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Melatonin Represses Metastasis in Her2-positive Human Breast Cancer Cells by Suppressing RSK2 Expression

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Abstract

The importance of the circadian/melatonin signal in suppressing the metastatic progression of breast and other cancers has been reported by numerous laboratories including our own. Currently, the mechanisms underlying the anti-metastatic actions of melatonin have not been well established. In the present study, the anti-metastatic actions of melatonin were evaluated and compared on the ER α -negative, Her2-positive SKBR-3 breast tumor cell line and ER α -positive MCF-7 cells overexpressing a constitutively active HER2.1 construct (MCF-7Her2.1 cells). Activation of Her2 is reported to induce the expression and/or phosphorylation-dependent activation of numerous kinases and transcription factors that drive drug resistance and metastasis in breast cancer. A key signaling node activated by the Her2/Mapk/Erk pathway is Rsk2, which has been shown to induce numerous signaling pathways associated with the development of epithelial-to-mesenchymal transition (EMT) and metastasis including: Creb, Stat3, cSrc, Fak, Pax, Fascin, and actin polymerization. The data demonstrate that melatonin (both endogenous and exogenous) significantly represses this invasive/metastatic phenotype through a mechanism that involves the suppression of EMT, either by promoting mesenchymal-to-epithelial transition (MET), and/or by inhibiting key signaling pathways involved in later stages of metastasis. These data, combined with our earlier in vitro studies, support the concept that maintenance of elevated and extended duration of nocturnal melatonin levels plays a critical role in repressing the metastatic progression of breast cancer.

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Keywords

Her2; Melatonin; Metastasis; Rsk2; Signaling Pathways

Introduction

Breast cancer, the most commonly diagnosed cancer in women, represents 29% of all new cases in 2013 (1). Although most breast cancers are diagnosed at relatively early stages, nearly 30% of them will eventually develop metastasis despite treatment (2). Metastasis, a hallmark of cancer, is the principal cause of death from breast cancer (3). However, the molecular mechanisms underlying the development of metastatic breast cancer remain unclear, and defining such mechanisms is of paramount importance. It is well known that activation of various signaling pathways promotes the epithelial-to-mesenchymal transition (EMT) and development of a metastatic phenotype in breast cancer cells (4). Human epidermal growth factor receptor 2 (Her2 or ErbB2) is a type I transmembrane receptor tyrosine kinase that belongs to the epidermal growth factor receptor (Egfr) family (5). Two additional members, Her3 and -4, complete this family. Overexpression of Her2 at the cell membrane has been reported to underlie a clinically aggressive metastatic form of breast cancer termed ErbB2/Her2-positive (6–8). Although Her2 is an orphan receptor that does not bind ligand, its canonical mechanism of activation includes homodimerization or heterodimerization with other members of the Her/ErbB family to stimulate its tyrosine (Tyr) kinase activity to induce the activation of downstream signaling pathways (8). In mammalian cells, various mitogenic stimuli, including epidermal growth factor (Egf), induce a Ras-dependent, mitogen-activated, and extracellular signal-regulated kinase (Mapk/Erk) cascade that results in the transcriptional activation of immediate early response genes including the Ser/Thr kinase ribosomal S6 kinase (Rsk) family (9–13). Of the Rsk family (Rsk1–4), Rsk2 is a substrate of Erk that has been implicated in mediating Erk-dependent transcriptional activation by phosphorylation of the transcription factor cAMP response element binding protein (Creb), signal transducer and activator of transcription 3 (Stat3), p53, and JUN, the protein that forms the activating protein one (AP-1) transcription factor (11–14).

Overexpression of Rsk2 has been associated with the development of multiple types of hematological and epithelial malignancies (13). Rsk2 levels are elevated in more than 50% of breast tumors (14) and its overexpression is associated with shorter disease-free survival (15). A potential role in cell migration was first proposed when Rsk2 was found to phosphorylate the cytoskeleton-associated protein filamin A (16). Recently, Rsk2 was identified as an effector of Ras/Erk-mediated EMT and its knockdown reduces metastasis in animal models (17,18). A genome-wide mRNA analysis revealed that Erk/Rsk signaling affects the expression of over 50 genes from diverse pathways responsible for cell motility and invasiveness (19). In addition, a number of growth factors and mitogens, including Egf (20,21), insulin (22), and Igf-1 (23), are reported to activate Rsk2. Activation Rsk2 stimulates tumor cell motility and invasion by phospho-activation of key transcription factors including Creb, Fos related antigen 1 (Fra1), an oncogenic member of the bZIP family, Stat3, and Fascin, a 55-kDa actin-bundling protein (24–26).

The transcription factor Creb has been implicated in tumor promotion and progression and its elevated expression can confer resistance to chemotherapy (25). Rsk2 can phospho-activate Creb at Ser-133 to promote gene transcription (24). Creb induces the expression of Stat3 which promotes the transcription of Fascin, which bundles F-actin into parallel arrays to play a central role in Rsk2's inductive influence on the metastatic progression of breast cancer (24,27–30). Elevated levels of Her2 also drive the expression and/or phospho-activation of Erk1/2, Rsk2, Creb, Stat3, and Fascin that correlate with an aggressive clinical outcome and poor prognosis in a variety of cancers, including breast cancer (31).

The potential inhibitory impact of the pineal hormone melatonin on the metastatic spread of cancer has been suggested for almost 50 years (32). Recently, we reported that “tissue-isolated” MCF-7 human breast tumor xenografts grown in circadian intact nude rats (i.e., expressing a robust nocturnal circadian/melatonin signal) exposed to dim light at night (dLAN) showed almost complete melatonin suppression accompanied by high nighttime expression of phospho-active Erk1/2, Rsk2, Creb, and Stat3, which was blocked when exogenous melatonin was reintroduced during dLAN (33,34). Here, we report that pro-invasive and -metastatic signaling pathway(s) such as Her2, Erk1/2, Rsk2, Creb, Stat3, and Fascin, when activated *in vitro*, also regulate the expression of urokinase plasminogen activator (uPA) and breast cancer metastasis suppressor protein 1 (BRMS-1) mRNA to drive invasion and metastasis in human breast tumor cells. In addition, melatonin induces BRMS1 mRNA expression to repress tumor cell invasion and metastasis *in vitro* and *in vivo*. Finally, *in vivo* studies employing poorly metastatic MCF-7 breast tumor xenografts grown in athymic female nude rats show that exposure to dLAN, via its disruption of the nocturnal circadian melatonin signal, drives the expression and/or phospho-activation of signaling nodes involved in metastasis including Her2, Her3, Erk1/2, Src, focal adhesion kinase (Fak), Paxillin (Pax), protein kinase C alpha (Pkc α), Rsk2, Creb, Stat3, and Fascin, all of which were suppressed in complete dark night when melatonin levels were elevated.

Materials and Methods

Chemicals, reagents, and cell lines

All chemicals and tissue culture reagents including Dulbecco's Modified Eagles Medium (DMEM) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) for cell culture was purchased from Gibco BRL (Waltham, MA). The MEK/ERK inhibitor UO126, the melatonin receptor antagonist Luzindole, and melatonin were purchased from Sigma-Aldrich (St. Louis, MO). The Rsk2 inhibitor SL0101-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A constitutively active (CA) RSK2 construct (35) was provided by Dr. Sue-Hwa Lin (MD Anderson, Houston, TX). Three human breast cancer cell lines were used in these studies. The Her2-positive SKBR-3 and estrogen receptor alpha positive (ER α +) MCF-7 human breast cancer cell were purchased from American Type Culture Collection (Manassas, VA). MCF-7_{Her2.1-RFP} cells stably transfected with the constitutively active Her2 variant (HER2.1) and expressing red fluorescent protein (RFP) were generously provided by Dr. Frank E. Jones (Tulane University, New Orleans, LA). All cell lines were cultured in DMEM medium supplemented with 10% FBS (Gibco BRL), 50 mM minimum essential medium non-essential amino acids, 1 mM sodium

pyruvate, 2 mM glutamine, 10 mM basal medium eagle, 100 mg/mL streptomycin, and 100 U/mL penicillin. These cell lines were routinely maintained at 37° C in a humidified atmosphere of 5% CO₂ and 95% air.

Red fluorescent protein (RFP) gene transduction of MCF-7/Her2.1 cell line

For retroviral transduction, the PT67 NIH3T3-derived packaging cell line, expressing the 10A1 viral envelope, was purchased from CLONTECH Laboratories Inc. (Mountain View, CA). PT67 cells were cultured in DME (Irvine Scientific, Santa Ana, CA) supplemented with 10% FBS. For vector production PT67 cells at 70% confluence were incubated with a precipitated mixture of DOTAP reagent (Roche Pharmaceuticals, Basel, Switzerland), and saturating concentration of the pLNCX2DsRed2 plasmid for 18 h. For selections, cells were cultured in the presence of 200–1,000 µg/ml of G418 (Life Technologies, Grand Island, NY) in a step-wise manner. For RFP gene transduction, MCF-7_{Her2.1} cells at approximately 60% confluence were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells and F12K medium containing 7% FBS. After 72 h cells were harvested in trypsin/EDTA and sub-cultured into selective media containing 200 µg/ml of G418, which was gradually increased to 1,000 µg/ml in a step-wise manner. Clones expressing RFP were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) by trypsin/EDTA and were amplified and transferred by conventional culture methods in the absence of G418.

Generation of SKBR-3 cells overexpressing a constitutively active myrRSK2

A cDNA encoding a constitutively active (CA) myrRSK2 (35) was inserted into bicistronic retroviral vector pBMN-I-GFP. SKBR-3/RSK2 cells were generated from SKBR-3 breast cancer cells transduced with retrovirus generated from pBMN-RSK-GFP vector and selected by FACS. SKBR-3 cells transduced with empty vector (SKBR-3/vector) were similarly generated.

In vitro cell invasion assays

Becton Dickinson BioCoat™ Matrigel™ Invasion Chambers (BD Biosciences, San Jose, CA) were used for the in vitro cell invasion assays according to the manufacturer's protocol. Briefly, the matrigel-coated chambers were rehydrated in a humidified tissue culture incubator at 37° C in a 5% CO₂ atmosphere. Cells (5×10⁴) were suspended in 500 µl of medium in each matrigel-coated transwell insert. The lower chamber of the transwell was filled with 500 µl of medium supplemented with 10% FBS (as a chemo-attractant). After 48 h, invaded and non-invaded cells were fixed and stained with a Diff-Quick staining kit (Siemens Healthcare Diagnostics, Deerfield, IL) and counted on an inverted scope at 100× magnification. Five fields per membrane were randomly selected and counted in each group. The percentage of invasive cells was determined by counting both non-invaded cells and invaded cells on the underside of the membrane. The relative invasion level was defined as the number of cells that had passed through the matrigel-coated membranes normalized to the total cell number, as previously described (36).

MCF-7 and MCF-7_{Her2.1-RFP} tumor xenograft development in nude mice

Ovariectomized athymic nude female mice (4–5 weeks old) were obtained from Charles River (Indianapolis, IN) and maintained in pathogen-free aseptic conditions with phytoestrogen-free food and water *ad libitum*. All mice were implanted with estrogen pellets (0.72 mg 17 β -estradiol 60-day release from Innovative Research of America, Sarasota, FL) three days prior to tumor inoculation. Exponentially growing parental MCF-7 (passage numbers 18–20) and MCF-7_{Her2.1-RFP} (passage number 26–28) cells were harvested and approximately 2×10^6 cells in 150 μ l of PBS-Matrigel mixture were orthotopically implanted into the mammary fat pads, as previously described (33). Tumor growth was monitored weekly by caliper measurements. Parental MCF-7 tumor xenografts were collected at the appropriate size, blocked, and transplanted into our circadian complete (melatonin circadian rhythm) female nude rats as described in the next section.

Upon development of MCF-7_{Her2.1-RFP} tumor xenografts, mice were maintained on a LD, 12:12 lighting schedule were randomly assigned to two groups of 7 mice each and administered either ethanolic water (0.001% ETOH) or melatonin in their nighttime drinking water such that they received approximately 50 μ g/ml melatonin daily based on their water intake. This concentration of melatonin simulates the high normal nighttime physiologic levels of melatonin (33). Animals were sacrificed after 5 weeks, as a number of mice looked stressed, and lungs were harvested in cold PBS and examined microscopically for metastatic foci using a fluorescent microscope (Nikon AZ-100 low power scope) with a fluorescent illuminator and objectives with appropriate filters. Pictures of ten fields were taken from each animal at a 10 $\times$ magnification and the number of tumor foci per field counted manually.

Athymic nude rats, housing conditions, arterial blood collection, and transplantation of tissue-isolated tumor xenografts

Female athymic, inbred nude rats (CrI:NIH- *Foxn1^{tmu}*) used in this study were purchased from Charles River Laboratories (Wilmington, MA) at 4–5 weeks of age. Upon arrival, animals were maintained in environmentally controlled rooms (25 $^{\circ}$ C; 50–55% humidity) with a controlled diurnal lighting schedule LD, 12:12 (300 lux; 123 μ W/cm 2 ; lights on at 0600 h and off at 1800 h). Animal rooms were completely devoid of light contamination during the dark phase (33,34). One week prior to parental MCF-7 tumor xenograft implantation, 6 animals were switched to a 12 h light:12 h dim light at night (dLAN) photoschedule, subjective night cycle (0.2 lux, with lights on at 0600 h and off at 1800 h, and dLAN on at 1800 h and off at 0600 h) and the other 6 maintained on a photo-schedule of LD, 12:12 in an AAALAC-accredited facility and in accordance with *The Guide*. Animals were given free access to food (Purina 5053 Irradiated Laboratory Rodent Diet, Richmond, IN) and acidified water as previously described (33,34). All procedures employed for animal studies were approved by the Tulane University Institutional Animal Care and Use Committee.

Diurnal plasma melatonin levels (pg/mL; mean $\pm$ 1 SD) of naïve, female nude rats (n = 12) maintained initially in the control LD, 12:12 cycle or in the dLAN cycle were measured as previously described (33). During the course of these studies blood was collected at six

circadian time points (0400-, 0800-, 1200-, 1600-, 2000, and 2400-hr) and melatonin levels measured using a radioimmunoassay kit (Alpco, Salem, NH), as previously described (33).

One week following arterial blood collection in the naïve nude rats described above, animals were implanted in a tissue-isolated fashion with MCF-7 human tumor xenografts obtained from the tumor xenografts initially developed in mice, as described previously (33,34). Once implanted and as tissue-isolated tumor xenografts reached a palpable size (approximately pea size) they were measured every day for estimated tumor weights, as previously described (33). After 28 days of growth for tumors in dLAN or 40 days of growth for tumors in rats housed in LD, 12:12, tumors were harvested at 2400 h under a dim red light, snap frozen in liquid nitrogen, and stored at -80°C until processed for Western blot and real time-polymerase chain reaction (qPCR) analyses.

Protein and mRNA extraction from tumor xenografts and cell lines

Frozen tumors were pulverized and manually homogenized in a 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.1% sodium deoxycholate, 1 mM Na_3VO_4 , 100 nM okadaic acid, and $1\times$ protease inhibitor cocktail-Set I (Calbiochem/EMD Biosciences, Billerica, MA). Cells from *in vitro* studies were harvested and then lysed in a protein extraction buffer containing Tris (50 mM, pH 7.4), EDTA (20 mM), NP-40 (0.5%), NaCl_2 (150 mM), phenylmethylsulfonyl fluoride (0.3 mM), NaF (1 mM), Na_3VO_4 (1 mM), dithiothreitol (1mM), aprotinin (1 $\mu\text{g}/\text{ml}$), leupeptine (1 $\mu\text{g}/\text{ml}$), and pepstatin (1 $\mu\text{g}/\text{ml}$). Tumor xenograft and cell line lysates were centrifuged at $15,300\times g$, for 20 min at 4°C and supernatants were aliquoted and stored at -80°C . Total RNA was isolated from the human breast cancer cell lines and the tissue-isolated MCF-7 breast tumor xenografts using TRIzol (Thermo Fisher, Waltham, MA) according to the manufacturer's instructions and mRNA was aliquoted and stored at -80°C until analyzed.

Western blot analysis

Protein concentrations of the total cellular protein extracts from tumor xenografts and cell line lysates were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Total protein (50 μg per sample) from either tumor xenograft or cell line lysates was electrophoretically separated on 10% SDS polyacrylamide gels and electro-blotted onto a Hybond membrane. After incubation with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20, the blots were probed with anti-phospho p-Erk1/2 (Thr²⁰²/Tyr²⁰⁴), p-Creb (Ser¹³³), p-Rsk2 (Ser³⁸⁶), p-Src (Tyr⁴¹⁶), p-Fak (Tyr^{576/577}), p-Pax (Y¹¹⁸), p-Pkca (S⁶⁵⁷), and p-Stat3 (Y⁷⁰⁵), polyclonal rabbit antibodies (Cell Signaling Technology Inc., Danvers, MA). The same blots were subsequently stripped and re-probed with anti-total (t) Her2, Her3, Erk1/2, cSrc, Creb, Fax, Pax, Pkca, Rsk2, Stat3, Fascin, E-Cadherin, β -Cadherin, Vimentin, Snail, and β -Actin antibodies (Cell Signaling Technology Inc.).

Quantitative real-time reverse transcriptase polymerase chain reaction (qPCR) analyses

Performance and optimal annealing temperatures of the PCR primers for uPA and BRMS1 were tested with gradient PCR. An initial DNA denaturation step at 95°C for 5 min was followed by 35 cycles of denaturation at 95°C for 30 s, primer annealing and extension at 50°C to 70°C for 20 s. The primers for uPA are forward 5'-

GGAGATGAAGTTTGAGGTGG-3' and reverse 5'-GGTCTGTATAGTCCGGGATG-3' and for BRMS1 are forward 5'-CTGCCGCC CAGCAAGA-3' and reverse 5'-GCCTTTTGGATGGCTGTCCA-3'. The primer specificity was confirmed by sequence searches against human DNA databases and analyzed electrophoretically on agarose gels. qPCR was performed using an iCycler iQ apparatus (Bio-Rad) associated with the iCycler Optical System Interface software (version 2.3; Bio-Rad). All PCR analyses were performed in triplicate in a volume of 20 µl, using 96-well optical-grade PCR plates and optical sealing tape (Bio-Rad). Differences in the expression of the uPA and BRMS1 transcripts were normalized with respect to GAPDH expression. The thermal cycling conditions used an initial DNA denaturation step at 95° C for 8 min followed by 35 cycles of denaturation at 95° C for 15 s, annealing and extension at 60° C for 1 min. The relative level of expression was calculated with the formula $2^{-\Delta\Delta C_t}$.

Statistical analysis

Data are represented as the mean $\pm$ the standard error of the mean. Statistical significance at 95% confidence level was determined by one-way ANOVA followed by Newman-Kuels post-hoc test analyses using the Statview software package (SAS Institute, Inc., Cary, NC). Melatonin serum levels presented in Figure 6C were compared by one-way ANOVA followed by Bonferroni multiple comparison test to evaluate differences (Prism, GraphPad Software, La Jolla, CA).

Results

Melatonin inhibits phospho-activation of Erk1/2 and Rsk2 in SKBR-3 and MCF-7_{Her2.1} breast cancer cell lines

Numerous studies have demonstrated that Her2-mediated activation of the Mapk/Erk signaling pathway promotes an invasive and metastatic phenotype in human breast cancer cells (37). We have reported that dLAN induces, but melatonin represses, Her2 and Her3 expression in MCF-7 human breast tumor xenografts (34). In the current study, we asked if melatonin regulates Her2-mediated activation of the Mapk/Erk signaling pathway. Figure 1A, shows that expression of the HER2.1 construct in MCF-7 cells significantly increases the expression of p-Rsk2^{S386}, p-Creb^{S133}, p-Stat3^{Y705}, and t-Fascin as compared to the parental MCF-7 cell line. Furthermore, Her2-positive SKBR-3 cells also showed strong expression of p-Rsk2, p-Creb, p-Stat3, and t-Fascin. Next, we examined whether melatonin regulates the Mapk/Erk signaling pathway in these cells. Figures 1B and 1C show that melatonin, at concentrations ranging from 10⁻⁶ M to 10⁻⁹ M, effectively suppresses Erk1/2 and Rsk2 phospho-activation 30 min after administration, with the most effective doses in both cell lines being between 10⁻⁸ M and 10⁻⁹ M. Thus, we chose to employ 10⁻⁸ M melatonin for the time course analysis of melatonin-mediated inhibition of Erk1/2 and Rsk2 phospho-activation. Figures 1D and E show that administration of 10⁻⁸ M melatonin significantly ($p < 0.05$) suppresses p-Erk1/2 and p-Rsk2 levels after 30' and 60', but not at 15', in SKBR-3 and MCF-7_{Her2.1} cells, respectively. Administration of Luzindole, a non-specific MT₁/MT₂ melatonin receptor antagonist, blocked the repression of p-Erk1/2 and p-Rsk2 by melatonin in both cell lines (Fig. 1F).

Melatonin inhibits the in vitro invasive capacity of SKBR-3 and MCF-7_{Her2.1} breast cancer cells

Given that phospho-activation of Rsk2 at Ser386 by Erk1/2 is associated with breast cancer metastasis, we next examined the effect of melatonin on the in vitro invasive capacity of SKBR-3 and MCF-7_{Her2.1} cells using matrigel-coated transwell invasion chamber assays. As seen in Figure 2, melatonin (10^{-8} M) significantly repressed the invasive potential of Her2 expressing SKBR-3 cells by 72% (Fig. 2a) and MCF-7_{Her2.1} cells by 43% (Fig. 2b) after 48 h. Furthermore, Luzindole blocked the inhibitory action of melatonin on the invasive capacity of these Her2+ breast cancer cell lines.

Melatonin represses the formation of lung metastasis from orthotopic MCF-7_{Her2.1}-RFP tumor xenografts

To evaluate the effects of melatonin on metastasis, we implanted athymic nude female mice with MCF-7_{Her2.1}-RFP-labeled breast cancer cells and allowed them to form tumor xenografts. We observed that all mice (7 controls and 7 melatonin-treated) formed minimal tumor xenografts, but demonstrated significantly different amounts of MCF-7_{Her2.1}-RFP metastatic foci in their lungs. Figure 3 shows a high incidence of RFP-labeled metastatic foci in the lungs (n=13 foci per field) of all control mice, while mice receiving nighttime melatonin in their drinking water had significantly fewer ($p < 0.05$) MCF-7_{Her2.1}-RFP metastatic lung foci (n = 6 foci per field).

Melatonin and the Mek/Erk inhibitor UO126 repress pRsk2 and downstream targets p-Creb, p-Stat3, and t-Fascin in SKBR-3 and MCF-7_{Her2.1} breast cancer cells

Activation of Rsk2 is reported to induce the phospho-activation of Creb and Stat3 to promote the transcriptional expression of Fascin, a key regulator of EMT and metastasis. Thus, we asked if melatonin, like the Mek/Erk inhibitor UO126, blocks or suppresses p-Creb, p-Stat3, or t-Fascin expression in these Her2+ breast cancer cells via repression of p-Rsk2. As shown in Figure 4A, melatonin (10^{-8} M) significantly suppresses ($p < 0.05$) the expression of p-Erk1/2, p-Rsk2, p-Creb, and p-Stat3, as well as t-Fascin after 3 h of treatment in SKBR-3 and MCF-7_{Her2.1} cells. A similar but less potent inhibition of p-Erk1/2 and p-Rsk2 was seen with the Mek/Erk inhibitor UO126 (100 ng/ml) as compared to melatonin in both cell lines. Interestingly, the combination of melatonin and UO126 was more effective than either agent alone at suppressing the phospho-activation of Erk1/2, Rsk2, Creb, and Stat3 and inhibiting Fascin protein expression.

Expression of a constitutively active Rsk2 significantly, but not completely, blocks melatonin-mediated inhibition of p-Creb, p-Stat3 and Fascin

To determine if expression of a constitutively active (CA)-Rsk2 can block the suppressive effects of melatonin on p-Creb, p-Stat3 and t-Fascin expression, we transfected SKBR-3 breast cancer cells with a CA-Rsk2, as previously described (35). As seen in Figure 4B, the expression of this CA-Rsk2 construct completely blocked UO126 inhibition of p-Creb, p-Stat3, and t-Fascin in SKBR-3 cells. However, expression of the CA-Rsk2 only partially blocked the melatonin-mediated suppression of p-Creb and p-Stat3 by 73% and 66%, respectively. Since the expression of a CA-Rsk2 did not completely block melatonin's effect,

these data suggest that melatonin also signals through other pathways to impact tumor metastasis.

Melatonin inhibits the expression of uPA mRNA and induces the expression of BRMS1 mRNA

Reportedly, Rsk2, via its phospho-activation of Stat3, can inhibit the transcription of the breast cancer metastasis suppressor protein Brms1 gene while inducing Fascin expression to stimulate uPA gene transcription (38). Thus, we asked if melatonin is able to regulate uPA and BRMS1 mRNA expression in SKBR-3 and MCF-7_{Her2.1} cells. Figure 5 shows that melatonin represses uPA mRNA expression but induces BRMS1 mRNA expression in a dose-dependent manner in SKBR-3 cells. The most effective dose of melatonin (10^{-9} M) was able to inhibit uPA mRNA expression by 80%, while inducing the levels of BRMS1 by 87%.

dLAN-induced disruption of the circadian melatonin signal promotes an EMT phenotype in tissue-isolated MCF-7 human breast tumor xenografts grown in female athymic nude rats

We evaluated the effect of circadian/melatonin on MCF-7 tissue-isolated breast tumor xenografts on nude rats housed in a dLAN or LD, 12:12 photo-schedule harvested at 2400 h (12 midnight) by Western blot analysis for the expression of markers of EMT and metastasis. Melatonin serum levels are presented in Figure 6A. As shown in Figure 6B, exposure to dLAN repressed the expression of t-E-Cadherin, an epithelial cell marker, but induced the expression of mesenchymal cell markers including p-Rsk2, t-Vimentin, t- β -Catenin, and t-Snail.

We have reported that MCF-7 breast tumor xenografts in athymic nude rats exposed to dLAN show increased expression of key signaling pathways (Erk1/2, cSrc, Fak, and Pax) involved in the metastatic progression of breast cancer (33,34). In the current study, we evaluated MCF-7 breast tumor xenografts from nude rats circadian/melatonin disrupted by dLAN and harvested at 2400 h for the expression of makers of EMT and metastasis. However, tumors isolated at midnight (elevated endogenous melatonin) from rats housed under a LD, 12:12 photo-schedule showed elevated expression of E-Cadherin but decreased expression of t-Vimentin, t- β -Catenin, and t-Snail.

In Figure 6C, MCF-7 breast tumor xenografts isolated from nude rats housed in a dLAN or a LD, 12:12 photo-schedule were harvested at 2400 h (12 midnight) and further evaluated by Western blot analysis for the expression of signaling pathways. Exposure to dLAN induced the expression of p-Her2, p-Her3, p-Creb, p-Pkca, p-Rsk2, p-Stat3, and t-Fascin in tumor xenografts during the dLAN period. Conversely, tumors isolated at 12 midnight from rats housed in a LD, 12:12 (dark night, with elevated endogenous nocturnal melatonin) showed either a complete blockade or a large suppression of the expression and/or phospho-activation of these kinases, transcription factors, and actin-binding proteins. The results from *in vivo* xenografts are consistent with those observed in the *in vitro* studies.

Discussion

Activation of Her2 is reported to induce the expression and/or phospho-activation of numerous kinases and transcription factors that drive drug resistance and metastasis in breast cancer (2–5). A key signaling node induced and activated by the Her2/Mapk/Erk pathway is Rsk2 (17,18), which, in turn, promotes numerous down-stream signaling pathways associated with the development of EMT and metastasis including integrin activation, the expression of matrix metalloproteinases, the expression/activation of Creb, Stat3, cSrc, Fak, Pax, and actin polymerization (13,14,39–43). Activation of these same signaling pathways is also seen in breast tumors from patients that have developed resistance to chemotherapy (24,25).

We recently reported that disruption of the circadian/melatonin signal by dLAN promotes the expression/phospho-activation of mitogenic and survival pathways associated with the development of drug resistance in ER α -positive breast tumor xenografts, including Erk1/2, Akt, cSrc, Fak, Pax, Creb, and Stat3 (33,34). In earlier studies, we reported that melatonin demonstrates potent anti-invasive actions in breast cancer cells *in vitro* that were mediated, in part, via its repression of p38 Mapk phosphorylation (36), and the circadian gating of Gsk3 β activity (44). Melatonin phospho-activation of Gsk3 β promotes the phosphorylation and ubiquitination of β -Catenin to suppress EMT in human breast cancer cells (44). To date, our studies of the anti-invasive and anti-metastatic actions of melatonin in breast cancer have been limited to the MCF-7 breast cancer cell line. In the current study we have evaluated and compared the anti-metastatic actions of melatonin on the ER α -negative, Her2-positive SKBR-3 breast tumor cell line and ER α -positive MCF-7 cells overexpressing a constitutively active HER2.1 construct (MCF-7_{Her2.1} cells). SKBR-3 cells express phospho-active Rsk2 that, in turn, phospho-activates Creb and Stat3, both of which can bind to the Fascin promoter to induce its expression (24). Fascin is reported to induce the expression of the protease uPA and suppress the expression of the metastasis suppressor BRMS-1 to promote a more metastatic phenotype (45). As shown in Figure 1, administration of melatonin to SKBR-3 or MCF-7_{Her2.1} cells repressed both Erk1/2 and Rsk2 phospho-activation in a dose and time dependent manner. The action(s) of melatonin in these cells is mediated through its MT₁ G protein-coupled receptor, as the melatonin receptor antagonist, Luzindole, blocks melatonin-mediated inhibition of Erk1/2 and Rsk2 in MT₁ expressing SKBR-3 cells.

The role of p-Erk1/2 and p-Rsk2 in driving the invasive/metastatic potential of SKBR-3 and MCF-7_{Her2.1} cells is demonstrated by the ability of melatonin to suppress their phospho-activation as well as the invasive capacity of these two cell lines in matrigel-coated transwell assays (Fig. 2). Melatonin also showed a greater anti-invasive action in SKBR-3 cells as compared to MCF-7_{Her2.1} cells. The reason(s) for this difference is currently not clear, but may relate to the fact that SKBR-3 cells endogenously express elevated levels of Her2 to drive Erk1/2 and Rsk2 expression/activation, while MCF-7_{Her2.1} cells have been modified to express a constitutively active Her2 mutant. Other differences may be related to the fact that MCF-7_{Her2.1} cells are ER α -positive while SKBR-3 cells are ER α -negative, or to differences in the levels of MT₁ receptor expressed in each line. What is clear, however, is that both cell

lines express MT₁ receptors (46) and are responsive to melatonin-mediated inhibition of p-Rsk2, p-Creb, p-Stat3, and t-Fascin expression.

Although the anti-invasive actions of melatonin in breast cancer have been reported (36,44,47), we have not yet determined if melatonin actually suppress the complex process of metastasis. We show in figure 3, that nighttime administration of melatonin to mice with metastatic MCF-7_{Her2.1}-RFP breast tumor xenografts significantly ($p<0.05$) suppresses the development of RFP-labeled metastatic foci in the lungs, demonstrating the full anti-metastatic actions of melatonin. Given that these tumor xenografts originated in the mammary fat pad, it is clear that the metastatic cells that colonized the lungs of these mice had to go through the entire process of metastasis. At present we do not yet know if melatonin inhibits a specific aspect or multiple aspects of metastasis; this will be addressed in future studies.

We previously reported (36) and now confirm that melatonin significantly inhibits this invasive/metastatic phenotype via suppression of EMT, either by promoting mesenchymal epithelial transition (MET), or by inhibiting key signaling pathways involved in the later stages of metastasis. For example, activation of Rsk2 by increased expression/or activation of Her2/Erk1/2 in MCF-7 breast cancer cells promotes the development of a highly metastatic phenotype in these cells that are typically poorly metastatic (44). Our studies show that elevated Her2 expression is associated with the phospho-activation of Rsk2 and its downstream targets p-Creb, p-Stat3, and Fascin in both MCF-7_{Her2.1} and SKBR-3 breast cancer cells. *In vitro* administration of melatonin or UO126 a Mek/Erk inhibitor to SKBR-3 cells and MCF-7_{Her2.1} cells greatly repressed the expression of p-Erk1/2, p-Rsk2, p-Creb, p-Stat3, and t-Fascin, confirming that Erk is a major upstream node in this signaling pathway that drives metastasis. However, the ability of melatonin to suppress the expression of p-Creb and p-Stat3 to a greater degree than UO126, combined with the fact that the combination of melatonin and UO126 was more efficacious at inhibiting p-Creb, p-Stat3, and t-Fascin than either agent alone, implies that at least some of the anti-metastatic effects of melatonin may be mediated independent of Rsk2. This concept is also supported by the ability of melatonin to partially block the expression of p-Creb, p-Stat3 and t-Fascin in SKBR-3 cells expressing a CA-Rsk2 construct (Fig. 4B).

Our studies confirm that activation of the Rsk2/Stat3/Fascin pathway can promote the expression of uPA mRNA (a known activator of MMP 2 and 9 and $\alpha_4\beta_5$ integrin) and inhibit the expression of the breast cancer metastasis suppressor, BRMS1 (Fig. 4). Interestingly, a consistent but opposite, dose-response effect of melatonin on the expression of uPA and BRMS1 mRNA was evident, with the physiological concentration of melatonin (1 nM) being more effective than higher concentrations for inhibiting uPA and inducing BRMS1 mRNA expression in SKBR-3 cells. This unusual dose response, also observed in our analysis of phospho-activated Erk1/2 and Rsk2, is consistent with our earlier report of the bell-shaped dose-dependent growth-inhibitory effect of melatonin on MCF-7 breast cancer cells (48,49). As the binding affinity of the MT₁ melatonin receptor for melatonin is in the picomolar/nanomolar range, these data, in concert with studies showing that the melatonin receptor antagonist Luzindole can block melatonin's inhibition of p-ERK1/2, p-RSK2 and SK-BR-3 and MCF-7_{Her2.1} invasive activity, confirm that the anti-metastatic

activity of melatonin is primarily driven through its activation of the MT₁ melatonin receptor.

The importance of the circadian/melatonin signal in suppressing the growth and invasive progression of breast and other cancers has been reported by us (36,46) and others (47,50). Unfortunately, to date, the mechanisms underlying the anti-invasive/metastatic actions of melatonin have not been well established. Our recent reports employing tissue-isolated MCF-7 breast tumor xenografts in circadian complete female athymic nude rats show that host exposure to dLAN induces the expression/activation of numerous key signaling nodes involved in tumor progression and metastasis including Erk1/2, Creb, Nf-kB, cSrc, Pax, Fak, and Stat3 (33,34). Conversely, the presence of endogenous melatonin during dark night (LD, 12:12) or exogenous administration of melatonin during dLAN blocks the expression/phospho-activation of these same signaling nodes. In new studies comparing tissue-isolated MCF-7 breast tumor xenografts from rats housed in either dLAN or LD, 12:12 photoperiods, we find that at the 12 midnight (2400 h) time point tumors in dLAN show increased levels of markers of EMT, including Vimentin, β -Catenin, and Snail, but decreased levels of the epithelial marker E-Cadherin. Whereas, tumors collected at 12 midnight from rats in a LD, 12:12 photoperiod, when endogenous melatonin is elevated, showed an epithelial phenotype expressing elevated E-Cadherin but decreased Vimentin, β -Catenin, and Snail. Our studies show that melatonin inhibition of Rsk2 inhibits the metastatic phenotype in breast cancer cells by suppressing EMT or by inhibiting other mechanisms/pathways that promote metastasis. These data strongly support the concept that exposure to dLAN, with its disruption of the circadian/melatonin signal, promotes EMT and, over time, could generate an enhanced metastatic phenotype in breast tumors.

The fact that some breast cancer cells do not express the MT₁ receptor and may be unresponsive to melatonin is important given the potent anti-cancer and anti-metastatic actions of melatonin. We would predict that cancer cells that do not express the MT₁ receptor may still respond to melatonin, particularly high physiologic or pharmacologic concentrations of melatonin, and that a melatonin may promote antioxidant mechanisms. However, it is most likely that cells that do not express the MT₁ receptor or respond to melatonin may show significantly enhanced tumor growth, progression, and metastatic spread.

These data, combined with our earlier *in vitro* studies by Mao et al. (36), support the concept that an extended duration of nocturnal melatonin production plays a critical role in suppressing the metastatic progression of breast cancer. Given the ability of dLAN to inhibit the nocturnal rise of melatonin in the blood and its associated induction of the expression and/or phospho-activation of various oncogenes including Akt, Her2, Ras, and cSrc (33,34,44), we now consider dLAN to be a key environmental promoter of metastasis. In this regard, dLAN, via its disruption of the circadian/melatonin signal, may represent a unique and previously unappreciated environmental risk factor that could account for the increasingly large number of patients presenting with more aggressive and metastatic breast cancer, leading to a shortened survival time and increased morbidity and mortality.

Eliminating, reducing, or slowing the development of metastasis could potentially be achieved by correcting circadian regulatory deficits through the use of circadian/melatonin-friendly lighting systems in the home, workplace, and hospital settings. Indeed, new lighting technologies are emerging that maintain visual efficacy while supporting the improvement of circadian regulatory dynamics, particularly the integrity of the nocturnal melatonin signal, by reducing wavelengths in the blue portion (~ 480 nm) of the visible light spectrum that are known to be melatonin-suppressive. Additional approaches may include emphasizing the importance of sleeping in a totally dark room to patients and/or the implementation of physiological, nocturnal melatonin replacement strategies (e.g., oral melatonin supplements) in conjunction with circadian-optimized chemotherapy.

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Implications

Melatonin inhibition of Rsk2 represses the metastatic phenotype in breast cancer cells suppressing EMT or inhibiting other mechanisms that promote metastasis; disruption of the melatonin signal may promote metastatic progression in breast cancer.

Figure 1.

Expression of Erk1/2, Rsk2, Creb, Stat3 and Fascin signaling nodes and their suppression by melatonin in MCF-7, MCF-7_{Her2.1}, and SKBR-3 breast cancer cells. (A) Expression of total (t) and phospho (p)-activated Erk, Rsk2, Creb, Stat3 and total Fascin in parental MCF-7, MCF-7_{Her2.1}, and SKBR-3 breast cancer cells. (B and C) SKBR-3 and MCF-7_{Her2.1} breast cancer cells were serum starved for 24 h and the dose-response effects of melatonin (MLT) treatment (10⁻⁶ to 10⁻¹⁰ M, for 1 hour) on the expression of total and phospho-active Erk and Rsk2 by Western-blot analysis as described in Materials and Methods. (D and E) SKBR-3 and MCF-7_{Her2.1} breast cancer cells were serum starved for 24 h and the time-course effects (15, 30, 60 min) of MLT treatment (10⁻⁸ M) on the expression of total and phospho-active Erk and Rsk2 were examined by Western blot analysis. (F) SKBR-3 and MCF-7_{Her2.1} breast cancer cells were serum starved for 24 h and the ability of the MLT-receptor antagonist Luzindole (LUZ) at a concentration of 10⁻⁶ M to block the effects of MLT treatment (10⁻⁸ M, for 1 h) on the expression of total and phospho-active Erk and Rsk2 was examined by Western blot analysis. Graphical data represents the mean $\pm$ standard deviation of 3 independent experiments.

Figure 2.

Melatonin-mediated inhibition of the invasive capacity of SKBR-3 and MCF-7_{Her2.1} breast cancer cells. **(A)** The effects of MLT treatment (10^{-8} M) or the combination of MLT and the MLT receptor antagonist LUZ (10^{-6} M) for 48 h on SKBR-3 cell invasion was analyzed using a Matrigel transwell invasion assay system as described in Materials and Methods. Cells were counted in six high-power fields per filter. **(B)** The effects of melatonin treatment (10^{-8} M) or the combination of MLT and LUZ (10^{-6} M) for 48 h on MCF-7_{Her2.1} cell invasion was analyzed using a matrigel transwell invasion assay system as described in Materials and Methods. Cells were counted by at least six high-power fields per filter. Graphical data in figures 2a and b represent the mean $\pm$ standard deviation of 3 independent experiments.

Figure 3. Melatonin mediation of lung metastasis in athymic nude mice

The effects of nighttime MLT administration (50 µg/ml) in the drinking water on the development of MCF-7_{Her2.1}-RFP lung metastases in female athymic nude mice five weeks following tumor cell inoculation in the mammary fat pad as described in Materials and Methods. Graphical data in figure represents the data from 6 different microscopic fields of lungs from 7 animals per group.

Figure 4.

Inhibition of the Erk, Rsk2, Creb, Stat3 and Fascin signaling pathways in Her2-positive breast cancer cells by melatonin and the Erk1/2 inhibitor UO126. **(A)** SKBR-3 and MCF-7_{Her2.1} breast cancer cells were grown in DMEM supplemented with 10% FBS and the effects of MLT (10^{-8} M), UO126 (100 ng/ml), or the combination of MLT and UO126 on the expression of total and phospho-active Erk, Rsk2, Creb, Stat3, and total Fascin, were determined by immune (Western)-blot analysis as described in Materials and Methods. **(B)** SKBR-3 breast cancer cells were grown in DMEM supplemented with 10% FBS and stably transfected with the CA-RSK2 construct and the effects of MLT (10^{-8} M), UO126 (100 ng/ml), or the combination of MLT and UO126 on the expression of total and phospho-active Rsk2, Creb, Stat3, and total Fascin, were determined by immune (Western)-blot analysis as described in Materials and Methods.

Figure 5. Melatonin inhibits the expression of uPA mRNA while inducing the expression of BRMS1 mRNA in SKBR-3 breast cancer cells. SKBR-3 breast cancer cells were grown in DMEM supplemented with 10% FBS and the effects of MLT (10^{-8} M) on the expression of uPA and BRMS1 mRNA expression were determined by qPCR analysis as described in Materials and Methods. Graphical data represent the mean $\pm$ standard deviation of 3 independent experiments.

Figure 6.

dLAN and melatonin effects on the expression of EMT markers and signaling nodes associated with metastasis. (A) dLAN promotes but MLT inhibits the expression of EMT markers in MCF-7 tissue-isolated breast tumor xenografts. Protein lysates (120 mg of protein per sample) from tissue-isolated (ERα+) MCF-7 human breast tumor xenografts from female nude rats exposed to LD, 12:12dLAN or a LD, 12:12 lighting schedule were analyzed by Western (immune)-blotting for expression of total and/or phosphorylated forms of E-Cadherin, Vimentin, β-Catenin, and Snail. β-actin was used as a control for equal

loading. All tumors were harvested at 24 h (mid-dLAN or mid-dark phase) from 3 animals in each group as described in Materials and Methods. **(B)** dLAN promotes but MLT inhibits the expression of metastatic signaling pathways in MCF-7 tissue-isolated breast tumor xenografts. Protein lysates (120 mg of protein per sample) from tissue-isolated (ER α +) MCF-7 human breast tumor xenografts from female nude rats exposed to LD, 12:12dLAN or a LD, 12:12 lighting schedule were analyzed by Western (immune)-blotting for expression of total and/or phosphorylated forms of Erk1/2, Akt, Nf-kB, cSrc, Fak, Pax, Pdk1, Rsk2, Pkc α , Creb, Stat3, and Fasn. β -actin was used as a control for equal loading. As noted above, all tumors were harvested at 2400 h (mid-dLAN or mid-dark phase) from 3 animals in each group as described in Materials and Methods. **(C)** Effect of dLAN versus LD 12:12 lighting schedules or administration of exogenous melatonin in the dLAN lighting schedule on the serum melatonin profile in female nude rats. Female nude rats with (ER α +) tissue-isolated breast tumor xenografts were housed under control (LD 12:12) or experimental, dLAN (with light at 0.2 lux) lighting schedules, or dLAN and supplemented with nighttime melatonin (MLT), and treated with diluent or 4OH-TAM. Plasma melatonin levels (pg/mL; mean $\pm$ 1 SD) of female nude rats maintained in a controlled LD 12:12 or experimental (dLAN) lighting cycle (n = 12/group) were measured as described in Materials and Methods. Data are double plotted to better visualize rhythmicity (n = 12/group). Asterisks (*) denote significant differences (P < 0.05) in plasma melatonin levels in rats under the different lighting schedules.