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Inhibition of breast cancer cell invasion by melatonin is mediated through regulation of the p38 mitogen-activated protein kinase signaling pathway

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Abstract

Introduction: The pineal gland hormone, melatonin, has been shown by numerous studies to inhibit the proliferation of estrogen receptor α (ERα)-positive breast cancer cell lines. Here, we investigated the role of melatonin in the regulation of breast cancer cell invasion.

Methods: Three invasive MCF-7 breast cancer cell clones: MCF-7/6, MCF-7/Her2.1, and MCF-7/CXCR4 cells, were employed in these studies. All three cell lines exhibited elevated phosphorylation of the ERK1/2 and p38 mitogen-activated protein kinase (MAPK) as determined by Western blot analysis. The effect of melatonin on the invasive potential of these human breast cancer cells was examined by matrigel invasion chamber assays. The expression and proteinase activity of two matrix metalloproteinases (MMPs), MMP-2 and MMP-9, were analyzed by Western blot analysis and gelatin zymography, respectively.

Results: Melatonin (10^{-9} M) significantly suppressed the invasive potential of MCF-7/6 and MCF-7/Her2.1 cells as measured by matrigel invasion chamber assays, and significantly repressed the proteinase activity of MMP-2 and MMP-9. In MCF-7/CXCR4 cells, melatonin significantly inhibited stromal-derived factor-1 (SDF-1/CXCL12) induced cell invasion and activity of MMP-9. Elevated expression of the MT1 melatonin receptor further enhanced, while luzindole, an MT1/MT2 antagonist, abrogated melatonin's anti-invasive effect, suggesting that melatonin's effect on invasion is mediated, principally, through the MT1 receptor. Furthermore, melatonin repressed the phosphorylation of p38 MAPK in MCF-7/Her2.1 cells and blocked stromal-derived factor-1 (SDF-1) induced p38 phosphorylation in MCF-7/CXCR4 cells. SB230580, a p38 inhibitor, was able to mimic, while transfection of the cells with a constitutively-active MKK6b construct blocked melatonin's effect on cell invasion, suggesting that the anti-invasive action of melatonin is mediated through the p38 pathway.

Conclusions: Melatonin exerts an inhibitory effect on breast cancer cell invasion through down-regulation of the p38 pathway, and inhibition of MMP-2 and MMP-9 expression and activity.

Introduction

Over the last several decades, melatonin's growth-inhibitory action in breast cancer has been extensively studied both *in vivo* and *in vitro*. In contrast, only a minimal amount of work has been done with regard to the role of melatonin in breast cancer invasion and metastasis. It has been observed in several early correlative studies that the plasma level of melatonin is significantly reduced in cancer patients with metastatic disease as compared to those without metastases [1, 2]. In 1998, Cos et al. reported that physiological concentrations of melatonin (10^{-9} M) significantly reduced the invasive capacity of MCF-7 human breast cancer cell as measured by Falcon invasion chamber assays, a modified Boyden chamber assay, and that melatonin could enhance the expression of the adhesion proteins, E-cadherin and β_1 integrin [3]. In addition, melatonin administration has been shown to reduce the incidence of metastases in several *in vivo* studies [4-6].

Collectively, these results suggest that melatonin may exert an inhibitory influence on breast cancer cell invasion and metastasis, possibly by decreasing cell attachment to the basement membrane. However, there has been no further exploration on melatonin's anti-invasive action and mechanism(s) since the work by Cos et al. in 1998. A major obstacle to a better understanding of melatonin's role in breast cancer invasion and metastasis is the lack of a cell line that exhibits a strong invasive potential but is also estrogen receptor α (ERα)-positive and melatonin-responsive. The ERα-positive MCF-7 cell, which has been well

characterized and extensively used in the *in vitro* studies examining melatonin's anti-proliferative effect and has been shown to be responsive to melatonin-mediated growth-inhibition, is well-regarded as poorly-invasive. Thus, the standard MCF-7 breast tumor cell line is not a good model for invasion/metastasis studies. Unfortunately, the highly invasive ERα-negative MDA-MB-231 cells are unresponsive to melatonin's growth-inhibitory actions and thus, are not a reasonable model to study melatonin's actions on invasion. Therefore, an alternative cell line that exhibits high invasive potential but still retains the melatonin-responsiveness, is essential for a model system in which to study melatonin's actions on breast cancer invasion.

Here we have used in our studies three invasive breast cancer cell lines. The MCF-7/6 cells were derived from parental MCF-7 cells by selection for metastatic potential by serial passaging in nude mice [7]. As compared to MCF-7/AZ line (a parental MCF-7 cell clone renamed by the group of Dr. M. Mareel), MCF-7/6 cells are invasive in the chick heart embryo invasion assay [7], and spontaneously metastasize in nude mice after subcutaneous injection [8]. These cells have been demonstrated to be ERα- and progesterone receptor (PR)-positive.

A second cell line used in our studies is the MCF-7/Her2.1 cell line, which has been stably transfected with and overexpresses the wild-type human Her2/neu (C-erbB2) receptor. According to previous studies, receptor tyrosine kinase Her2/neu plays an important role in the malignant progression of breast cancer [9]. Amplification and overexpression of Her2/neu occur in approximately 15~30% of primary breast tumors, and correlate with the nodal metastases and poor prognosis [10]. It has been previously shown that Her2/neu overexpression in breast cancer cells frequently leads to hyper-activation of MAPK signaling pathways [11]. Moreover, overexpression of Her2/neu not only induces the invasive capacity in mammary epithelial cells, but also promotes the invasiveness of breast cancer cells *in vitro* [12, 13] and induces metastasis in animal models

[13, 14]. Although the effect of Her2/neu on metastasis is well documented, the mechanism underlying the effect of Her2/neu on breast cancer invasion and metastasis is not fully understood. A recent study by Ke et al. [15] has shown that, in human mammary epithelial cells, expression and activity of MMP-2 and MMP-9, two members of the matrix metalloproteinases (MMPs) family which play an important role in degradation of the extracellular matrix, were up-regulated in response to overexpression of Her2/neu, and that the regulation of MMP-2 and MMP-9 by Her2/neu may be mediated through the p38 MAPK and PI3K signaling pathways. These results suggest that the effects of Her2/neu on breast cancer cell invasion may impinge on target molecules to orchestrate the degradation of the extracellular matrix through simultaneous activation of multiple signaling pathways.

Also utilized in our studies is a MCF-7 clone (MCF-7/CXCR4) that overexpresses the chemokine receptor CXCR4, a recently described key regulator of breast cancer invasion and metastasis [16]. Through interaction with its cognate ligand, the chemokine stromal-derived factor-1 (SDF-1/CXCL12), CXCR4 is proposed to direct homing of breast cancer cells to particular sites of metastases [16]. It has been demonstrated that down-regulation of CXCR4 inhibits *in vitro* invasiveness of breast cancer cell and blocks breast cancer metastasis *in vivo* [17, 18]. Several lines of evidence suggest that, the effect of the SDF-1/CXCR4 axis on cell invasion may involve activation of multiple signaling pathways including the p38 MAPK pathway [19-21].

Although MCF-7/6, MCF-7/Her2.1 and MCF-7/CXCR4 breast cancer cells acquire their malignant phenotypes through different mechanisms and approaches, their invasive potential may be enhanced through activation of a common intracellular signaling pathway which plays an essential role in regulating cancer cell invasion and metastasis. According to previous studies [22-25], the p38 MAPK is a central kinase in a common pathway that plays an important role in

breast cancer invasion and metastasis by modulating the expression and activity of molecules involved in the degradation of extracellular matrix (i.e. MMP-2 and MMP-9). Additionally, p38 MAPK has been reported to be activated by both the Her2/neu and SDF-1/CXCR4 pathways [15, 19-21]. Moreover, p38 MAPK activity has been shown to be regulated by cAMP [26-30]. Based on the above results and the fact that melatonin, via its MT1 receptor, regulates the intracellular concentration of cAMP in breast cancer cells [31], we hypothesize that, melatonin plays an inhibitory role in breast cancer cell invasion by modulating the activation of the p38 MAPK pathway. Our results demonstrated that melatonin suppresses the *in vitro* invasive potential of breast cancer cells by altering the phosphorylation of p38 MAPK and the downstream activity of MMP-2 and MMP-9.

Materials and methods

Chemicals and reagents. All chemicals and tissue culture reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Cell culture medium, DMEM/F12 (1:1), RPMI 1640, and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). The FuGENE 6 transfection reagent was purchased from Roche (Indianapolis, IN). SDF-1/CXCL12 was purchased from Research Diagnostics Incs (Concord, MA).

Cell lines and cell culture. Eight human breast cancer cell lines were used in these studies. The MDA-MB-231 human breast cancer cell was purchased from American Tissue Type Culture Collection (Rockville, MD). The parental MCF-7 cell was obtained from the laboratory of late William L. McGurie (San Antonio, TX). The MCF-7/6 and MCF-7/AZ (control MCF-7 cell for MCF-7/6) cells were kindly provided by Dr. Marc Mareel (Gent University, Belgium). The

MCF-7/Her2.1 (stably transfected with Her2/neu in pcDNA3.1 vector and overexpresses wild-type Her2/neu) and its control cell MCF-7/vec were generously provided by Dr. Frank E. Jones (University of Colorado Health Sciences Center, Colorado). The MCF-7/CXCR4 cell line (stably transfected with CXCR4 in pcDNA3 vector and overexpresses CXCR4) and its control counterpart MCF-7/pcDNA3 were gift from Dr. Matthew E. Burow (Tulane University, LA). All cell lines, except MCF-7/6 and MCF-7/AZ, were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) [Gibco BRL, Grand Island, NY], 50 mM MEM non-essential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 10 mM basal medium eagle (BME), 100 mg/ml streptomycin, and 100 U/ml penicillin. The MCF-7/6 and MCF-7/AZ cells were cultured in DMEM/F12 (1:1) with the same supplements described above. These cell lines were routinely maintained at 37° C in a humidified atmosphere of 5% CO_2 and 95% air.

Total RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR) analysis. RT-PCR analysis was performed to confirm the overexpression of MT1 in MT1-transfected cells. Total cellular RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad) according to the manufacturer's instructions. Reverse transcription was performed on 1 μg of total RNA using Superscript II RNase H- reverse transcriptase (Invitrogen, CA) and 200 ng random hexadeoxynulceotide primers in 20 μl reaction volumes containing 3 mM $MgCl_2$, 10 mM DTT, 75 mM KCl and 0.5 mM dNTP. The polymerase chain reaction (PCR) amplification was conducted using primer sets as previously described [32]. Aliquots of the PCR reaction products were separated on a 1% agarose gel.

Real time RT-PCR analysis. Real time RT-PCR was performed to determine the mRNA levels of MMP-9 in MCF-7 cells transiently transfected with CAMKK6b. Total cellular RNA was isolated using the PerfectPure RNA Cultured Cell Kit (5 Prime, Gaithersburg, MD) according to

the manufacturer's instructions. Reverse transcription was performed on 1 μg of total RNA using Superscript II RNase H- reverse transcriptase (Invitrogen, CA) and 200 ng random hexadeoxynulceotide primers in 20 μl reaction volumes containing 3 mM $MgCl_2$, 10 mM DTT, 75 mM KCl and 0.5 mM dNTP. Real time PCR was carried out in 20 μl of PCR mixture containing 10 μl of 2× iQ SYBR Green Supermix and 1 μl of each cDNA sample on an iCycler iQ real time detection system (Bio-Rad) in triplicates, and recorded in real time and analyzed using the accompanying program, iCycler iQ real time PCR detection system software Version 3.0A (Bio-Rad). Level of the 18s ribosomal RNA was also determined by real time RT-PCR in each cDNA sample to normalize the expression of MMP-9. The primers used were as follows: human MMP-9: forward primer, 5'-TGACAGCGACAAGAAGTG-3', and reverse primer, 5'-CAGTGAAGCGGTACATAGG-3'; 18s ribosomal RNA: forward primer, 5'-TTGACGGAAGGGCACCACCAG-3', and reverse primer, 5'-GCACCACCACCCACGGAATCG-3'. Melt curve analysis was performed at the end of each PCR to confirm the specificity of the PCR product. Ct values of MMP-9 among samples were compared after correction for 18s expression. The ratio of MMP-9 versus the corresponding 18s of each sample was determined based on the equation MMP-9/18s = $2^{Ct(18s) - Ct(MMP-9)}$. The ratio of MMP/18s was compared among samples, and the -fold change of MMP-9 expression was obtained by setting the values from vector-transfected cells to one.

Cell proliferation assay. For melatonin response studies, MCF-7, MCF-7/AZ, MCF-7/6 cells, MCF-7/vec, MCF-7/Her2.1 and MDA-MB-231 cells were seeded at a density of 2.0×10^4 cells per well, serum-starved for 24 hours, and treated with melatonin (10^{-9} M), or diluent (0.00001% ethanol), in complete medium supplemented with 10% FBS. Cells were counted after 6 days of melatonin exposure on a hemacytometer using trypan blue to select for viable cells.

Protein extraction and Western blot analysis. Cells 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 (150 mM), phenylmethylsulfonyl fluoride (0.3 mM), NaF (1 mM), $NaVO_4$ (1 mM), dithiothreitol (1mM), aprotinin (1 μg/ml), leupeptine (1 μg/ml), and pepstatin (1 μg/ml). The cell lysates were centrifuged for 10 min at 10,000 × g, at 4° C. Protein concentrations of the supernatants were determined using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Total protein (50 μg per sample) was electrophoretically separated on a 10% SDS polyacrylamide gel, and electroblotted onto a Hybond membrane. After incubation with 5% nonfat milk in a Tris-buffered saline containing 0.1% Tween, the immunoblots were probed with antibodies to MT1, Her2/neu (Santa Cruz Biotechnology, Santa Cruz, CA), CXCR4 (Abcam, Cambridge, MA), phospho-p38 (Thr180/Tyr182), phospho-p44/42 MAPK (Thr183/Tyr185) [Cell Signaling, Beverly, MA], MKK6 (Millipore, Billerica, MA), or phospho-ETS1 (Thr38) [Invitrogen, Carlsbad, CA]. The same blots were stripped and reprobed with antibodies to β-actin (Sigma, St. Louis, MO), p38, p44/42 MAPK (Cell Signaling, Beverly, MA), GAPDH, or ETS1 (Millipore, Billerica, MA), respectively. For MMP-2 and MMP-9 expression studies, the enriched conditioned medium from each treatment group was electrophoretically separated on a 10% SDS polyacrylamide gel, and the blots were probed with anti-MMP-2 and -MMP-9 antibody (Chemicon, CA).

Transient transfection. MCF-7/6 or MCF-7/Her2.1 cells were seeded in 150 mm^2 cell culture flasks at a density of 3.16×10^6 cells per flask in DMEM/F12 (1:1) medium or RPMI-1640 supplemented with 10% FBS. After 24-hour serum-starvation, cells were then transfected with 7.9 μg of pcDNA3.1-MT1 or pcDNA3.1 empty vector, or 7.9 μg of CA-MKK6b (a dominant-positive MKK6b construct provided by Dr. Matthew Burow, Tulane University, LA) or pcDNA3 empty vector, using the FuGENE 6 transfection reagent. Twenty-four hours following transfection, these

cells were seeded for matrigel invasion assays.

Matrigel invasion chamber assay. The invasive potential of breast cancer cells was assessed *in vitro* in matrigel-coated invasion Chambers (BD BioCoat Matrigel Invasion Chamber, Becton Dickinson Labware, Franklin Lakes, NJ) according to the manufacturer's instructions. Briefly, cells in log-phase of growth were serum-starved for 24 hours prior to seeding, detached by brief trypsinization, and resuspended in medium containing the appropriate treatment. The matrigel invasion inserts were rehydrated and prepared as described in the manufacture's instructions. Cells (5×10^4 cells/ml in 0.5 ml serum-free medium) were added in suspension to the upper chamber, and medium (0.75 ml, supplemented with 10% FBS as chemoattractant) containing the same treatment was added to the bottom well. After incubation for 4 or 6 days, the non-invasive cells were removed from the upper surface of the membrane, and the invasive cells on the under surface of the membrane were stained with Diff-Quick staining kit (Dade Behring, Newark, DE) and counted microscopically at 100 × magnification. Five fields per membrane were randomly selected and counted in each group. The percentage of invasive cells was calculated as the percent invasion through the matrigel membrane relative to the migration through the control membrane, as described in the manufacture's instructions. Because Cos et al. [3] reported that treatment of MCF-7 cells with melatonin for 6 days reduced cell invasion, we first confirmed his results by incubating the cells for 4 or 6 days (as described above) when examining melatonin's effect on the invasion of MCF-7/6 and MCF-7/Her2.1 cells. These results were confirmed in the more common 2-day invasion assays (data not shown). In these assays, the underside of the membrane was pre-coated with fibronectin (20 ng/ml, as chemoattractant) for 1 hour at 37° C after rehydration, and the cells were seeded at a higher density ($2\text{-}3 \times 10^6$ cells/ml) and incubated for 2 days. As similar results were obtained using these different time frames, we chose to employ the 2-day

invasion assays in our subsequent experiments.

Preparation of conditioned medium. MCF-7/6 or MCF-7/CXCR4 cells were cultured in DMEM/F12 (1:1) or RPMI-1640 (respectively) supplemented with 10% FBS until confluency. The cells were washed with PBS three times, and then incubated in serum-free medium containing the appropriate treatments. The conditioned medium was collected and an equal amount of the medium (0.5 ml) was concentrated approximately 10-fold by centrifugation at 14,000 x g using Microcon YM-30 columns (Millipore, Billerica, MA). The volume of the concentrated medium was measured, and a normalized volume of it (15 μl) was stored at -70º C until used for Western blot analysis or gelatin zymography.

Gelatin Zymography. The activity of MMP-2 and MMP-9 in the conditioned medium was determined by gelatin zymography. Briefly, the conditioned medium (15 μl per sample) was mixed with an equal volume of 2 × SDS sample buffer (Invitrogen, Carlsbad, CA) and subjected under non-reducing conditions to SDS-polyacrylamide gel polymerized with 1 mg/mL gelatin. Following electrophoresis, the gels were incubated in a renaturing buffer (2.5% Triton X-100) with gentle agitation to remove SDS and then incubated in a developing buffer [50 mM Tris-HCl buffer (pH 7.4), 10 mM $CaCl_2$] overnight at 37° C. Gels were then stained with SimplyBlue Safestain (Invitrogen, Carlsbad, CA) and destained in the Gel-Drying solution (Invitrogen, Carlsbad, CA). Gelatinase activity was visualized as clear bands against the blue-stained background. Molecular sizes were determined from mobility using gelatin zymography standards (Chemicon, CA).

Statistical Analysis. Data are represented as the mean $\pm$ the standard error of the mean. The statistical significance at 95% confidence level was determined by one-way ANOVA followed by a Student-Newman-Keul's post-hoc test analysis using the Statview software.

Results

Characterization of MT1 expression and melatonin-responsiveness of a panel of human breast cancer cells with various invasive potentials. Three invasive MCF-7 variants were used in our following studies: the MCF-7/6, MCF-7/Her2.1 and MCF-7/CXCR4 cell lines. Among these three cell lines, MCF-7/Her2.1 is highly invasive as compared to parental MCF-7 cells (6-fold elevated invasion); MCF-7/6 and MCF-7/CXCR4 cells are moderately to highly invasive as compared to MCF-7 cells showing changes in invasive capacity by 2.6- and 4.9-fold, respectively (Figure 1a). Western blot analysis demonstrated that MT1 is expressed in MCF-7, MCF-7/6, MCF-7/Her2.1, and MCF-7/CXCR4 cells, although MCF-7/6 and MCF-7/CXCR4 cells exhibit lower levels of MT1 protein than MCF-7 and MCF-7/Her2.1 cells (Figure 1b). In addition, melatonin treatment after 6 days significantly suppressed cell growth (by 30-40%) in all cells examined except the ERα-negative MDA-MB-231 cells where no growth inhibition was observed (Figure 1c).

Phosphorylation of ERK1/2 and p38 MAPKs is up-regulated in human breast cancer cells with elevated expression of the Her2/neu or CXCR4. Expression of the Her2/neu and CXCR4 receptor was examined by Western blot analysis. MCF-7/Her2.1 cells showed markedly enhanced expression of Her2/neu as compared to MCF-7/vec cells; Interestingly, Her2/neu expression was also elevated in MCF-7/6 cells, as compared to their parental cell line MCF-7/AZ. (Figure 2a, top). In addition, CXCR4 expression is elevated by approximately 2-fold in MCF-7/CXCR4 cells as compared to the MCF-7/pcDNA3 cell line (Figure 2b).

To further investigate if Her2/neu overexpression leads to up-regulation of downstream MAPKs, we examined the phosphorylation of ERK1/2 and p38 MAPK by Western blot analysis.

Phosphorylation of ERK1/2 was elevated in MCF-7/Her2.1, MCF-7/CXCR4 and MCF-7/6 cells by 2.8-, 2.4- and 1.2-fold, respectively, as compared to their respective control cells (Figure 2a, middle). Moreover, phosphorylation of p38 MAPK (Figure 2a, bottom) was markedly elevated in MCF-7/CXCR4 and MCF-7/Her2.1 cells (5.8- and 6.5-fold, respectively), as compared to their respective control cell lines. The MCF-7/6 cells also exhibited increased p38 phosphorylation (2.4-fold), as compared to MCF-7/AZ cells.

Melatonin represses breast cancer cell invasion. Here, we examined the effect of melatonin on the *in vitro* invasion of MCF-7/6, MCF-7/Her2.1 and MCF-7/CXCR4 cells by matrigel invasion chamber assays. Melatonin significantly repressed the invasive potential of all three cell lines. In MCF-7/6 cells, melatonin (10^{-9} M) significantly suppressed cell invasion by 32% by day 4 and 71% by day 6 (control set as 100%). The same concentration of melatonin inhibited the invasion of MCF-7/Her2.1 cells by 72% by day 4, and 62% by day 6 (Figure 3a). In MCF-7/CXCR4 cells, treatment with 10^{-9} M melatonin for 2 days significantly decreased cell invasion by 66% (Figure 3b).

According to previous studies, invasion of CXCR4-expressing breast cancer cells is enhanced in response to SDF-1/CXCL12 [33]. To investigate whether melatonin inhibits SDF-1-induced invasion of MCF-7/CXCR4 cells, SDF-1 (300 ng/ml) was added to the bottom well of the matrigel invasion chambers as a chemo-attractant. SDF-1 increased the invasive potential of MCF-7/CXCR4 cells by 100% as compared to the non-stimulated control (no SDF-1 added to the bottom well and diluent-treated, set as 100%), and this effect was completely blocked by melatonin administration (Figure 3b).

MT1 receptor mediates melatonin's action on breast cancer cell invasion. We have previously reported that the MT1 but not the MT2 receptor is expressed in MCF-7 cells [32], and

that MT1 mediates melatonin's growth-suppressive effect in MCF-7 cells [32, 34, 35]. We further examined if the anti-invasive effect of melatonin is also mediated through the MT1 melatonin receptor. As shown in Figure 3c, melatonin treatment induced a significantly enhanced inhibition of cell invasion (73%) in cells transiently transfected with and overexpress MT1, as compared to sham-tranfected and vector-transfected cells (approximately 40% inhibition). Moreover, pre-treatment of cells with Luzindole, an MT1/MT2 antagonist, for 15 minutes prior to the addition of melatonin, completely abrogated melatonin's anti-invasive effect (Figure 3d). These results suggest that the anti-invasive action of melatonin is mediated via the MT1 receptor.

Melatonin suppresses the expression and activity of MMP-2 and MMP-9. To investigate whether melatonin regulates breast cancer cell invasion by altering the expression of matrix metalloproteinases, particularly MMP-2 and MMP-9, we examined the effect of melatonin on MMP-2 and MMP-9 protein expression in the conditioned medium using MCF-7/6 cells. As determined by Western blot analysis, expression of MMP-9 and MMP-2 was reduced in response to melatonin treatment (10^{-9} M) (Figure 4a).

We further examined the effect of melatonin on the activity of MMP-2 and MMP-9 by gelatin zymography. As shown in Figure 4b, a significant 60% decrease in the activity of MMP-9 was observed in cells treated with melatonin (10^{-9} M) for 48h (control set as 100%). Similarly, melatonin also induces a significant decrease in MMP-2 activity (70%) at 48 h.

Previous studies have shown that one of the mechanisms underlying SDF-1-induced cancer cell invasion is activation of MMP-9 and MMP-2 [33]. Therefore, we further investigated whether melatonin suppresses SDF-1-induced MMP-9 and MMP-2 activity. As determined by gelatin zymography, MMP-9 activity was significantly increased (1.5-fold) after administration of SDF-1 (100 ng/ml) for 24 hours as compared to diluent-treated control (set as 1 fold). The

SDF-1-mediated induction of MMP-9 activity was completely blocked by simultaneous treatment with melatonin (Figure 4c). However, melatonin did not block SDF-1-induced MMP-2 activity (data not shown).

Melatonin's anti-invasive actions are mediated through the p38 MAPK pathway. To investigate if the MAPKs, ERK1/2 and p38, play a role in melatonin regulation of breast cancer invasion, Western blot analyses were performed to determine if melatonin regulates the phosphorylation of ERK1/2 and p38 MAPK. Our results demonstrated that melatonin treatment (10^{-9} M) significantly suppressed p38 phosphorylation in MCF-7/6 cells (Figure 5a) and in MCF-7/Her2.1 cells (data not shown). The melatonin-repression of p38 phosphorylation was abrogated by a 45-minute pre-treatment with H89 (10 μM), a PKA inhibitor, suggesting that the effect of melatonin on p38 phosphorylation is mediated through PKA (Figure 5c). Furthermore, a robust induction of p38 phosphorylation was observed in MCF-7/CXCR4 cells after 2-minute stimulation with SDF-1 (100 ng/ml), which was blocked by a 5-minute pre-treatment with melatonin (Figure 5b). Conversely, melatonin treatment did not affect the phosphorylation of ERK1/2 in MCF-7/6 cells (data not shown), suggesting that the suppressive effect of melatonin on p38 phosphorylation is specific.

We subsequently conducted matrigel invasion chamber assays to determine if SB230580, a p38 inhibitor, could mimic melatonin's action on the invasion of MCF-7/6 cells. Treatment of MCF-7/6 cells with melatonin (10^{-9} M) induced a significant 50% reduction in the invasive potential of MCF-7/6 cells, as compared to diluent-treated controls (ETOH, set as 100%). Similarly, SB230580 significantly inhibited the invasion of MCF-7/6 cells by 50% compared to diluent-treated group (DMSO). (Figure 5d).

The activity of p38 MAPK is regulated by the upstream kinase, MKK6 [36]. To investigate if

up-regulation of p38 activity by transfection with a constitutively-active MKK6 DNA construct (CAMKK6b) could reverse melatonin's effect on MCF-7/Her2.1 cell invasion, we conducted invasion assays and phosphorylation analyses of p38 by Western blot analyses. As demonstrated in Figure 5e, p38 phosphorylation was up-regulated in CAMKK6b-transfected cells. In vector-transfected cells, melatonin treatment significantly suppressed cell invasion by 70%, as compared with ethanol-treated control (set as 100%). In contrast, melatonin did not have significant effect on the invasive potential of CAMKK6b-transfected cells (Figure 5e).

CAMKK6 up-regulates MMP-9 mRNA expression and ETS1 phosphorylation. MMP-9 has been shown to be a target gene of the transcription factor ETS1 [37]. There is evidence that p38 can phosphorylate and potentially activate ETS1 [38]. To investigate if p38 regulates the transcription of MMP-9, real time RT-PCR analyses were performed to determine the mRNA expression of MMP-9 in MCF-7 cells transiently-transfected with CAMKK6b. As shown in Figure 6a, MMP-9 mRNA levels were significantly increased in CAMKK6b-transfected cells as compared to vector-transfected cells. This induction of MMP-9 mRNA expression was accompanied by an increase in the phosphorylation of ETS1 as determined by Western blot analyses (Figure 6b).

Discussion

To define the role of melatonin in breast cancer cell invasion and metastasis, instead of using the poorly-invasive MCF-7 breast cancer cells, we utilized three invasive breast cancer cell clones that were derived from the parental MCF-7 cells, the MCF-7/6, MCF-7/Her2.1, and MCF-7/CXCR4 cells. These cells were selected because they are highly invasive and metastatic

as compared to the parental MCF-7 cells while still retaining many characteristics of the parental MCF-7 cells.

As shown in Figure 1, MT1 is expressed at protein level in all three cell lines, with, MCF-7/Her2.1 cells exhibiting highest level of MT1. In addition, both MCF-7/6 and MCF-7/Her2.1 cells are responsive to melatonin's growth-suppressive effect, with melatonin (10^{-9} M) showing equal effectiveness in suppressing the growth of these cells as it does in parental MCF-7 cells (Figure 1). When it comes to the ability to invade and metastasize, MCF-7/6, MCF-7/Her2.1 and MCF-7/CXCR4 cells all exhibited elevated invasive potential as compared to the parental MCF-7 cells.

The enhanced invasive potential in MCF-7/Her2.1 and MCF-7/6 cells, respectively, may be a direct result of Her2/neu overexpression. According to previous reports, overexpression of Her2/neu and constitutive-activation of the Her2/neu signaling pathway can constitutively activate MAPK [11], while promoting breast cancer cell invasion and metastastasis [12-14]. As expected, expression of Her2/neu is markedly up-regulated in MCF-7/Her2.1 cells which have been transfected with the wild-type Her2/neu. Interestingly, Her2/neu is also overexpressed in MCF-7/6 cells which have been selected for metastatic potential by serial passaging in nude mice. This data suggest that up-regulation of the Her2/neu pathway may be one of the early events in the progression toward invasiveness.

Although the precise mechanisms by which the Her2/neu pathway regulates breast cancer invasion and metastasis are not yet fully understood, it has been suggested that multiple signaling pathways, including ERK1/2 and p38, act as the downstream effectors to promote the invasive potential of these breast cancer cells [15]. Therefore it is possible that, MCF-7/Her2.1 and MCF-7/6 cells acquire their invasive capacities through Her2/neu-induced constitutive-activation

of ERK1/2 and p38 MAPK. Recent studies have revealed that the chemokine receptor CXCR4 also plays a critical role in the regulation of breast cancer cell invasion and metastasis [16-18]. The molecular mechanisms underlying the action of CXCR4 on breast cancer cell invasion are currently under intense investigations. Given that activation of CXCR4 leads to activation of multiple signaling pathways including ERK1/2 and p38 in several cell types [19-21], MCF-7/CXCR4 cells may acquire their invasiveness and metastatic potential through CXCR4-mediated up-regulation of the ERK1/2 and p38 MAPK signaling pathways.

To define the role of melatonin in breast cancer cell invasion, the effects of melatonin on breast cancer cell invasion were tested on MCF-7/6, MCF-7/Her2.1, and MCF-7/CXCR4 cells by *in vitro* matrigel invasion chamber assays. In agreement with the data reported by Cos et al. [3], we have demonstrated that, melatonin, at physiological concentrations (10^{-9} M), significantly inhibits the invasion of MCF-7/6, MCF-7/Her2.1 and MCF-7/CXCR4 breast cancer cells. Furthermore, melatonin also blocked the SDF-1/CXCL12-induced invasive potential of MCF-7/CXCR4 cells. Melatonin's anti-invasive action is also reflected by its suppressive effects on the expression of MMP-2 and MMP-9, two major matrix metalloproteinases mediating the degradation of the extracellular matrix. Several reports have indicated that melatonin regulates the activity of MMP-9 during protection against ethanol-induced gastric ulcer and endometriosis [39, 40]. In our study, melatonin treatment not only reduces the protein expression, but also represses the enzymatic activity of MMP-2 and MMP-9. These results suggest that melatonin's anti-invasive action is mediated, at least in part, through diminishing the ability of breast cancer cells to degrade the components of extracellular matrix, by modulating MMP-2 and MMP-9 expression and activity. Moreover, previous studies have shown that the activities of MMPs are regulated by a group of endogenous molecules, namely, the tissue inhibitors of matrix

metalloproteinase (TIMPs) [41]. According to our un-published data from cDNA microarray analysis, TIMP-3 expression is up-regulated in melatonin-treated MCF-7 cells overexpressing the MT1 receptor, suggesting that TIMP-3 may be another target of melatonin's anti-invasive action.

To delineate the signaling pathway(s) utilized by melatonin to affect the invasive capacity of breast cancer cells, we first investigated whether melatonin's anti-invasive action is an MT1 receptor-mediated event. We have previously reported that only the MT1 but not the MT2 receptor is expressed in MCF-7 cells [32], and that MT1 mediates melatonin's growth-suppressive effect in MCF-7 cells [32, 34, 35]. It appears that MT1 is also involved melatonin's anti-invasive actions. As demonstrated in our studies, enhanced MT1 expression potentiated melatonin-mediated inhibition of cell invasion in MCF-7/6 cells. In contrast, this inhibitory effect was abolished by pre-treatment of the cells with Luzindole, an MT1/MT2 antagonist. The above data suggest that, melatonin's anti-invasive action is mediated, at least in part, through the G-protein-coupled MT1 receptor.

A well-established key intracellular signaling pathway downstream of the MT1 receptor is the cAMP/PKA pathway. As we previously reported, the MT1 receptor, by coupling to Gi-proteins upon melatonin binding, blocks the accumulation of cAMP, and potentially, inhibits the activity of PKA in MCF-7 cell [31]. Given that the cAMP/PKA pathway cross talks with diverse signaling pathways including the ERK1/2 and p38 MAPK, and inspired by our observation that constitutive-activation of ERK1/2 and p38 MAPK appears to be the driving force to promote the invasion of MCF-7/6, MCF-7/CXCR4 and MCF-7/Her2.1 cells, we hypothesized that melatonin inhibits the invasion of these cells by interacting with the ERK1/2 and p38 MAPK pathways.

Melatonin regulation of MAPKs has been observed in several cell types including the COS-7 cell and brain tissue, where melatonin has been reported to increase the phosphorylation of

ERK1/2 and JNK (c-Jun N-terminus kinase) [42, 43]. In human adult mesenychymal stem cells, the phosphorylation of MEK1/2 and ERK1/2 is increased by acute melatonin exposure, but inhibited by the long-term melatonin administration [44]. However, our studies show that, the anti-invasive action of melatonin is mediated through the p38 MAPK pathway, not the ERK1/2 pathway, since melatonin treatment (10^{-9} M) repressed p38 phosphorylation in both MCF-7/6 (Figure 5a) and MCF-7/Her2.1 (data not shown) breast cancer cells, but had no effect on ERK1/2 phosphorylation (data not shown). Furthermore, the p38 inhibitor, SB230580, mimicked melatonin's effect on cell invasion in MCF-7/6 cells, significantly reducing the invasive potential of these cells (Figure 5d), while expression of the constitutively-active MKK6 upstream of p38 blocking melatonin's effect on MCF-7/Her2.1 cell invasion (Figure 5e).

Interestingly, expression of CAMKK6 did not significantly induce the invasive potential of MCF-7/Her2.1 cells as we had expected. This may be due to the hyper-activation of p38 MAPK in these Her2/neu-overexpressing cells (6.5-fold elevated phosphorylation compared to control cell line), which has reached a maximum for its stimulatory effect on cell invasion such that further elevation of p38 activity beyond the maximum does not necessarily lead to further increase in the invasive potential of these already highly-invasive breast cancer cells.

Several lines of evidence suggest that p38 MAPK regulates the expression of MMP-9 [25, 45]. We have shown that expression of CAMKK6 leads to increased MMP-9 mRNA expression and elevated ETS1 phosphorylation in MCF-7 cells (Figure 6). Given that MMP-9 is an ETS1 target gene [37], these results suggest that the MKK6/p38 pathway may regulate MMP-9 transcription through phosphorylation and potentially activation of ETS1.

In summary, the above data suggest that melatonin, via its MT1 receptor, plays an inhibitory role in breast cancer cell invasion, possibly by specifically down-regulating the p38 MAPK

signaling pathway. Although the precise mechanism(s) underlying melatonin's action on p38 MAPK remains unknown, one possibility is that, melatonin regulation of p38 phosphorylation is mediated through Gi-protein-induced changes in cAMP level and PKA activity, since melatonin's effect on p38 phosphorylation is attenuated by PKA inhibitor H89 (Figure 5c).

In recent years, the p38 MAPK has emerged as a key signaling molecule in the regulation of cancer invasion and metastasis by modulating the expression and activity of molecules governing the degradation of extracellular matrix (i.e. urokinase plasminogen activator, MMP-2 and MMP-9) [22-25, 45]. Our studies show that phosphorylation of p38 MAPK, but not ERK1/2, is dramatically elevated in both Her2/neu-overexpressing (MCF7/6 and MCF-7/Her2.1) and CXCR4-overexpressing (MCF-7/CXCR4) cells. This data indicates that although MCF-7/6, MCF-7/Her2.1 and MCF-7/CXCR4 cells acquire their malignant phenotypes through different approaches, constitutive-activation of p38 MAPK may be a common mechanism driving the transition of these cells from the poorly/non-invasive to an invasive phenotype (Figure 7). By inhibiting p38 phosphorylation, melatonin has emerged as a promising anti-invasion factor which may be useful in future cancer therapeutics not only in prevention of breast cancer [46], but also prevention of the "non-invasive-to-invasive" transition. In addition, cancer metastasis is a multi-step event. Although our studies have clearly demonstrated that melatonin significantly inhibits breast cancer cell invasion *in vitro*, it is critical to further define the role of melatonin in regulating breast cancer metastasis *in vivo* to evaluate the clinical significance of melatonin's anti-invasive effect.

Conclusions:

In the present studies, we investigated the role of melatonin in the regulation of breast cancer cell

invasion. Our results demonstrated that melatonin, via its MT1 receptor, plays an inhibitory role in breast cancer cell invasion, possibly by specifically down-regulating the p38 MAPK signaling pathway, and the downstream activity of MMP-2 and MMP-9.

Abbreviations: ERα (estrogen receptor α); MMP (matrix metalloproteinase); TIMP (tissue inhibitors of matrix metalloproteinase); SDF-1 (stromal-derived factor-1); JNK (c-Jun N-terminus kinase).

Competing Interests

The author(s) declare that they have no competing interests.

Author's contributions:

LM participated in the overall study design, carried out the molecular studies, and drafted the manuscript. LY carried out the RT-PCR analysis. LS performed some of the Western blot analyses. FJ and MB critically revised the manuscript. SH conceived the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Figure 1. Melatonin-responsiveness and MT1 receptor expression of a panel of human breast cancer cells. (**a**) The relative invasive potential of MCF-7, MCF-7/AZ, MCF-7/6, MCF-7/Her2.1 and MCF-7/CXCR4 breast cancer cells. Data is presented as fold of control (MCF-7, set as 1). *, $P < 0.05$ vs. MCF-7 cells. (**b**) Western blot analysis of MT1 protein expression in MCF-7, MCF-7/CXCR4, MCF-7/Her2.1, and MCF-7/6 cells. GAPDH protein levels are shown as loading control. (**c**) Effect of melatonin on cell proliferation in MCF-7, MCF-7/AZ, MCF-7/6, MCF-7/Her2.1, MDA-MB-231 cells. Cells were serum-starved for 24 hours and treated with melatonin (Mlt, 10^{-9} M), or diluent (Control, 0.00001% ethanol). Cell numbers were determined on day six by hemacytometer cell count. Data is presented as percent of mean cell count in vehicle-treated cells (100%). One-way ANOVA followed by Student-Newman-Kuel's post-hoc test analyses was used to determine statistically significant differences in the cell number between melatonin and vehicle-treated groups. *, $P < 0.05$ vs. vehicle-treated cells, n = 3.

Figure 2. Elevated phosphorylation of ERK1/2 and p38 MAPK in MCF-7/CXCR4, MCF-7/6 and MCF-7/Her2.1 cells. (**a**) Western blot analysis of Her2/neu receptor (*top*), phospho-ERK1/2 (p-ERK1/2) [*middle*], and phospho-p38 (p-p38) [*bottom*] in MCF-7/vec, MCF-7/Her2.1, MCF-7/AZ, MCF-7/6, MCF-7/pcDNA3, and MCF-7/CXCR4 breast cancer cells. The band intensity of Her2/neu, phospho-p38 and phsopho-ERK1/2 was normalized to that of β-actin, total p38 and ERK1/2, respectively, and expressed as fold of control (MCF-7/vec, MCF-7/AZ, and MCF-7/pcDNA3, set as 1). (**b**) Western blot analysis of CXCR4 expression in MCF-7/pcDNA3 and MCF-7/CXCR4 cells. Levels of GAPDH are shown as loading control. The band intensity of CXCR4 was normalized to that of GAPDH, and expressed as fold of control (MCF-7/pcDNA3, set as 1).

Figure 3. Melatonin, via MT1, suppresses the invasive potential of human breast cancer cells. (**a**) Effect of melatonin on the invasion of MCF-7/6 and MCF-7/Her2.1 cells. Cells were plated onto matrigel invasion chambers after being serum-starved for 24 hours and treated with melatonin (Mlt, 10^{-9} M), or diluent (Control, 0.00001% ethanol) for 4 and 6 days. Data is represented as percent of control (100%). *, $P < 0.05$ vs. diluent-treated cells, n = 3. (**b**) Effect of melatonin on SDF-1-induced invasion of MCF-7/CXCR4 cells. Cells were treated with melatonin (Mlt, 10^{-9} M), or diluent (Control, 0.00001% ethanol) for 2 days, with or without SDF-1 in the bottom chamber as chemo-attractant [(+)/(–) SDF-1, 300 ng/ml]. Data is presented as percentage of control [(–) SDF-1, vehicle-treated cells, 100%]. *, $P < 0.05$ vs. diluent-treated cells, n = 3. (**c**) Effect of MT1 overexpression on melatonin-regulated invasion of MCF-7/6 cells. Cells were transiently transfected with either pcDNA3.1 empty vector (V) or pcDNA3.1-MT1 plasmid (MT1), or sham-transfected (P). Twenty-four hours later, cells were plated onto matrigel invasion chambers and treated with melatonin (Mlt, 10^{-9} M), or diluent (Control, 0.00001% ethanol) for 4 days. Data is presented as percentage of the sham-transfected diluent-treated control (100%). RT-PCR analysis of MT1 expression is shown to demonstrate the overexpression of MT1 in pcDNA3.1-MT1-transfected cells. *, $P < 0.05$ vs. sham-transfected vehicle-treated control cells (P-Control); **, $P < 0.05$ vs. vector-transfected melatonin-treated cells (V-Mlt), n = 3. (**d**) Effect of Luzindole on melatonin-regulated invasion of MCF-7/6 cells. Cells were treated with diluent (Control, 0.00001% ethanol), melatonin (Mlt, 10^{-9} M), Luzindole (LZD, 10^{-8} M), or Luzindole (10^{-8} M) for 15 minutes followed by melatonin (10^{-9} M) treatment [LZD + Mlt] for 2 days. Data is presented as percentage of ethanol-treated control (100%). *, $P < 0.05$ vs. vehicle-treated control cells; **, $P < 0.05$ vs. melatonin-treated cells, n = 3. One-way ANOVA followed by

Student-Newman-Kuel's post-hoc test analyses was used to determine statistically significant differences in the percentage of invasive cells among different treatment groups.

Figure 4. Melatonin suppresses the expression and activity of MMP-2 and MMP-9 in human breast cancer cells. Conditioned medium was collected and concentrated as described in Materials and methods. (**a**) Melatonin's effect on the protein expression of MMP-2 and MMP-9. MCF-7/6 cells were treated with melatonin (Mlt, 10^{-9} M) or diluent (Control, 0.00001% ethanol) for 48h. Expression of MMP-2 and MMP-9 in the conditioned medium was analyzed by Western blot analysis using anti-MMP-2 and anti-MMP-9 antibody. (**b**) The effect of melatonin on the activity of MMP-2 and MMP-9. MCF-7/6 cells were treated with melatonin (Mlt, 10^{-9} M) or diluent (Control, 0.00001% ethanol) for 48h. The gelatinase activity of MMP-9 and MMP-2 in the conditioned medium was determined by gelatin zymography. The band intensity of MMP-9 and MMP-2, respectively, is presented in the graph as percent of control (set as 100%). *, $P < 0.05$ vs. diluent-treated control, n = 4. (**c**) Effect of melatonin on SDF-1-induced activity of MMP-9 in MCF-7/CXCR4 cells. MCF-7/CXCR4 cells were treated with diluent (control, 0.00001% ethanol), SDF-1 (100 ng/ml), or melatonin (10^{-9} M) and SDF-1 (100 ng/ml) simultaneously [Mlt + SDF-1], for 24h. The gelatinase activity of MMP-9 in the conditioned medium was determined by gelatin zymography. The band intensity of MMP-9 is presented in the graph as percent of control (set as 100%). *, $P < 0.05$ vs. diluent-treated control; **, $P < 0.05$ vs. SDF-1-treated group, n = 3. Figures shown in (**a**), (**b**) and (**c**) are representative Western blots or gelatin zymograms of three independent studies, respectively.

Figure 5. The anti-invasive effects of melatonin are mediated through the p38 MAPK signaling pathway. **(a)** The effect of melatonin on the phosphorylation of p38 MAPK. MCF-7/6 cells were serum-starved for 24h, and treated with diluent (Control, 0.00001% ethanol) or melatonin (Mlt,

10^{-9} M) for 30 min in fresh medium supplemented with 10% FBS. Expression of phospho-p38 (p-p38) and total p38 (p38) was analyzed by Western blot analysis. (**b**) The effect of melatonin on SDF-1-induced p38 phosphorylation. MCF-7/CXCR4 cells were serum-starved for 48h, pre-treated with melatonin (10^{-9} M) or diluent (0.00001% ethanol) for 5min followed by SDF-1 (100 ng/ml) stimulation for 2 min [Mlt + SDF-1]. (**c**) The effect of H89 on melatonin-regulation of p38 phosphorylation. MCF-7/6 cells were serum-starved for 24h, and pre-treated with H89 (10 μM) for 45 min followed by a 30-minute treatment with melatonin (10^{-9} M). Figures shown in (**a**), (**b**), and (**c**) are representative Western blots from three independent studies, respectively. In (**a**) and (**c**) The band intensity of phospho-p38 was normalized to that of total p38, and expressed in the graph as percent of control (set as 100%). *, $P < 0.05$ vs. diluent-treated control, n = 3. (**d**) The effect of SB230580 on the invasive potential of MCF-7/6 breast cancer cells. MCF-7/6 cells were plated onto matrigel invasion chambers after 24-hour serum-starvation and incubated in the medium containing diluent (ETOH, 0.00001% ethanol; DMSO), melatonin (Mlt, 10^{-9} M), or SB230580 (20 μM). Data is presented as percentage of ethanol-treated control (100%). *, $P <$ 0.05 vs. vehicle-treated control cells. (**e**) Effect of CAMKK6b on melatonin's anti-invasive action. MCF-7/Her2.1 cells were transiently transfected with empty vector (V) or CAMKK6b plasmid for 24 h, plated onto matrigel invasion chambers and treated with diluent (Control, 0.00001% ethanol) or melatonin (Mlt, 10^{-9} M) for 2 days. Data is presented as percentage of vector-transfected, diluent-treated control (100%). *, $P < 0.05$ vs. vehicle-treated control cells. Phosphorylation of p38 MAPK (p-p38) in vector- and CAMKK6b-transfected cells was analyzed by Western blot analysis. Expression of total p38 (p38) was used as loading control.

Figure 6. CAMKK6 up-regulates MMP-9 mRNA expression and ETS1 phosphorylation. (**a**) Effect of CAMKK6b on MMP-9 mRNA expression. MCF-7 cells were transiently transfected with empty vector or CAMKK6b plasmid for 24 h. Real-time RT-PCR analyses were performed to examine the expression of MMP-9 mRNA in vector- and CAMKK6b-transfected MCF-7 cells. The MMP-9 mRNA expression was normalized to levels of 18s ribosomal RNA. *, $P < 0.05$ vs. vector-transfected cells. Figure shown is a representative real time RT-PCR analysis from three independent studies. (**b**) Effect of CAMKK6b on ETS-1 phosphorylation. MCF-7 cells were transiently transfected with empty vector or CAMKK6b plasmid. Phosphorylation of ETS1 (p-ETS1) in vector- and CAMKK6b-transfected cells was analyzed by Western blot analysis. Expression of total ETS1 (t-ETS1) was used as loading control. Figure shown here is a representative Western blot from three independent studies.

Figure 7. Model of melatonin regulation of breast cancer cell invasion. Ligand-dependent activation of the membrane-associated G-protein-coupled receptor MT1 leads to coupling of Gi2 protein to MT1 receptor. As a result, the Gαi2 subunit dissociates from the Gβγ subunits and inhibits the activity of adenylcyclase (AC), which leads to decrease in the intracellular level of cAMP and inhibition of PKA activity. The cAMP/PKA pathway cross talks with the p38 pathway through PKA. In response to the reduced cAMP level, activity of p38 is suppressed, which further causes down-regulation of MMP-9 expression via repression of ETS1 transcriptional activity, and potentially, down-regulation of MMP-2 transcription.

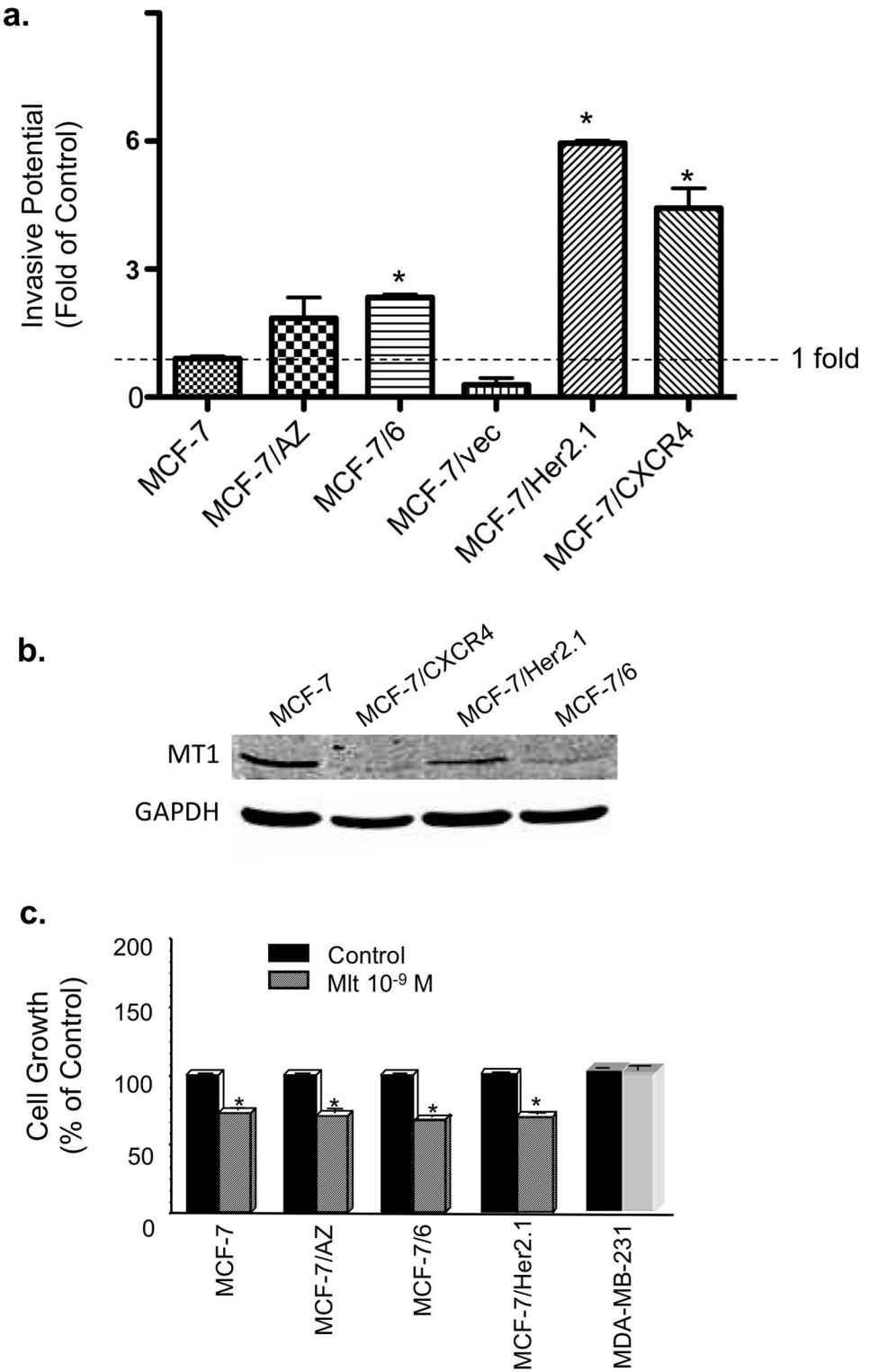

a.
Invasive Potential
(Fold of Control)
6
3
0
1 fold
*
*
*
MCF-7
MCF-7/AZ
MCF-7/6
MCF-7/vec
MCF-7/Her2.1
MCF-7/CXCR4
b.
MCF-7
MCF-7/CXCR4
MCF-7/Her2.1
MCF-7/6
MT1
GAPDH
c.
Cell Growth
(% of Control)
200
150
100
50
0
Control
Mlt 10-9 M
*
MCF-7
MCF-7/AZ
MCF-7/6
MCF-7/Her2.1
MDA-MB-231

Figure 1

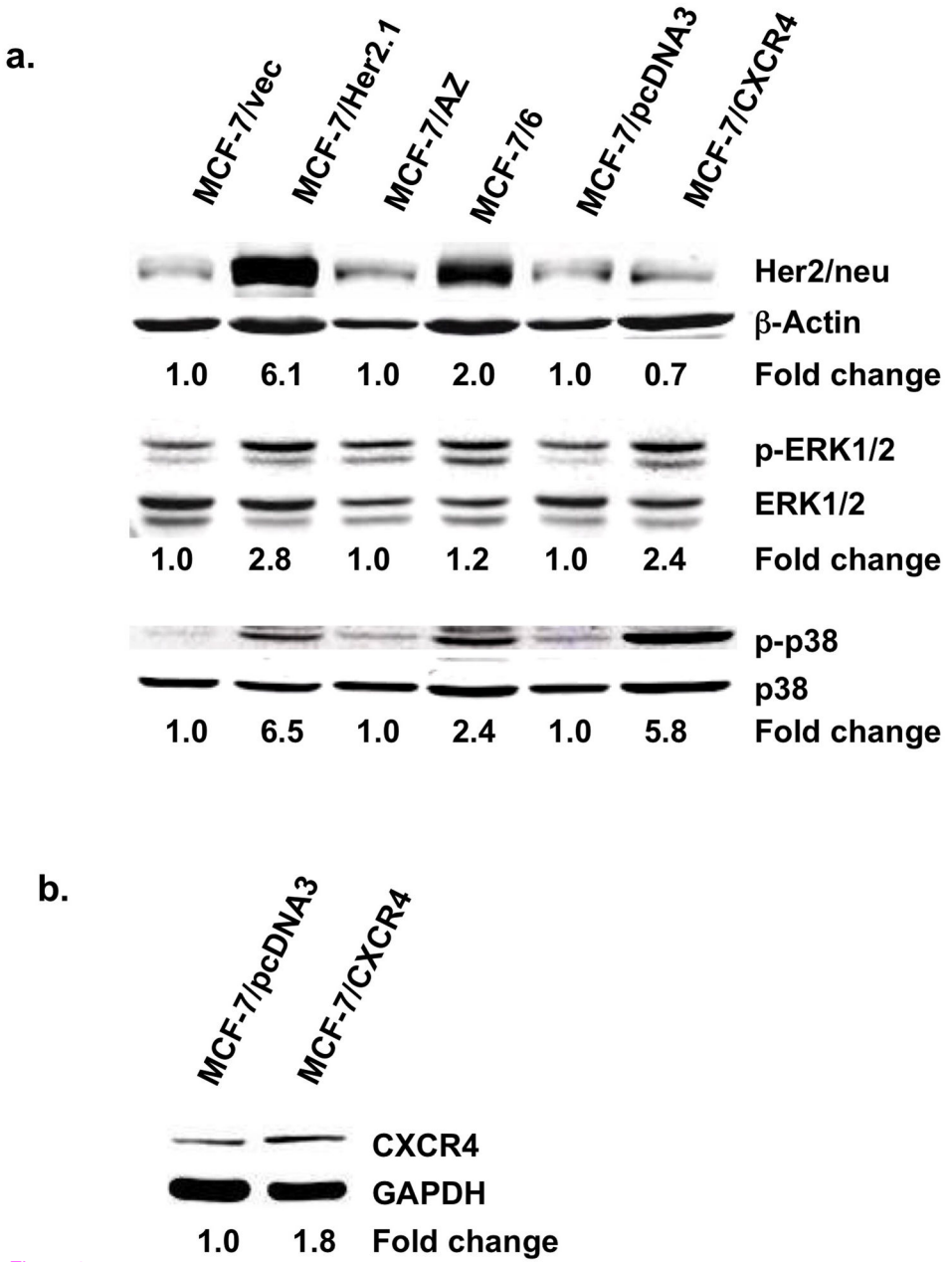
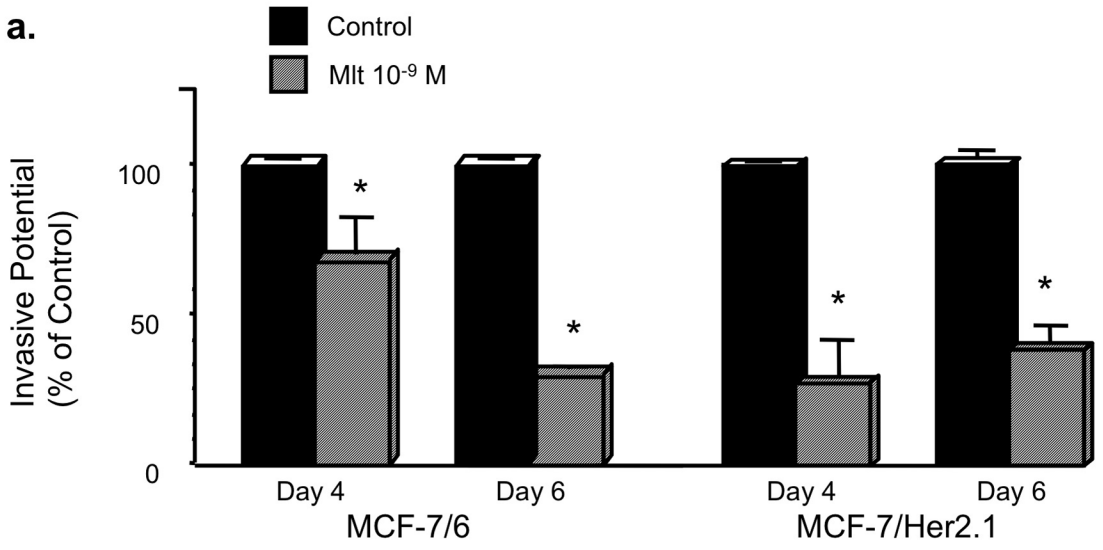
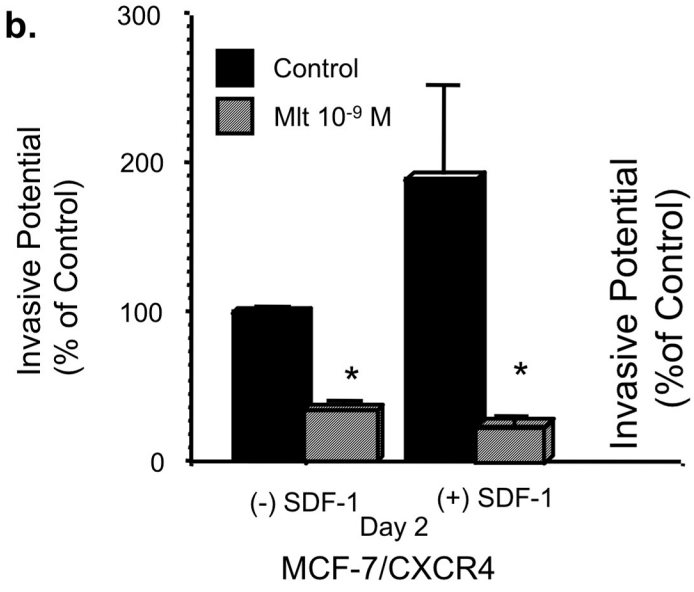
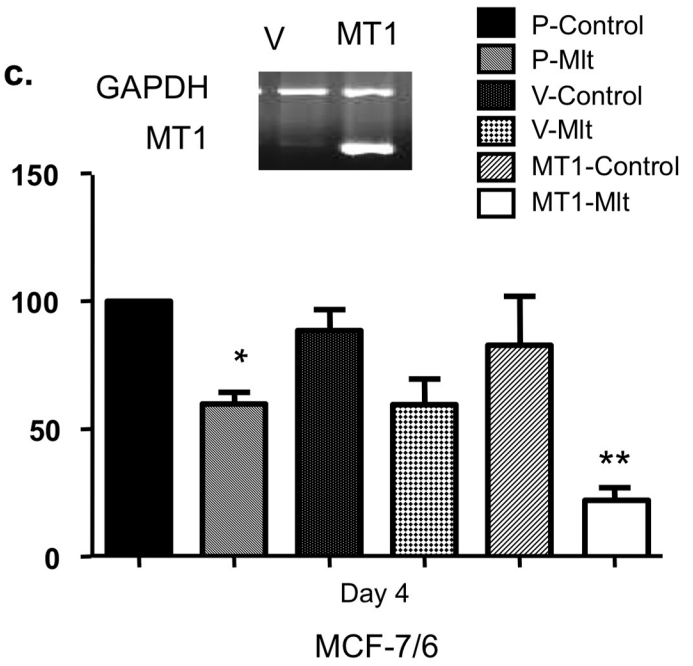

a.
MCF-7/vec
MCF-7/Her2.1
MCF-7/AZ
MCF-7/6
MCF-7/pcDNA3
MCF-7/CXCR4
Her2/neu
β-Actin
1.0 6.1 1.0 2.0 1.0 0.7 Fold change
p-ERK1/2
ERK1/2
1.0 2.8 1.0 1.2 1.0 2.4 Fold change
p-p38
p38
1.0 6.5 1.0 2.4 1.0 5.8 Fold change
b.
MCF-7/pcDNA3
MCF-7/CXCR4
CXCR4
GAPDH
1.0 1.8 Fold change

Figure 2


a.
Control
Mlt 10-9 M
Invasive Potential
(% of Control)
100
50
0
*
*
*
*
Day 4
Day 6
Day 4
Day 6
MCF-7/6
MCF-7/Her2.1


b.
300
200
100
0
Control
Mlt 10-9 M
Invasive Potential
(% of Control)
*
*
(-) SDF-1
(+) SDF-1
Day 2
MCF-7/CXCR4

Invasive Potential
(%of Control)


c.
V
MT1
GAPDH
MT1
P-Control
P-Mlt
V-Control
V-Mlt
MT1-Control
MT1-Mlt
150
100
50
0
*
**
Day 4
MCF-7/6

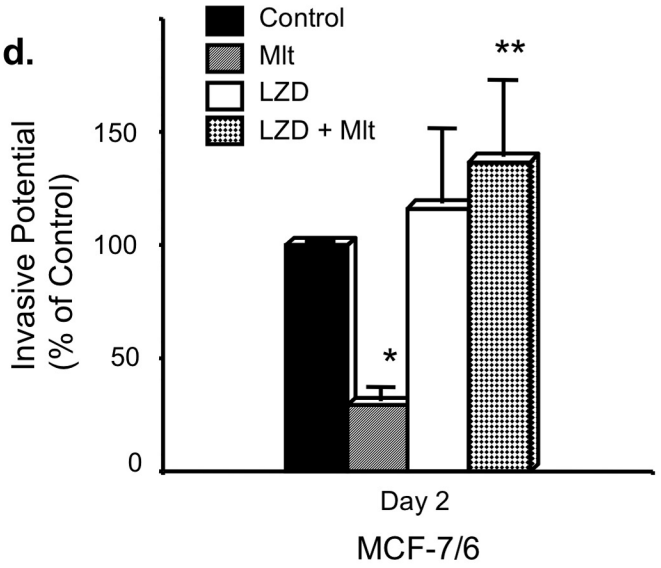

d.
Control
Mlt
LZD
LZD + Mlt
Invasive Potential
(% of Control)
150
100
50
0
*
**
Day 2
MCF-7/6

Figure 3

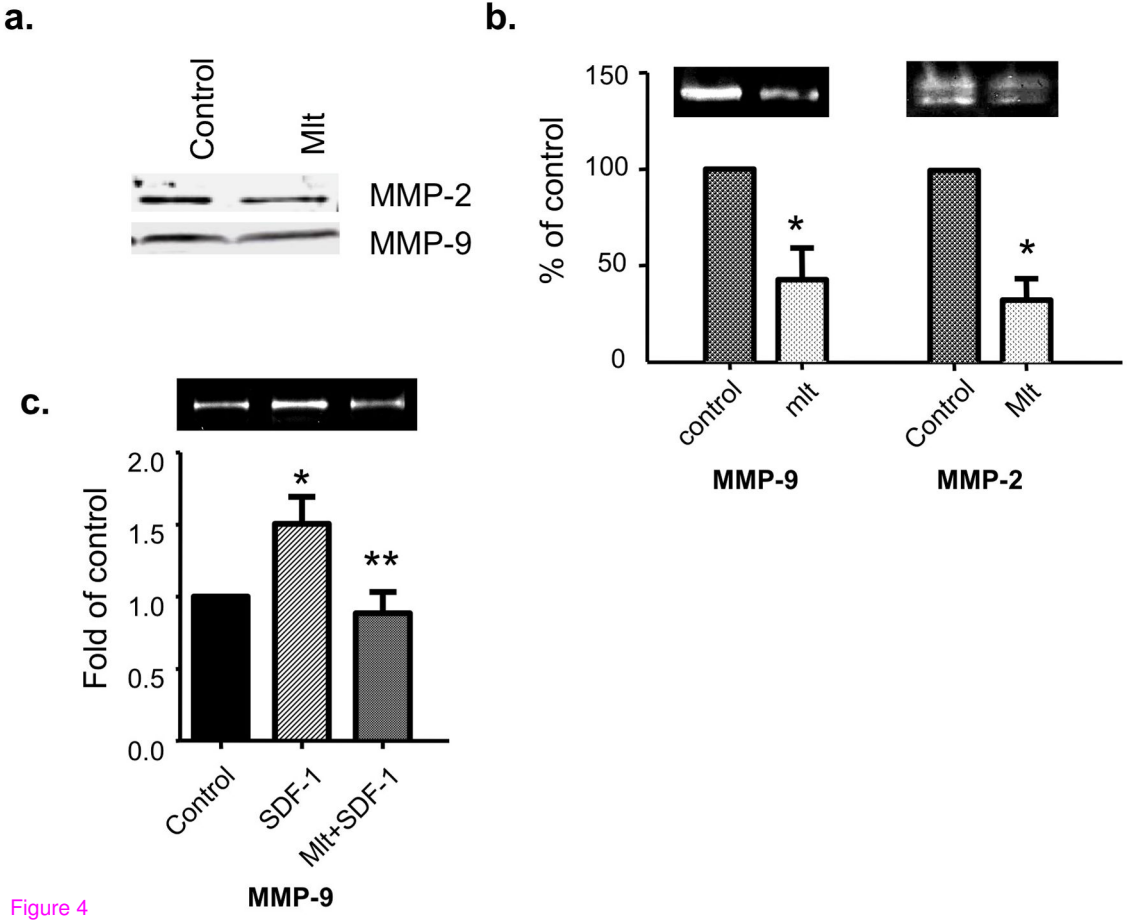

a.
Control
Mlt
MMP-2
MMP-9
b.
150
100
50
0
% of control
*
*
control
mlt
Control
Mlt
MMP-9
MMP-2
c.
2.0
1.5
1.0
0.5
0.0
Fold of control
*
**
Control
SDF-1
Mlt+SDF-1
MMP-9

Figure 4

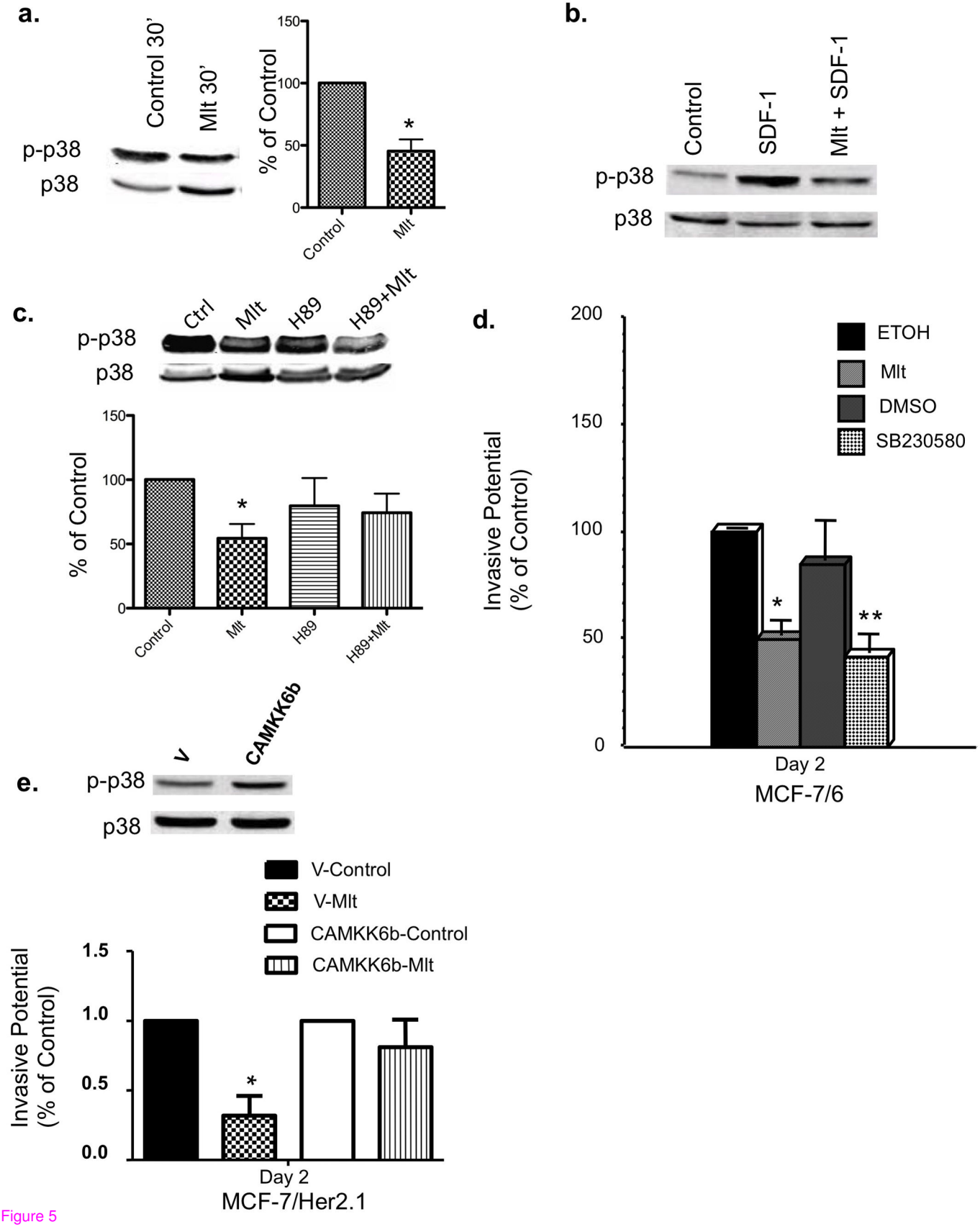
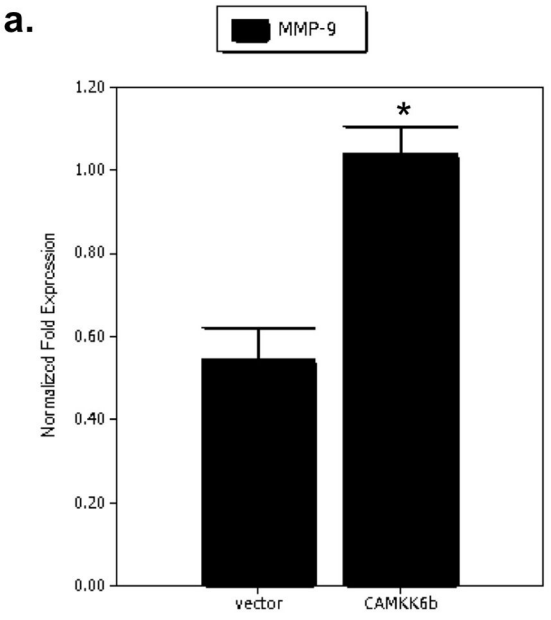

a.
Control 30'
Mlt 30'
p-p38
p38
% of Control
150
100
50
0
*
Control
Mlt
b.
Control
SDF-1
Mlt + SDF-1
p-p38
p38
c.
Ctrl
Mlt
H89
H89+Mlt
p-p38
p38
% of Control
150
100
50
0
*
Control
Mlt
H89
H89+Mlt
d.
Invasive Potential
(% of Control)
200
150
100
50
0
ETOH
Mlt
DMSO
SB230580
*
**
Day 2
MCF-7/6
e.
V
CAMKK6b
p-p38
p38
V-Control
V-Mlt
CAMKK6b-Control
CAMKK6b-Mlt
Invasive Potential
(% of Control)
1.5
1.0
0.5
0.0
*
Day 2
MCF-7/Her2.1

Figure 5


a.
MMP-9
Normalized Fold Expression
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
vector
CAMKK6b

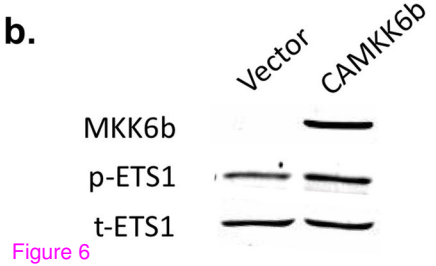

b.
Vector
CAMKK6b
MKK6b
p-ETS1
t-ETS1

Figure 6

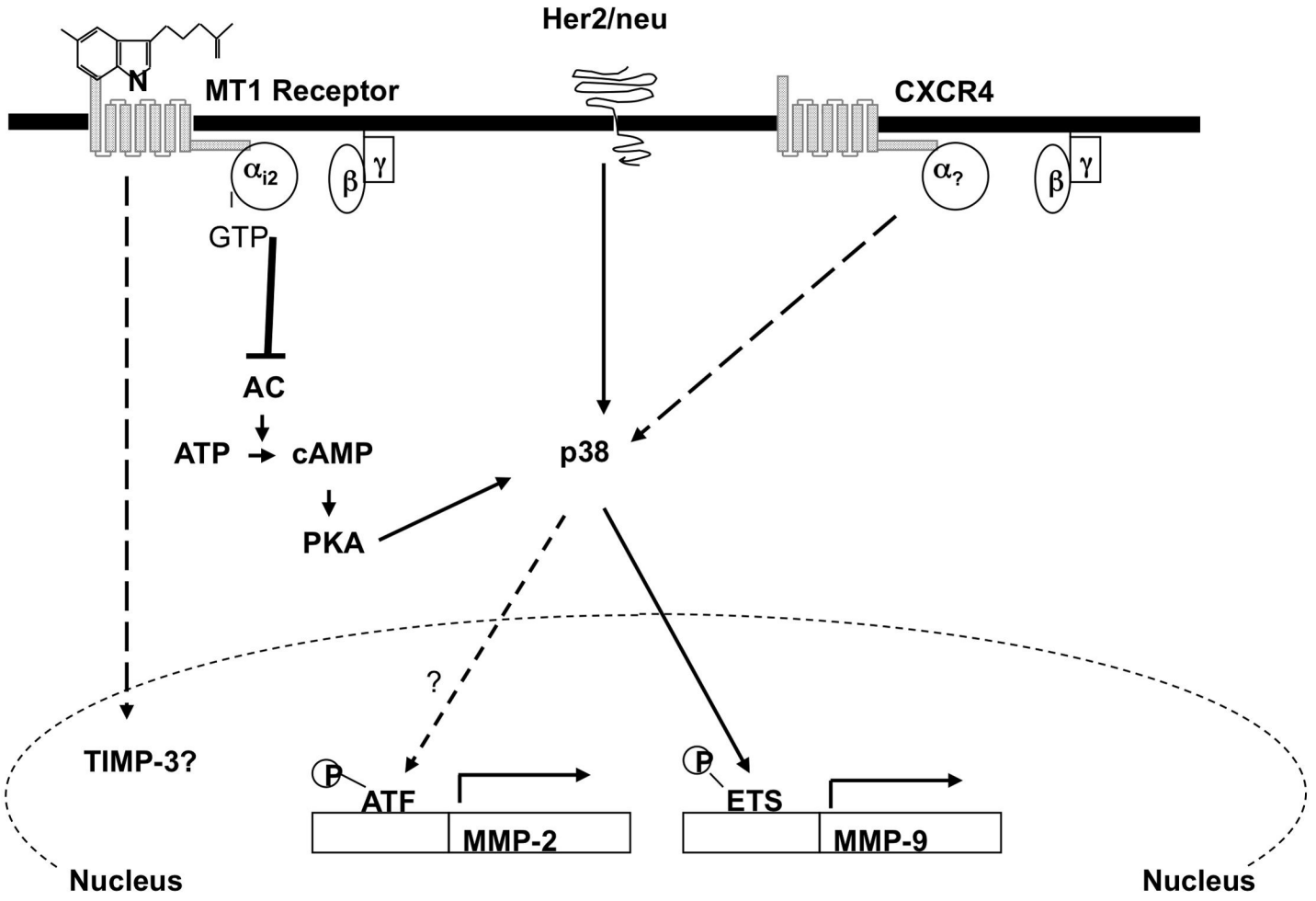

Her2/neu
N
MT1 Receptor
CXCR4
α_{i2}
β
γ
$\alpha_?$
β
γ
GTP
AC
ATP
cAMP
PKA
p38
?
TIMP-3?
P
ATF
MMP-2
P
ETS
MMP-9
Nucleus
Nucleus

Figure 7