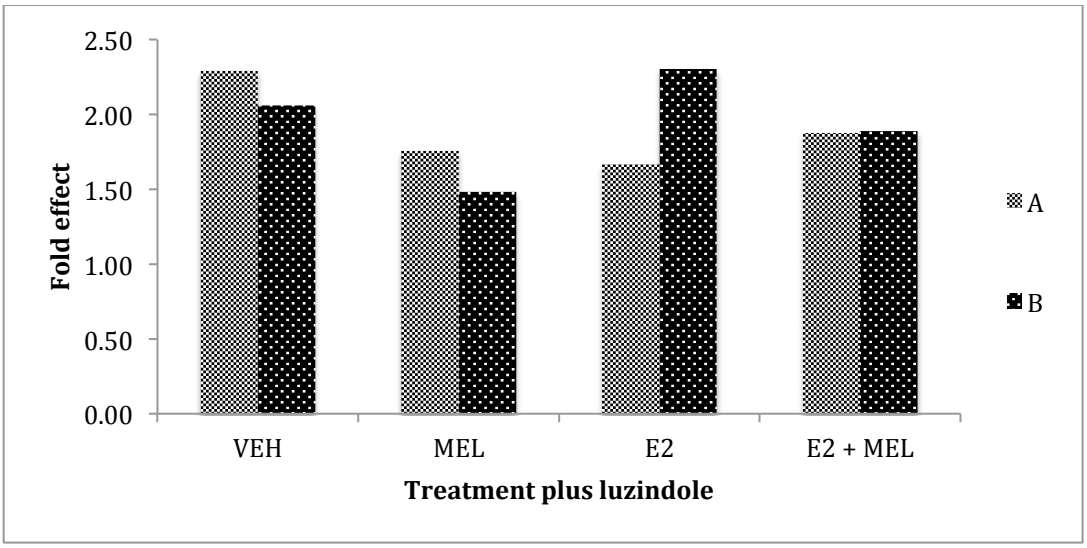

Figure 10: Fold effect of ERK phosphorylation in MT1 inhibited MCF-7 cells. Graphical representation of the fold effect of ERK 1/2 phosphorylation in cells treated with luzindole over that of cells treated with luzindole vehicle. Fold effect quantified by ImageJ colorimetric analysis as seen in Figure 6. Fold effect is calculated as the ratio between the amount of ERK1/2 phosphorylation from total ERK 2 expressed in luzindole treated cells, and the amount of ERK1/2 phosphorylation from total ERK 2 expressed in vehicle treated cells (see Equation 1 in Appendix).

Discussion

Evidence shows a strong correlation between circadian disruption and increased risk of cancer, likely due to the repression of melatonin production as a result of nighttime light exposure (Vollrath, 2001). Studies in human and animal models provide persuasive evidence of the antitumoral effects of melatonin (Shah et al., 1984). *In vivo* studies likewise show that melatonin has a significant antiproliferative effect on MCF-7 breast cancer cells, potentially through action on the cell cycle or the regulation of cellular clock genes (Crespo et al., 1994; Xiang et al., 2012). In addition, melatonin has been shown to induce modulation in the expression of the MAPK family proteins ERK 1 and 2, in the MAPK family through the MT1/ 2 receptors (Bondi et al., 2007). As MAP kinases play a large role in the regulation of processes such as proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis, further inquiry into the interrelation between melatonin and ERK activity is justified.

Cell proliferation

In the experiments described above, I confirmed that melatonin exerts an antiproliferative effect on the growth of MCF-7 cells after 5 days of exposure to nighttime physiological levels (10nM) in largely estrogen-free media. In addition, melatonin appeared to work against the proliferative effects of 17ß-estradiol (E2) treatment, reducing cell number significantly within this same time window. No significant effects were seen before day 5, suggesting that the action of melatonin on cell cycle necessitates chronic exposure. This conclusion suggests further exploration into the mechanism of melatonin effects on the cell cycle in MCF-7 cells, in particular the notion that melatonin counteracts the cell cycle - accelerating action of estrogen treatment should be reassessed. As recent literature implicates the role of melatonin in the regulation of the cellular clock, further study into how melatonin might influence clock genes, and through these the cell cycle cells, is warranted.

Due to the presence of growth factors and estrogen precursors in fetal bovine serum (FBS) commonly used in cell culture, I used low (1%) levels of FBS during and before treatment in an effort to avoid the confounding effects of other hormones. Based on the work of Briand and Lykkesfeldt (1984), charcoal stripped FBS levels would have to be below 0.05% to reasonably ensure the absence of estrogen in the media. My preliminary work demonstrated that levels this low are associated with high apoptosis and thus are not ideal for longer-term cell culture; in the present work 1% charcoal stripped serum was used instead. It is possible that the serum level could be further optimized or completely serum-free artificial medium could be developed in future projects.

Though the AlamarBlue fluorescence assay is frequently used to measure cell number, it should be noted that only the metabolic activity of the cells is measured. The assumption is that total cellular dehydrogenase activity is similar across treatments. If true, a reasonable estimate of cell number can be made. As previously discussed, both melatonin and E2 treatments caused morphological changes in the cell, such as increased size and surface area. It is possible that the calculated cell numbers presented in the 'Results' are skewed, and differences in fluorescence values are due to changes in cell morphology or metabolism. However, direct observation of the wells under a light microscope subjectively confirmed different cell numbers between treatments, which lends confidence to the AlamarBlue-derived values. Additional complications of the AlamarBlue assay include the introduction of human error, such as variations in pipetting technique.

Based on earlier experimentation, it was determined that the effects of melatonin would be most visible at a minimum of three days post-treatment (data not shown). Likewise, previous proliferation experiments suggested that measurement every 48 hours would sufficiently demonstrate cell growth trends. Because the AlamarBlue assay gave an indication of metabolic activity, it was essential that all measurements be taken at the same time relative to feeding. An unplanned measurement was taken at 6.5 days as a result of suspected experimental error, but was ultimately discarded from the final analysis due to this asynchronicity from the feeding schedule. Day 7 data was also excluded due to the presence of mild unknown bacterial contamination. An additional possible source of error was the unorthodox use of halved cell culture plates. In order to

reduce labwork to a reasonable level, only 24 wells (consisting of 4 treatment groups, N=6) were measured at any given timepoint. It is possible that the excess time outside of the incubator introduced error into later measurements on days 7 and 9.

Despite these issues, other results provide confidence in the above experiment. No overcrowding of wells was observed. The standard curve was likewise reliable, featuring small variation between three separate trials, and yielding a highly reasonable linear trend ($R^2 = 0.990$). Due to repeated preliminary experimentation, the seeding technique was eventually refined to produce highly reproducible baseline values among wells for the final cell proliferation experiment (Figure 5). Thus, the trends seen in the proliferation experiment are very likely due to treatment effects. Further, the observed fold effects of E2 treatment on proliferation were close to expected effects based upon reported doubling rates in the literature.

ERK 1/2 activation and luzindole MT1/2 receptor inhibition

Western blot analysis of cell lysate from MCF-7 cells treated for 24 hours with melatonin and/or E2 showed that relative ERK 1/2 phosphorylation was lowest in vehicle treated cells, with slightly higher activity in melatonin treated cells, and then comparably higher levels in E2 and E2+melatonin treatments. Total ERK expression between treatments did not vary to a notable degree, with the exception of one E2 treated sample where a bubble had likely prevented proper protein transfer during western blotting. Because melatonin treatment had no effect on ERK 1/2 phosphorylation, it may be that the single 24 hour time point was not optimal. E2 treatment was expected to increase ERK1/2 phosphorylation (Toffey, 2012). Evidence from the proliferation experiment indicates that melatonin effects are delayed and any effects of melatonin on the ERK1/2 phosphorylation may not be evident at 24 hours,

Surprisingly, the addition of luzindole to inhibit the MT-1 receptor resulted in decreased expression of total ERK and increased ERK 1/2 phosphorylation in all treatments including the vehicle controls. Initially, experimental error was suspected because of the apparent precipitation of luzindole during treatment, but the addition of luzindole caused a clear down-regulation of total ERK in all treatments. This effect was modest in vehicle-treated cells, and notably increased across the three treatment groups.

Both samples of luzindole plus melatonin treated cells had 3 to 4-fold less total ERK 2 expression than non-inhibited cells, as visually appraised. This effect was even more pronounced in one sample of E2 treated cells, though the previously mentioned bubble obscured results from the replicate sample. These results could be due to off-target effects of luzindole. A previous report documented that luzindole inhibited outward K+ current via direct association with the channel protein (Mi-ou et al., 2003). To determine the full effect of luzindole on cellular physiology, further experimentation is required.

The observation that MT1/2 inhibition dramatically raised the amount of ERK phosphorylation invites speculation about possible tonic effects of MT-1 receptors in MCF-7 cells. Perhaps MT1/2 receptor action enforces a suppressive effect on the MAPK pathway under normal circumstances. Additional, more specific MT-1/2 inhibitors should be employed to test this notion. As a technical positive control, lysate from cells treated with EGF were included, but not subjected to luzindole inhibition. In future experimentation, MT1/2 inhibition of EGF treated cells could help elucidate the mechanism of action.

Semi-quantitative densitometry

In addition to visual analysis, densitometry using Image J freeware was performed on each Western blot. Though it is possible that useful quantification of relative density values can be obtained under certain circumstances, the methods associated with densitometry have recently come under significant criticism. Common methods of capturing blot images and processing the resulting colorimetric data are not standardized across the scientific community. In a statistical analysis of a variety of methods used to analyze western blots, Gassmann et al., (2009) determined that several common procedures applied to the same western blot produced correlational P values ranging from 0.000013 to 0.76, indicating a need for standardization and optimization of procedures for acquiring band densities (Gassmann et al., 2009). Due to the unreliability of densitometry, I did not perform statistical analysis on the relative band densities obtained from Image J analysis of the phsopho ERK1/2 western blots. However, the numbers provide a helpful addendum to visual analysis, and were therefore included in the Results. According to the Gassmann analysis, the ImageJ algorithm for obtaining area

under the peak is near ideal. A high-quality gel image reader was used to photograph the membranes. A full description of my densitometric methods is located in the Appendix.

…

The data presented here confirm previous work illustrating the potential antiproliferative action of melatonin in breast cancer and raise new questions about melatonin receptor action. Extensive evidence links the effects of light-at-night to modulation of the circadian rhythm, and through this, the health consequences associated with the suppression of such regulatory signals as melatonin. Regulatory physiology is likely the downstream target of melatonin. In an age where technology and light are illuminating our very future, perhaps it is wise to remain aware that the pace of change in our own physiology is orders of magnitude slower than technological revolutions.

Appendix A:

Semi-quantitative densitometry method

An online protocol for Western blot analysis was followed in order to analyze the relative densities of each protein band.

(http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/)

A FluorChemE gel imager (Cell Biosciences, Santa Clara, CA) was used to capture multiple exposures of each Western blot membrane. Care was taken to select an exposure that resulted in neither overexposure (areas of pure white), nor underexposure (underrepresentation of bands). An appropriate image (8bit) was selected, and opened in ImageJ. Using the gel analysis tools, identical rectangular selections of each band of interest were taken. In the case of the double p44/p42 stained areas, both stripes were treated as a single band. These rectangular areas were then graphed as peaks representing the total intensity and vertical width of the band (Figure 11). Care was taken to ensure that no peak flattened at the top, indicating overexposure and saturation. Background was eliminated by cutting off the very bottom of the curve at the level of noise. The area under the curve was then measured, yielding arbitrary values representing the relative intensity of each band compared to the others in the same image.

In order to prepare figures as seen in Results, the original white-on-black membrane images were color inverted, and the troglodyte program Paintbrush (Macintosh) was used to crop lanes, and correct for slight vertical unevenness in lanes. No alteration of value or size was performed.

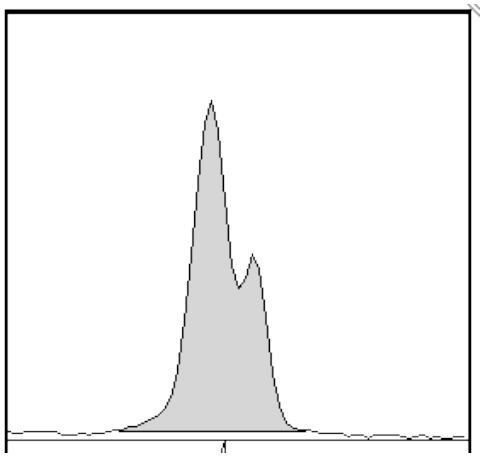

Figure 11: Sample of a peak obtained from ImageJ representing the measured intensity of a double phospho-ERK band.

Note the artificial line cutting off the very bottom of the peak, at approximate level with the background signal. Placement of this line was subject to human error, and a possible cause of erroneous variation with this method. Gray shading represents the area integrated, and produced an arbitrary reading of 6779.933 in this test scenario.

In order to calculate a "fold effect" in ERK phosphorylation, arbitrary blot intensity values obtained in ImageJ were compared to indicate relative levels of ERK activity. To do this, the ratio of P-ERK/total ERK was first calculated by dividing the intensity of the phosphorylated ERK band by the intensity of the corresponding ERK band. In the case of comparison between non-luzindole inhibited treatments (Figure 7), this ratio was then normalized so that the fraction of P-ERK/total ERK in each treatment (MEL, E2+MEL) was expressed relative to the vehicle treatment (Figure 8)

To compare the ratio of P-ERK/total ERK between luzindole treated- to untreated cells, Equation 1 was used.

$$\text{Eq. 1} \quad \text{+LUZ/no luz} = \frac{\text{(P-ERK/total ERK) in +LUZ cells}}{\text{(P-ERK/total ERK) in no luz cells}}$$

Some pitfalls exist with any method of densitometric reading, as detailed in the Discussion section.

Useful tools:

Randomize subjects to treatment plans: Graph pad quickcalcs

http://www.graphpad.com/quickcalcs/randomize1/

R software: Free statistical analysis tool

http://www.r-project.org/

f.lux: free software to impose a circadian rhythm on your computer screen. f.lux dims the display at sunset, shifting the emitted light further towards the amber spectrum to reduce strain on eyes, and may help reduce the effects of computer light at night.

http://stereopsis.com/flux/

Excellent for students who stay up all night writing theses.

…

http://tinyurl.com/in-reposed0

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