

Efficacy of melatonin, IL-25 and siIL-17B in tumorigenesis-associated properties of breast cancer cell lines



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ARTICLE INFO

Article history:

Received 20 March 2017

Received in revised form 7 June 2017

Accepted 13 June 2017

Available online 15 June 2017

Keywords:

Breast cancer

Melatonin

Interleukin-25

Interleukin-17E

Interleukin-17B

Apoptosis

VEGF

ABSTRACT

Mammary tumorigenesis can be modulated by melatonin, which has oncostatic action mediated by multiple mechanisms, including the inhibition of the activity of transcription factors such as NF- κ B and modulation of interleukins (ILs) expression. IL-25 is an active cytokine that induces apoptosis in tumor cells due to differential expression of its receptor (IL-17RB). IL-17B competes with IL-25 for binding to IL-17RB in tumor cells, promoting tumorigenesis. This study purpose is to address the possibility of engaging IL-25/IL-17RB signaling to enhance the effect of melatonin on breast cancer cells. Breast cancer cell lines were cultured monolayers and 3D structures and treated with melatonin, IL-25, siIL-17B, each alone or in combination. Cell viability, gene and protein expression of caspase-3, cleaved caspase-3 and VEGF-A were performed by qPCR and immunofluorescence. In addition, an apoptosis membrane array was performed in metastatic cells. Treatments with melatonin and IL-25 significantly reduced tumor cells viability at 1 mM and 1 ng/mL, respectively, but did not alter cell viability of a non-tumorigenic epithelial cell line (MCF-10A). All treatments, alone and combined, significantly increased cleaved caspase-3 in tumor cells grown as monolayers and 3D structures ($p < 0.05$). Semi-quantitative analysis of apoptosis pathway proteins showed an increase of CYTO-C, DR6, IGFBP-3, IGFBP-5, IGFBP-6, IGF-1, IGF-1R, Livin, P21, P53, TNFR1, XIAP and hTRA proteins and reduction of caspase-3 ($p < 0.05$) after melatonin treatment. All treatments reduced VEGF-A protein expression in tumor cells ($p < 0.05$). Our results suggest therapeutic potential, with oncostatic effectiveness, pro-apoptotic and anti-angiogenic properties for melatonin and IL-25-driven signaling in breast cancer cells.

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1. Introduction

The breast tumor microenvironment is composed of several cell types, including inflammatory and endothelial cells, fibroblasts and adipocytes, among others [1–3]. Mutual interactions between malignant epithelial cells and the surrounding microenvironment, in part mediated by cytokines and their receptors, modulate the behavior of malignant cells and drive tumor progression [3–6]. Thus, it is important to identify these microenvironmental mediators of tumor progression as well as the strategies to successfully target them [7].

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Melatonin (*N*-acetyl-5-methoxytryptamine), an endogenous molecule, is a conserved indolamine synthesized from tryptophan which is mainly produced by the pineal gland and other nonendocrine organs [8]. Different mechanisms of melatonin anti-tumor action have been proposed, regulating a variety of cellular pathways. These include oncostatic and anti-inflammatory effects, increased local immunity [8–11], cellular antioxidant capacity [12,13], upregulation of tumor suppressor gene expression [14], control of cell differentiation and proliferation [15,16], and induction of apoptosis [17]. Melatonin also blocks the activity of transcription factors such as nuclear factor- κ B (NF- κ B), inhibiting the expression of cytokines, metalloproteinases and the vascular endothelial growth factor (VEGF) [18–20]. Furthermore, Alvarez-García et al. [10] and Ordoñez et al. [8] have shown that melatonin also modulates pro-inflammatory cytokines, and this might impact the tumor microenvironment.

A family of six pro-inflammatory cytokines comprised of interleukin (IL) 17A–F [21–24], have been considered potent activators of innate immunity, promoting a protective tumor immunity [25]. Unlike the pro-

Abbreviations

ACTB	beta-actin
ANOVA	analysis of variance
au	arbitrary unit
APAF1	apoptotic protease activating factor 1
Bax	Bcl-2-like protein 4
BCA	bicinchoninic acid
Bcl-2	B-cell lymphoma 2
cAMP	receptor protein
cDNA	complementary DNA
CYTO-C	cytochrome C
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagles' medium
DMSO	dimethylsulfoxide
DR6	death receptor
3D	tridimensional
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPCR	G protein-coupled receptors
G13D	mutation in an amino acid substitution at position 13
HIF-1 α	hypoxia-inducible factor 1-alpha
HRP	horseradish peroxidase
hTRA	family of serine proteases
HUMEC	basal serum-free medium
IF	immunofluorescence
IGF-1	insulin-like growth factor 1
IGFBPs	insulin-like growth factor-binding protein
IGFR-1	insulin-like growth factor 1 receptor
ILs	interleukins
ILR	interleukin receptor
K-ras	kirsten rat sarcoma viral oncogene homolog
MCF-7	human breast adenocarcinoma cell line estrogen positive
MDA-MB-231	human breast adenocarcinoma cell line triple negative
MAPK	mitogen-activated protein kinases
MCF-10A	human breast epithelial cell line
MOD	mean optical density
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
MT1	melatonin receptor-1
MT2	melatonin receptor-2
mRNA	messenger RNA
NF-kB	factor nuclear kappa B
NRP2	neuropilin 2
PARP	enzyme poly ADP ribose polymerase
PBS	phosphate buffered saline
PLAU	plasminogen activator, urokinase
p21	cyclin-dependent kinase inhibitor 1
p53	tumor protein
RNA	ribonucleic acid
PKA	protein kinase A
PKC	protein kinase C
RT-PCR	reverse transcription polymerase chain reaction
SFB	fetal bovine serum
siRNA	small interfering RNA
TNFR1	tumor necrosis factor receptor 1
TNFR2	tumor necrosis factor receptor superfamily II
VEGF-A	endothelial growth factor
VEGFRs	receptors for vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis protein

inflammatory effects associated with the IL-17 family, IL-17E (also known as IL-25) appears to be a unique pleiotropic cytokine that engages a systemic Th2-like response with tissue-specific immunological and pathological changes [21,22]. These changes include, but are not limited to, expression of IL-4, 5 and 13. Thus, overexpression of IL-25 results in profound alterations in the immune system [22].

IL-25 is secreted by non-malignant mammary epithelial cells and referred to as anti-tumoral cytokine [26]. Anti-inflammatory effects have been associated with IL-25 in both *in vitro* and *in vivo* studies [21]; besides, this cytokine can induce breast cancer cell apoptosis when its receptor IL-25R (also called IL-17RB), which is composed of IL-17RB and IL-17RA heterodimer [26,27], is available on the target cell.

Furuta et al. [26] observed that another ligand, IL-17B, competes for the same receptor site of IL-25 in malignant tissues, which contributes to tumorigenic potential. IL-17B was reported to bind to IL-17RB with an affinity of 7.6 nM, while IL-25 binds to IL-17RB with an affinity in the range of 1.1–1.4 nM. The IL-17RB/IL-17B transduces pro-survival signaling, and their combined expression has been associated with poor prognosis in breast cancer patients [27].

No studies have previously addressed whether melatonin in combination with IL-25 could be a better strategy to target breast cancer cells. Here we explore the therapeutic potential of such a combinatory approach by assessing the effect of melatonin, IL-25, and IL-17B gene silencing, alone or in combination, on cell viability, differential mRNA and protein expression of apoptosis and angiogenesis mediators in malignant human breast cells.

2. Materials and methods

2.1. Reagents and antibodies

Melatonin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Purified IL-25 was from ProSpec (East Brunswick, NJ, USA). Primary antibodies included: cleaved caspase-3 (Sigma-Aldrich, St. Louis, MO, USA), IL-17RB and VEGF-A (both from Santa Cruz Biotechnology, Dallas, TX, USA).

2.2. Cell lines culture

Triple negative (MDA-MB-231) [American Type Culture Collection (ATCC), Manassas, VA, USA] and estrogen receptor (ER) positive (MCF-7) (ATCC, Manassas, VA, USA) human breast cancer cell lines, purchased from the ATCC, were cultured in 75 cm² culture flasks (Sarstedt, Nümbrecht, Germany) with Dulbecco's modified Eagle's medium (DMEM) (Cultilab, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (FBS) (Cultilab, Campinas, SP, Brazil), penicillin (100 IU/mL) and streptomycin (100 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) in a humidified incubator at 5.0% CO₂ at 37 °C until they were 80–90% confluent.

The human non-tumorigenic breast epithelial cell line (MCF-10A) (ATCC, Manassas, VA, USA), purchased from the ATCC, was cultured in 1:1 DMEM: Ham's F-12 (Cultilab, Campinas, SP, Brazil) media supplemented with 5% donor equine serum (Thermo Fisher Scientific, Waltham, MA, USA), epidermal growth factor (20 ng/mL) (Sigma-Aldrich, St. Louis, MO, USA), hydrocortisone (500 ng/mL) (Sigma-Aldrich, St. Louis, MO, USA), insulin (0.01 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA), cholera toxin (100 ng/mL) (Sigma-Aldrich, St. Louis, MO, USA), penicillin (100 IU/mL) and streptomycin (100 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA) in a humidified incubator at 5.0% CO₂ at 37 °C until they were 80–90% confluent.

2.3. Three-dimensional (3D) Matrigel culture assay

MDA-MB-231 and MCF-7 cells were maintained under standard culture conditions as aforementioned. Subconfluent monolayers were trypsinized in a solution of 0.05% Trypsin-EDTA, washed once with DMEM plus 10% FBS, and resuspended in assay media at a density of

3.5×10^4 cells/mL. Assay media consisted of Basal Human Mammary Epithelial Cell (HuMEC) medium supplemented with the HuMEC Supplement Kit (both from Gibco® - Life Technologies, Eugene, OR, USA) and 2% Matrigel® (Becton Dickinson, Franklin Lakes, NJ, USA). Five hundred microliters of the cell suspension were plated on individual wells of 8-well chamber slides (Sarsted, Newton, NC, USA) previously covered with a 1-mm thick layer of laminin-rich extracellular matrix. The cells were maintained in a humidified incubator at 5% CO₂ and 37 °C for eight days until the formation of established 3D structures, with treatments replenished every two days (48 h) (1 mM of melatonin, 1 ng/mL of IL-25, 10 nM of siIL-17B, combined treatments and specific control groups). These concentrations were previously selected based on results from the cell viability assay (method described below). The apoptosis of 3D structures was analyzed using immunofluorescence and confocal microscopy, as indicated below.

2.4. Cell viability assessment by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

For MTT assay, individual wells of 0.31 cm² (96-well plate) were inoculated with 100 µL of regular growth medium containing 5×10^4 cells and incubated in this media overnight, after which the media was changed to 2.0% FBS, containing increasing concentrations of melatonin (0.00001 mM, 0.0001 mM, 0.001 mM, 0.01 mM, 0.1 mM and 1 mM) and different concentrations of purified IL-25 (1 ng/mL, 10 ng/mL and 50 ng/mL). Control wells, corresponding to 0 ng/mL of either treatment, contained the highest concentration of the vehicle for the corresponding treatment, which was 1% dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) for melatonin, and 0.001% e-pure water for IL-25. Following 48 h of the aforementioned treatments, 10 µL of MTT solution from the Vibrant MTT Cell Proliferation Assay Kit (Invitrogen - Life Technologies, Eugene, OR, USA) was added to each well and the plates were incubated at 37 °C for an additional 4 h. To solubilize the MTT formazan crystals, the cells were incubated with 50 µL of DMSO (100%) and then incubated again at 37 °C for 10 min. Absorbance was measured at 540 nm using an ELISA plate reader (Thermo Fisher Scientific - Waltham, MA USA). Medium alone was used as a blank and the corresponding optical density was subtracted from the samples. Cell viability (%) was calculated for all groups relative to control samples. All treatments were done in triplicate.

2.5. Gene silencing of interleukin-17B

For IL-17B gene silencing, four different siRNA were initially tested (SI00106127, SI00106134, SI02640652 and SI02640659) (ProSpec, East Brunswick, NJ, USA), selected from preserved gene regions and thermodynamic stability according to inventoried assay (Qiagen, Valencia, CA, USA). Individual wells of 1.88 cm² (24-well plate) were inoculated with 500 µL of normal growth medium containing 8×10^4 cells. Subsequently, cells were transfected using siRNA Human/Mouse Starter Kit (Qiagen, Valencia, CA, USA), which included the positive control MAPK-1 siRNA (Qiagen, Valencia, CA, USA), a siScramble negative control (Qiagen, Valencia, CA, USA) and the Gene Kit Solution targeting siIL-17B (Cat No. 1027416 - Qiagen, Valencia, CA, USA), all at 10 nM in a 0.5% HiPerfect solution (Qiagen, Valencia, CA, USA). Cells were incubated with these reagents for 48 h, after which total cellular RNA was isolated using the Trizol method (Invitrogen - Life Technologies, Eugene, OR, USA) and purified using the RNeasy Kit extraction columns (Qiagen, Valencia, CA, USA).

2.6. Relative mRNA quantification by real-time (qRT-PCR)

The concentration of RNA from each sample was determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific - Waltham, MA USA). cDNA was obtained by RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction) using the High Capacity cDNA Kit (Applied Biosystems, Foster City, CA, USA).

The qRT-PCR reaction was performed to assess the efficiency of gene silencing of IL-17B, as well as the effect of the treatment on caspase-3 and VEGF-A levels, using a StepOne Plus Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Specific primers included: IL-17B - sense (5' GCAGCTGTGGATGTCCAACA 3') antisense (5' GGGTCGTGGT TGATGCTGTAG 3') and MAPK-1 - sense (5' TCCAACCTGCTGCTCAACAC 3') antisense (5' TCATGGTCTGGATCTGCAACA 3'), inventoried TaqMan assays caspase-3 (Hs0023487_m1), VEGFA (Hs00900055_m1), and the housekeeping genes beta-actin (ACTB; 4333762F) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 4333764F), all at a concentration of 100 ng for each cDNA sample.

The amplification was performed in cycles at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for one minute. The samples were tested in triplicate and each experiment included a negative control. The value of the relative expression of the genes of interest was determined with DataAssist 3.0 software (Applied Biosystems, Foster City, CA, USA) by $\Delta\Delta C_t$ method [28]. The RQ value for each respective control group was initially established as 1.0 au to assess relative changes. The analysis was ultimately performed on values converted to a logarithmic scale, which sets the expression value for control groups as 0 (zero). Treatments were interpreted as underexpressed or overexpressed relative to controls.

2.7. Immunofluorescence staining

Previously treated 3D structures grown on matrigel and treated monolayer cells were washed once with PBS, fixed in 4.0% paraformaldehyde solution in PBS for 20 min at room temperature, added a 0.5% Triton in PBS solution and blocked with 10% donkey serum solution for 1 h at room temperature. The specific primary antibodies were then added and incubated overnight at 4 °C. After washing three times with immunofluorescence (IF) buffer (0.1% FSB, 0.2% triton and 0.05% Tween 20), a secondary Alexa Fluor 488 anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO, USA) for both cleaved caspase-3 and VEGF-A was added per 1 h at room temperature. Following three time washing with IF buffer, the cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) solution (Life Technologies, Eugene, OR, USA) and mounted with Prolong Gold® (Life Technologies, Eugene, OR, USA). Images from 3D structures were captured and processed using a confocal microscope and associated software (ZEISS, model LSM 710, software ZEN 2010, Thornwood, NY, USA) and monolayer cells were captured and processed using a standard fluorescence microscope and associated software (OLYMPUS, model BX53, software Image-Pro Plus version 7.0, Center Valley, PA, USA).

2.8. Evaluation of immunofluorescence staining

For apoptosis analysis, all cleaved caspase-3 positive cells were counted in three different photomicrographs (100× magnification) per treatment group. Results were quantified as percent of apoptotic cells in monolayer and 3D structures. The number of cleaved caspase-3 positive cells was normalized to the area of the photomicrography.

IL-17RB and VEGF-A proteins were quantified according to Jardim-Perassi et al. [29]. In summary, three different photomicrographs were taken at 100× magnification and the intensity of the staining was quantified by Image J Software (NIH, Bethesda, MD, USA). Each photograph was divided into four quadrants, and 20 spots (small circular ROI) were randomly selected (avoiding the nucleus) in each photomicrograph. A negative control section of the corresponding staining was used for measuring background activity. The values were obtained in arbitrary units (au) and showed the mean optical density (MOD) to each sample.

2.9. Protein extraction

In order to reaffirm melatonin action in apoptosis pathway in triple negative breast cancer cells, we performed a membrane array analysis in MDA-MB-231 cells. Cells were plated in individual wells of 1.88 cm²

(24-well plate) at a 0.5×10^6 cell density and inoculated with 500 μ L of normal growth medium overnight. Thereafter, we treated cells with or without 1 mM of melatonin for 48 h and performed protein extraction of adherent and supernatant cells. Cells were washed in ice-cold PBS and lysed with MILLIPLEX® MAP lysis buffer supplemented with 1 mM of phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, EUA) and 1:10 of protease inhibitor (Sigma-Aldrich, St. Louis, MO, EUA). After incubation for 30 min with intermittent vortexing, the cell lysate was centrifuged and the proteins collected on supernatant. Concomitantly the supernatant medium was collected and we used ultrafiltration (Amicon®, EMD Millipore, Billerica, MA) to concentrate the proteins. The supernatant was included in columns containing filter of 3 kDa and centrifuged. Larger proteins that were retained in filter were extracted and subsequently quantified.

Protein extract was quantified by the bicinchoninic acid (BCA) protein assay kit (PIERCE - Thermo Scientific - Thermo Fisher Scientific, Waltham, MA, USA).

2.10. Membrane array

The membrane *Human Apoptosis Array C1* (RayBiotech, Norcross, GA, EUA) (Table 1) was incubated with 2 mL of $1 \times$ blocking solution buffer (RayBiotech, Norcross, GA, EUA) for 30 min. Treated and control samples were added at a concentration of 600 μ g (300 μ g of lysate cells and 300 μ g of supernatant cells) and incubated on the membrane at 4 °C overnight. Next, the membrane was washed three times with wash buffer $1 \times$ (RayBio I, RayBiotech, Norcross, GA, EUA) for five minutes each. Biotin conjugate anti-cytokines (RayBiotech, Norcross, GA, EUA) were added and the samples were incubated at 4° overnight. The membrane was washed again and then incubated with horseradish peroxidase (HRP) streptavidin 1000 \times (RayBiotech, Norcross, GA, EUA) solution at 4 °C overnight. Finally, the membrane was washed and incubated with detection solution (RayBiotech, Norcross, GA, EUA) for two minutes and exposed to ChemiDoc system (BioRad, Hercules, CA, EUA).

Optical density reference to protein expression was normalized with positive control and quantification was performed using ImageJ Software (NIH, Bethesda, MD, USA) as image analyzer. The values were obtained in arbitrary units and represented as the MOD to each sample. All samples were included in duplicate plus positive and negative membrane controls.

2.11. Statistical analysis

All data was expressed as mean \pm standard error of mean (SEM). All statistical analyses were done using GraphPad Prism 4 (San Diego, CA, USA). Raw data was initially subjected to descriptive analysis to determine the normal range. Normal range data was analyzed by two-way ANOVA, followed by Bonferroni test for MTT results and Student's *t*-test was used for other analyses. A *p*-value ≤ 0.05 was considered significant.

3. Results

3.1. Melatonin and IL-25 reduce viability of breast cancer cells but do not affect non-transformed MCF-10A cells

Breast cancer cell lines and MCF-10A were subjected to MTT cell viability testing, after being treated with IL-25 (breast cancer cells) or melatonin + IL-25 (MCF-10A). Jardim-Perassi et al. [29,30] previously showed that the MDA-MB-231 and MCF-7 cells were sensitive to 1 mM of melatonin after 24 h of incubation, showing a statistically significant reduction in cell viability compared to control groups (*p* < 0.05). Similar results were found by Borin et al. [31] following 48 h of treatment. As the 1 mM concentration showed a significant reduction of viability, it was adopted as the standard dose for the current study.

Table 1

Human apoptosis array C1 (RayBiotech) for analysis of protein expression profile of MDA-MB-231 cells after melatonin treatment.

Apoptotic factors	Melatonin treatment	Apoptotic factors	Melatonin treatment
BAD	ns	BAX	ns
CD40	ns	BCL-2	ns
CD40 LIGAND	ns	BCL-W	ns
BIRC-3	ns	BID	ns
CYTO C	$\uparrow p = 0.04$	BIM	ns
DR6	$\uparrow p = 0.02$	CASPASE-3	$\downarrow p = 0.04$
FAS	ns	CASPASE-8	ns
FAS LIGAND	ns	HSP27	ns
IGFBP-1	ns	HSP60	ns
IGFBP-2	ns	HSP70	ns
IGFBP-3	$\uparrow p = 0.03$	hTRA	$\uparrow p = 0.02$
IGFBP-4	ns	IGF-1	$\uparrow p = 0.02$
IGFBP-5	$\uparrow p = 0.01$	IGF-2	ns
IGFBP-6	$\uparrow p = 0.01$	LIVIN	$\uparrow p = 0.04$
IGF-1R	$\uparrow p = 0.05$	P21	$\uparrow p = 0.03$
TNF-RII	$\uparrow p = 0.04$	P27	ns
TNF- α	ns	P53	$\uparrow p = 0.04$
TNF- β	ns	SMAC	ns
TRAIL-R1	ns	SURVININ	ns
TRAIL-R2	ns	TNF-RI	ns
TRAIL-R3	ns	XIAP	$\uparrow p = 0.04$
TRAIL-R4	ns		

BAD: Bcl-2-antagonist of cell death; BAX: Bcl-2-associated X protein; BCL-2: B-cell lymphoma 2; BCL-W: Bcl-2-like protein 2; BID: BH3 interacting-domain death agonist; BIM: Bisindolylmaleimide-based protein kinase C (PKC) inhibitors; BIRC-3: Baculoviral IAP repeat-containing protein 3; CASPASE-3: Cysteine-aspartic acid protease 3; CASPASE-8: Cysteine-aspartic acid protease 8; CD40: Cluster of differentiation 40; CD40 LIGAND: Cluster of differentiation 40 ligand; CYTO C: Cytochrome c; DR6: Death receptor 6; FAS: First apoptosis signal; FAS LIGAND: First apoptosis signal ligand; HSP27: Heat shock protein 27; HSP60: Heat shock protein 60; HSP70: Heat shock protein 70; hTRA: High-temperature requirement A serine peptidase; IGF-1: Insulin-like growth factor 1; IGF-2: Insulin-like growth factor 2; IGFBP-1: Insulin-like growth factor-binding protein 1; IGFBP-2: Insulin-like growth factor-binding protein 2; IGFBP-3: Insulin-like growth factor-binding protein 3; IGFBP-4: Insulin-like growth factor-binding protein 4; IGFBP-5: Insulin-like growth factor-binding protein 5; IGFBP-6: Insulin-like growth factor-binding protein 6; IGF-1R: Insulin-like growth factor receptor 1; Livin; P21: Cyclin-dependent kinase inhibitor 1; P27: Cyclin-dependent kinase inhibitor 1B; P53: Tumor protein p53; SMAC: Second mitochondria-derived activator of caspases; Survivin; TNF- α : Tumor necrosis factor alpha; TNF- β : Tumor necrosis factor beta; TNF-RI: Tumor necrosis factor receptor 1; TNF-RII: Tumor necrosis factor receptor 2; TRAIL-R1: TNF-related apoptosis-inducing ligand receptor 1; TRAIL-R2: TNF-related apoptosis-inducing ligand receptor 2; TRAIL-R3: TNF-related apoptosis-inducing ligand receptor 3; TRAIL-R4: TNF-related apoptosis-inducing ligand receptor 4; XIAP: X-linked inhibitor of apoptosis protein. All antibodies are prepared in duplicate. ns: no significant.

Cell viability was tested with IL-25 at 1, 10, and 50 ng/mL for 48 h, and there was a reduction in cell viability in both breast cancer cell lines. For MDA-MB-231 cells, a biphasic effect was observed, with 1 ng/mL and 50 ng/mL of IL-25 causing a significant 26% - 27% reduction in viability compared to the control group ($74.0 \pm 10.2\%$ and $72.5 \pm 8.5\%$, respectively; *p* < 0.05). For MCF-7 cells, the lowest dose of 1 ng/mL of IL-25 significantly reduced cell viability by 61% ($39.0 \pm 8.9\%$; *p* < 0.05) (Fig. 1A and B).

Neither melatonin nor IL-25 treatments caused a significant reduction in viability compared to control in MCF-10A cells, when the effective doses for MDA-MB-231 and MCF-7 cells were chosen (*p* > 0.05) (Fig. 1C). These results suggest that melatonin and IL-25 preferentially target the transformed cells.

3.2. Effective IL-17B silencing

In order to test our hypothesis that a reduction in IL-17B level may lead to increased tumor cell apoptosis by enhancing IL-25 binding to the IL-17RB receptor, IL-17B gene silencing was performed, as there is no specific inhibitor for this interleukin. Silencing of the positive control, MAPK-1, was carried out to optimize the concentration and incubation period of the transient transfection. The positive control was effective at 10 nM in both breast cancer cell lines with 74.0% for MDA-MB-231 cells and 67.0% for MCF-7 cells 48 h after transfection (data not

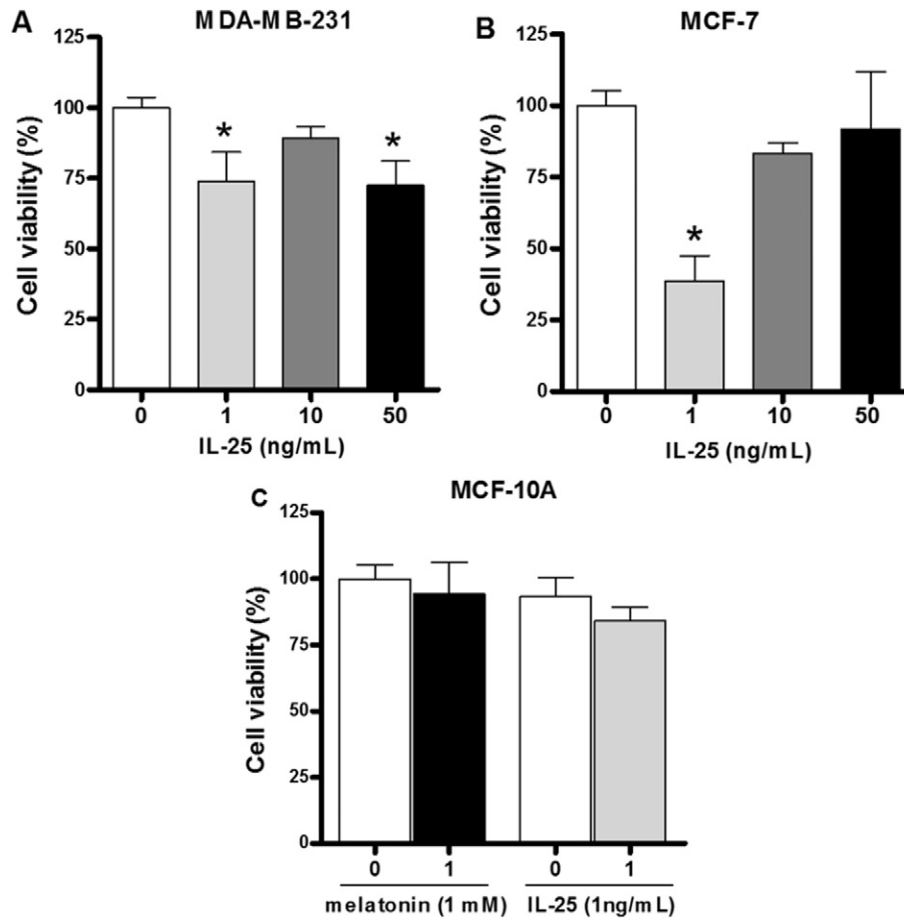


Fig. 1. Effect of melatonin and interleukin-25 on viability of cell lines. A) MDA-MB-231 and B) MCF-7 cells were treated with 1 ng/mL, 10 ng/mL and 50 ng/mL of IL-25 for 48 h and cell viability was measured by MTT assay. C) MCF-10A cells were treated with 1 mM of melatonin and 1 ng/mL of IL-25 for 48 h and cell viability was measured by MTT assay. The white column corresponds to control group. Each column represents the mean \pm standard error of triplicate experiments. (* $p < 0.05$). Statistical significance compared to control group was determined by ANOVA followed by Bonferroni test.

shown). siRNA #2 and #3 were effective for IL-17B silencing for MDA-MB-231 cells with the best silencing (44.0%) obtained with siIL-17B #2 (Fig. 2A). For MCF-7 cells, gene silencing was effective for siRNA #2, #3 and #4, with 66.0% of silencing for siIL-17B #2 (Fig. 2B).

3.3. Induction of apoptosis by melatonin, IL-25 and siIL-17B treatment of monolayer and three-dimensional breast cancer cell cultures

To test the hypothesis that IL-25 signaling engagement added to melatonin enhances breast cancer cell apoptosis, breast cancer cell lines were cultured with 1 mM of melatonin, 1 ng/mL of IL-25 and 10 nM of

siIL-17B #2 as well as their respective vehicles, for 48 h. Caspase-3 mRNA expression was evaluated by real-time PCR, while cleaved caspase-3 was examined by immunofluorescence (see Methods for details).

The three treatments separated and in combination, were effective at increasing the caspase-3 gene expression in MDA-MB-231 cells. The difference in caspase-3 expression between control and treated cells was 0.3 au (± 0.01 au; $p < 0.0001$) with melatonin, 0.3 au (± 0.02 au; $p = 0.0002$) with IL-25, 0.2 au (± 0.01 au; $p = 0.0001$) with siIL-17B, and 0.4 au (± 0.03 au; $p = 0.0002$) after combined treatment. Furthermore, MDA-MB-231 cells treated for 48 h showed a ~3-fold higher average of positive apoptotic cells compared to their control group for each

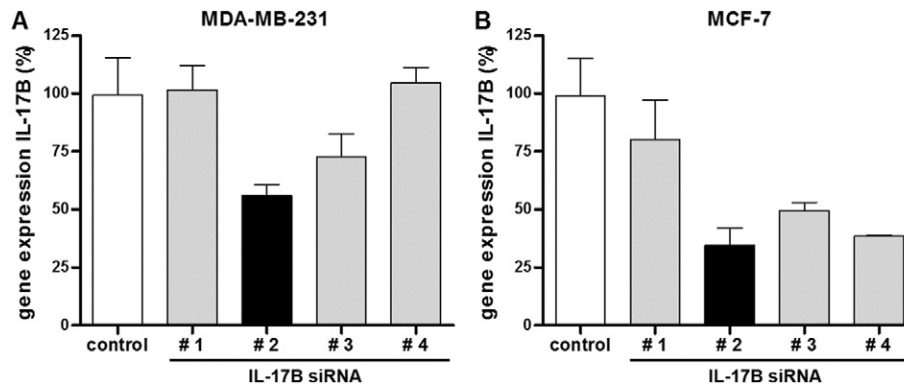


Fig. 2. Gene silencing of IL-17B. Gene expression was measured by qRT-PCR at 48 h post-transfection of cells with 4 different IL-17B siRNAs (#1 to #4) or a Scramble siRNA (Control; white column). A) MDA-MB-231 cells showed 44% gene silencing with 10 nM of siIL-17B #2. B) MCF-7 cells showed 66% gene silencing with 10 nM of siIL-17B #2.

treatment ($p < 0.05$ in all cases), as determined by cleaved caspase-3 immunofluorescence. The combined treatments did not further increase apoptosis compared to individual treatments (Fig. 3).

Similarly, melatonin, IL-25 and siIL-17B treatments for 48 h, alone and combined, were effective in increasing the caspase-3 gene expression in MCF-7 cells. The difference in caspase-3 expression between control and treated cells was 1.0 au (± 0.05 au; $p = 0.002$) for melatonin, 0.3 au (± 0.04 au; $p = 0.01$) for IL-25, 0.8 au (± 0.1 au; $p = 0.01$) for siIL-17B, and 1.4 au (± 0.2 au; $p = 0.002$) for combined treatments. The average percent of apoptotic cells after 48 h of individual treatments was ~3–4 times higher than their respective control groups ($p < 0.05$). Importantly, for MCF-7 cells, the combined treatment showed pro-apoptotic advantage compared to melatonin alone, as it resulted in an average 4.4 fold enhancement of the percentage of apoptotic cells, as compared to the 3 fold enhancement observed with melatonin alone (Fig. 4).

As an alternative to classical *in vitro* studies with cells grown as monolayers, the 3D culture on reconstituted basement membrane mimics tissue architecture *in vivo*, possibly predicting better cellular response in actual tumors [32]. 3D culture permits cells to explore the three dimensions of the space thereby increasing cell-cell interactions, as well as interactions with the microenvironment [32]. Thus, we aimed to characterize the increased expression of cleaved caspase-3 in 3D structures of breast cancer cells.

Positive nuclear staining of cleaved caspase-3 in different sequential plane images of MDA-MB-231 and MCF-7 cells was observed in all groups, including controls (Fig. 5A and B). Compare to respective control group, treatment with 1 mM of melatonin enhanced the proportion of apoptotic cells by 31% for MDA-MB-231, and by 24% for MCF-7 ($p = 0.05$; $p = 0.002$, respectively). IL-25 treatment enhanced apoptotic cells by 20% for MDA-MB-231, and by 26% for MCF-7 ($p = 0.04$; $p = 0.01$, respectively). siIL-17B was more effective at enhancing the proportion of apoptotic cells; 68% for MDA-MB-231 and 74% for MCF-7 ($p < 0.0001$; $p < 0.0001$, respectively). Combined treatments did not increase the percentage of apoptotic cells in MDA-MB-231 3D structures, while it has a similar effect to that of melatonin alone in MCF-7 3D structures (30% combined treatments versus 11% control group; $p = 0.01$) (Fig. 5C and D).

3.4. Melatonin modulates expression of apoptosis mediators in MDA-MB-231 cells

Apoptosis-associated proteins were assessed in MDA-MB-231 cells using a membrane protein array. As compared to control group, treatment with 1 mM of melatonin for 48 h showed a significant increase of cytochrome c (CYTO-C), death receptor 6 (DR6), insulin-like growth factor-binding protein-3 (IGFBP-3), -5 (IGFBP-5), -6 (IGFBP-6), insulin-like growth factor-1 (IGF-1), IGF-1 receptor (IGF-1R), Livin, P21, P53,

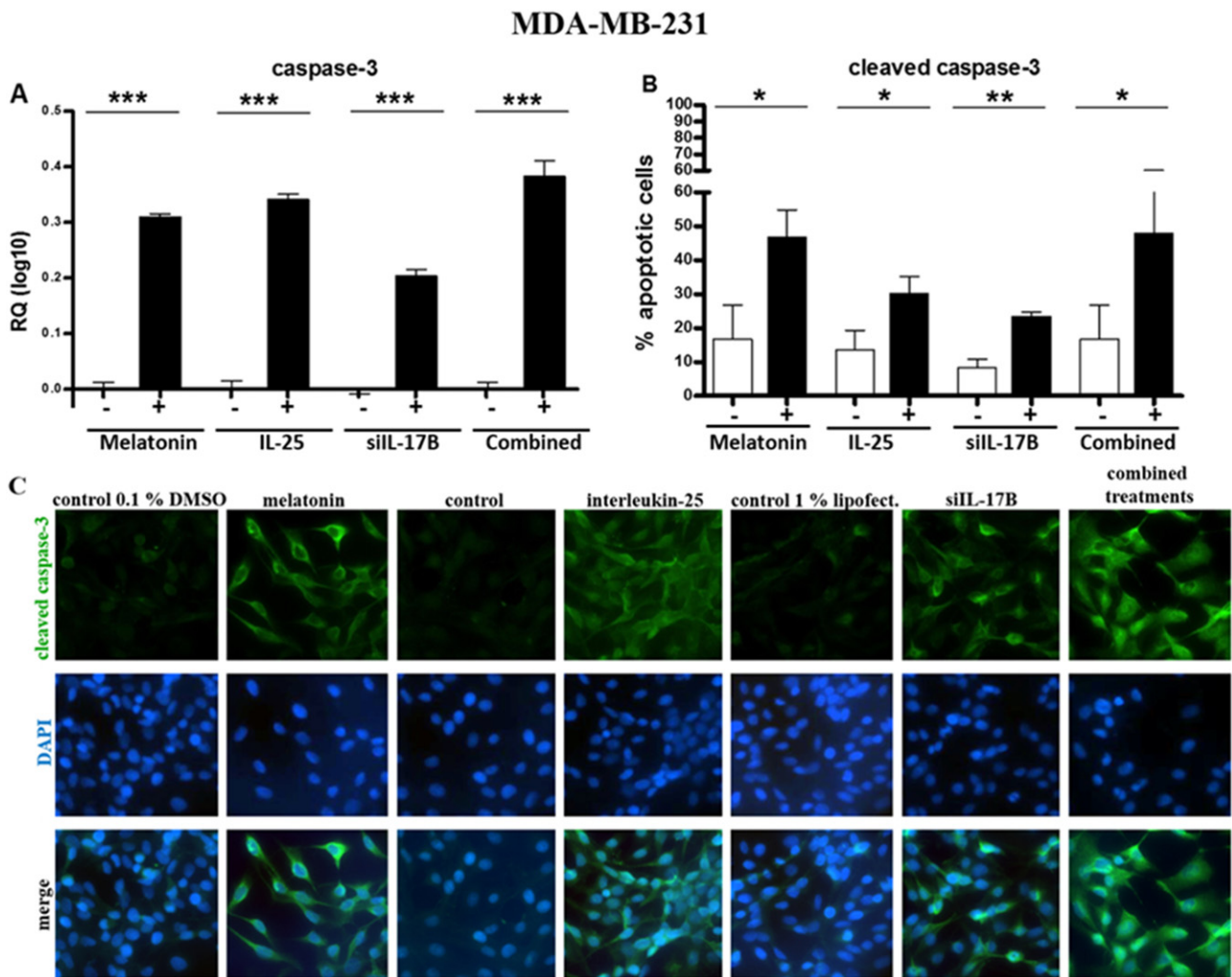


Fig. 3. Cleaved caspase-3 expression in MDA-MB-231. A) Increase gene expression of caspase-3 after treatments compared to control groups. B) Average percentage of apoptotic cells for each treatment. C) Photomicrographs of immunofluorescence staining for cleaved caspase-3 in MDA-MB-231 cells. Magnification = 100 \times . Cleaved caspase-3 (green) and nuclei DAPI (blue). Each column represents the mean \pm standard error of triplicate experiments ($^*p \leq 0.05$). Significance was determined by Student's *t*-test. $^*p < 0.01$ $^{**}p < 0.001$ $^{***}p < 0.0001$.

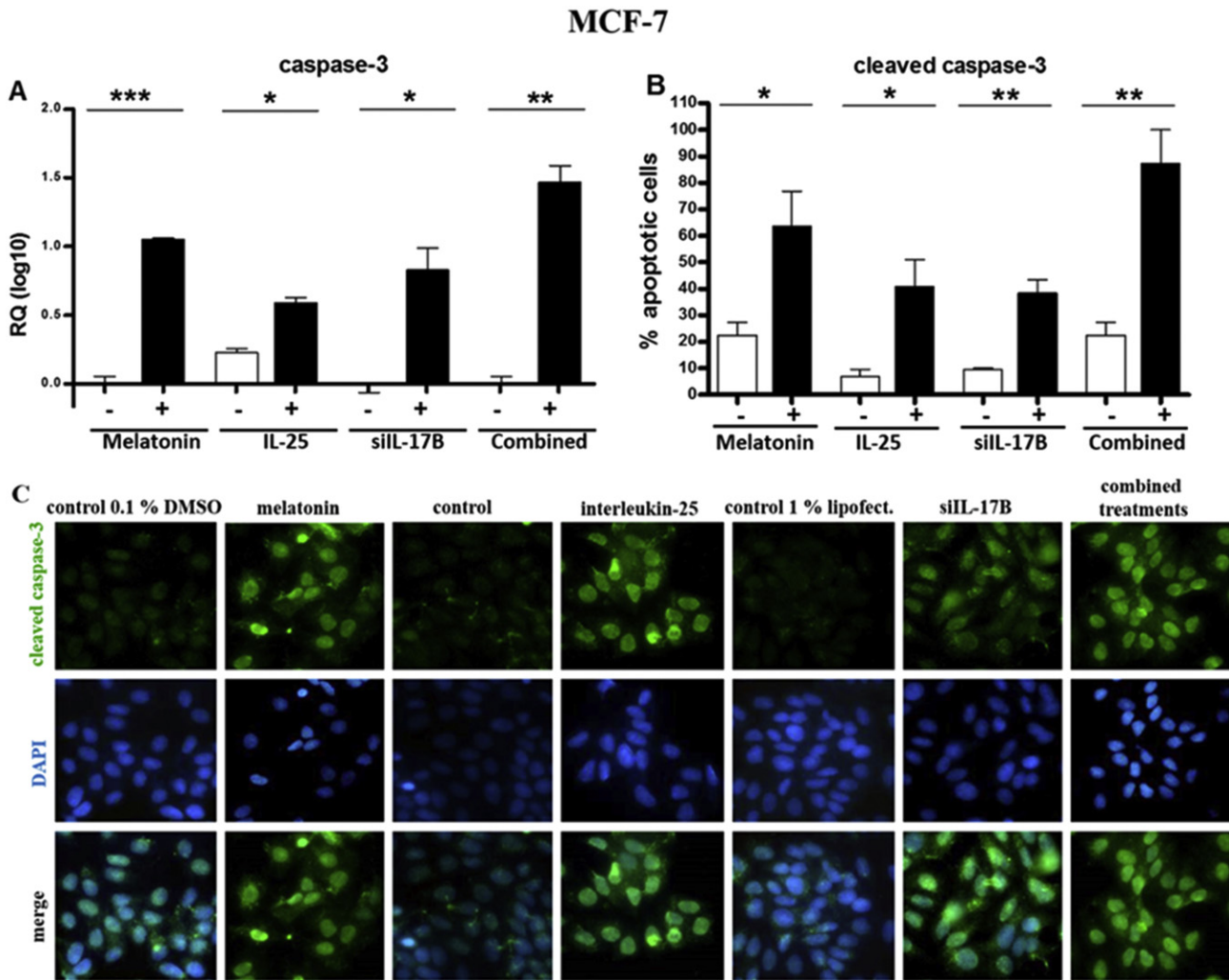


Fig. 4. Cleaved caspase-3 expression in MCF-7. A) Increase gene expression of caspase-3 after treatments compared to control groups. B) Average percentage of apoptotic cells for each treatment. C) Photomicrographs of immunofluorescence staining for cleaved caspase-3 in MCF-7 cells. Magnification = 100 \times . Cleaved caspase-3 (green) and nuclei DAPI (blue). Each column represents the mean \pm standard error of triplicate experiments (* $p < 0.05$). Significance was determined by Student's *t*-test. * $p < 0.01$ ** $p < 0.001$ *** $p < 0.0001$.

tumor necrosis factor receptor II (TNFR2), apoptosis inhibitory protein X (XIAP) and high temperature required A (hTRA) ($p < 0.05$). A significant reduction of caspase-3 was also observed ($p = 0.04$) (Fig. 6).

3.5. Decreased expression of VEGF after treatment with melatonin, IL-25 and siIL-17B

Next, we tested the influence of 48 h treatment with melatonin, IL-25 and siIL-17B separately or in combination, on VEGF-A mRNA and protein expression.

We observed a significant increase of VEGF-A mRNA expression in MDA-MB-231 cells after treatments with IL-25, siIL-17B and when the treatments were combined. The difference in VEGF-A mRNA expression between control and treated cells with IL-25 was 0.07 au (± 0.01 au; $p = 0.007$), with siIL-17B treatment was 0.08 au (± 0.01 au; $p = 0.002$) and after associated treatments was 0.1 au (± 0.02 au; $p = 0.006$). In contrast, the protein levels of this factor were significantly reduced by treatments with melatonin (16.0 ± 0.6 au, compared to control group 30.0 ± 1.0 au; $p < 0.0001$), IL-25 (10.7 ± 0.3 au, compared to control group 28.2 ± 0.8 au; $p < 0.0001$), siIL-17B (14.0 ± 0.5 au, compared to control group 22.0 ± 0.5 au; $p < 0.0001$) and combined treatments (21.1 ± 0.7 au, compared to control group 30.0 ± 1.0 au; $p < 0.0001$) (Fig. 7). In comparison with all other treatments, IL-25 was the most potent at reducing

VEGF protein levels and the combined treatment was less effective than IL-25 alone (Fig. 7).

For MCF-7 cells, VEGF-A mRNA decreased after treatment with melatonin, with a difference of -0.18 au between control group (± 0.05 au; $p = 0.02$). In contrast, IL-25, siIL-17B and combined treatments increased VEGF-A gene expression. The difference in VEGF-A expression between control and treated cells with IL-25 was 1.5 au (± 0.02 au, $p < 0.0001$), siIL-17B was 0.5 au (± 0.01 au, $p < 0.0001$) and when treatments were combined the observed difference was 0.9 au (± 0.04 , $p < 0.0001$). VEGF-A protein levels were modulated after 48 h of incubation, with all treatments showing a reduction after melatonin treatment (17.1 ± 0.6 au, compared to control group 22.5 ± 0.9 ; $p < 0.0001$), IL-25 (14.4 ± 0.4 au, compared to control group 25.3 ± 1.3 ; $p < 0.0001$), siIL-17B (18.0 ± 0.7 au, compared to control group 21.1 ± 0.8 ; $p = 0.004$) and after combined treatments (15.0 ± 0.6 au, compared to control group 22.5 ± 0.9 ; $p < 0.0001$) (Fig. 8). In comparison with all other treatments, IL-25 was the most potent at reducing VEGF protein levels and the combined treatment was not better than IL-25 alone (Fig. 8).

4. Discussion

This study confirms previous findings that pharmacological levels of melatonin reduce cell viability of triple negative and ER-positive breast

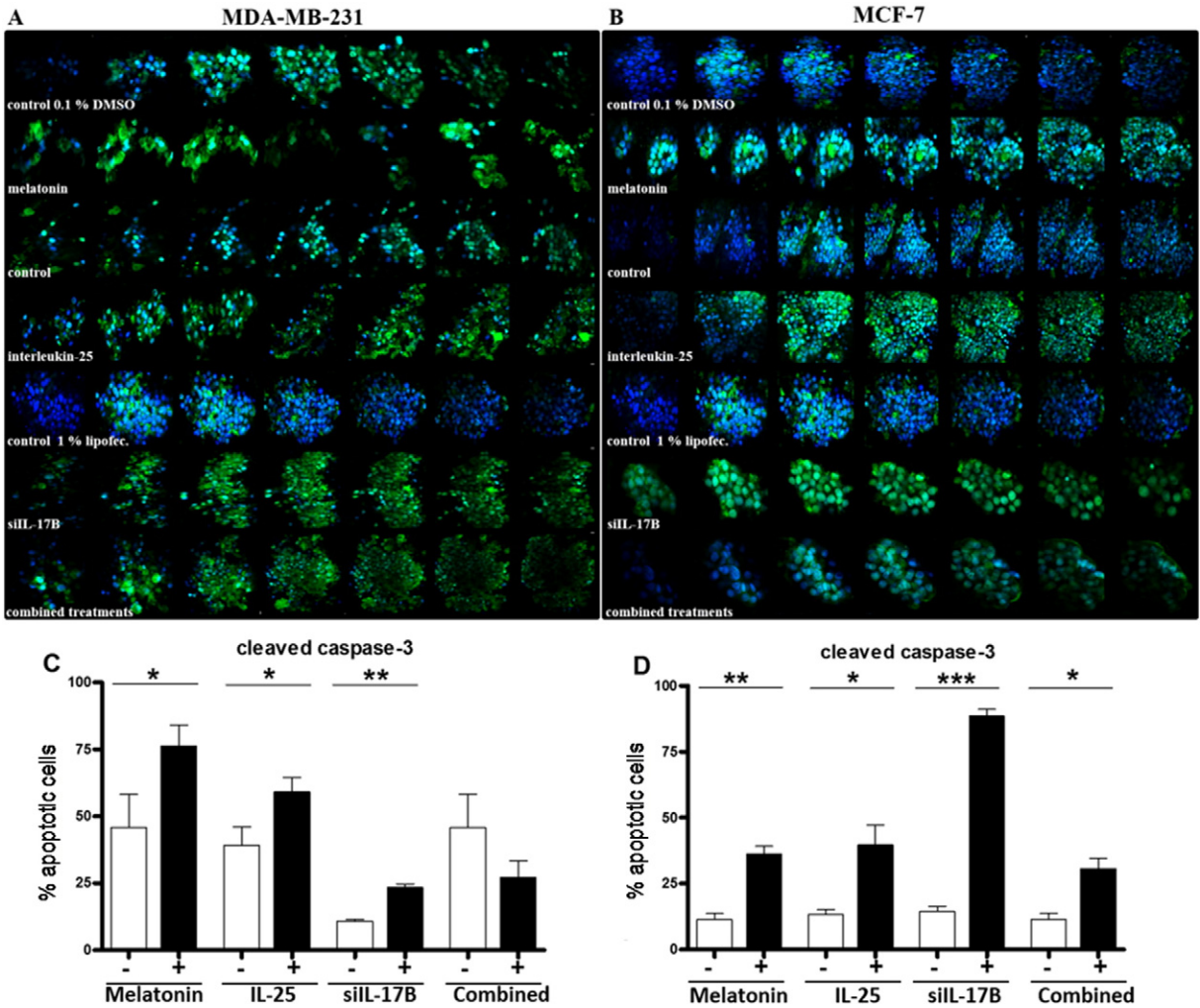


Fig. 5. Cleaved caspase-3 expression in MDA-MB-231 and MCF-7 3D structures. Representative sequential images of positive immunofluorescence staining for cleaved caspase-3 in MDA-MB-231 (A) and MCF-7 (B) 3D structures. C) Average percentage of apoptotic cells for each treatment in MDA-MB-231 3D structures and D) MCF-7 3D structures. Magnification = 100×. Each column represents the mean ± standard error of triplicate experiments (*p ≤ 0.05). Significance was determined by Student's *t*-test. *p < 0.01 **p < 0.001 ***p < 0.0001.

cancer cells [29,33–40]. Administration of different dosages of melatonin in humans (20–40 mg/day, as single agent or in combination with other drugs) is safe and associates with a significant reduction in risk of death within 1 year for a range of solid cancers [15].

Two major mechanisms have been reported for melatonin's biological activity: G-protein coupled receptor (GPCR) - mediated activity (MT1 and MT2 receptors) and non-receptor-mediated antioxidant activity, because melatonin is highly liposoluble [41,42]. MT1 engagement inhibits the activity of adenylyl cyclase, decreasing the production of adenosine 3',5'-cyclic monophosphate (cAMP). This in turns modulates the

activity of selected protein kinases (PKC, PKA, MAPK), and downstream expression of genes involved in proliferation, angiogenesis, cell differentiation and migration [43]. Both MDA-MB-231 (triple negative subtype) and MCF-7 (estrogen receptor/ER-positive subtype) breast cancer cell lines express MT1 receptor, although lower MT1 expression was reported for triple negative as compared to ER-positive breast cancers [43,44]. In addition, pharmacologic concentrations of melatonin may activate other non-receptor mediated pathways [45], which could explain the observed significant inhibition caused by melatonin in the viability of MDA-MB-231 cells.

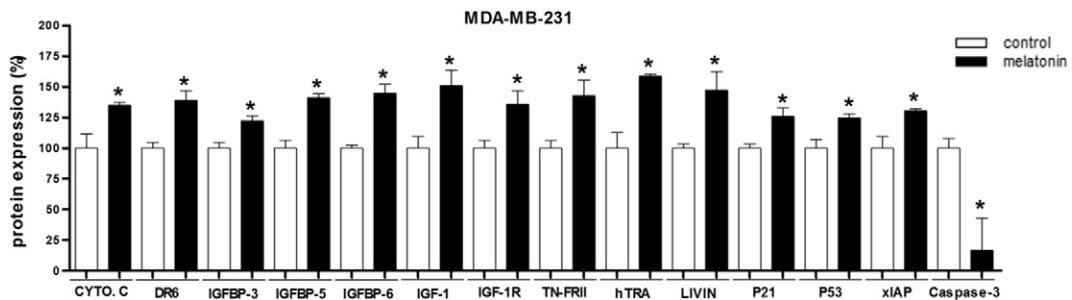


Fig. 6. Differentially apoptotic protein expression in MDA-MB-231 cells treated with 1 mM of melatonin. The white column corresponds to control groups. Each column represents the mean ± standard error of duplicate experiments. (*p ≤ 0.05). Significance was determined by Student's *t*-test.

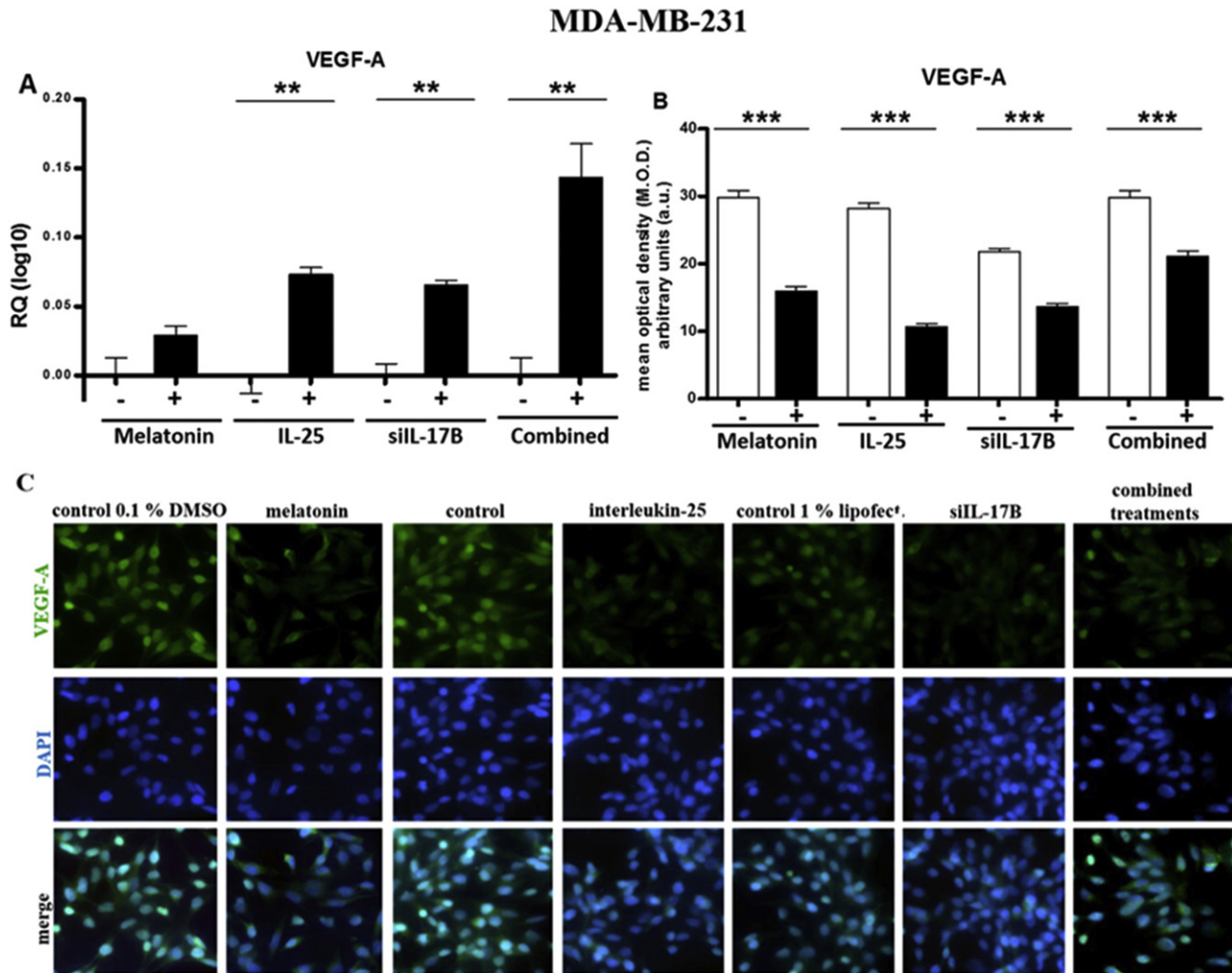


Fig. 7. VEGF-A expression in MDA-MB-231. A) Decrease VEGF-A gene expression after treatments compared to control groups. B) Quantification of VEGF-A protein expression showing decreased cytoplasm labeling after treatments, compared to control groups for each treatment. C) Photomicrographs of immunofluorescence staining for VEGF-A in MDA-MB-231 cells. Magnification = 100 \times . VEGF-A (green) and nuclei DAPI (blue). Each column represents the mean \pm standard error of triplicate experiments ($*p \leq 0.05$). Significance was determined by Student's *t*-test. $*p < 0.01$ $**p < 0.001$ $***p < 0.0001$.

We also found that 1 ng/mL of IL-25 reduced the viability of both MDA-MB-231 and MCF-7 tumor cells. The dose-dependent reduction in cell viability of MDA-MB-231 by IL-25 treatment has not been reported until now; although Furuta et al. [26] showed that MDA-MB-468, a highly metastatic and ER-negative cell line, and MCF-7 cells, are responsive to treatment with 10 ng/mL of IL-25, a 10-fold higher concentration than the one we used. In addition, IL-25 and a stroma IL-25 inducing agent were shown to significantly hamper breast cancer progression *in vivo* [46].

On the other hand, we found the absence of an effect of both melatonin and IL-25 on MCF-10A viability; suggesting no cytotoxic potential for these agents in normal luminal breast cells. The results with IL-25 are in agreement with those of Furuta et al. [26], which showed virtually absent expression of IL-25R and resistance to IL-25 treatment in MCF-10A cells. Whether or not the same is true for other normal epithelial cells in the body (thus indicating a reduced probability of side effects) remains to be determined. In this regard, studies with eosinophils indicate that these cells do have receptors for IL-25, and this cytokine enhance their viability and survival [47,24].

Demonstrating the effect of the proposed treatments on apoptosis, we observed enhanced cleaved caspase-3 protein after each treatment alone and the three combined, in both MDA-MB-231 and MCF-7 monolayer and 3D cultures. Melatonin was previously shown to reduce viability of cancer cells by different mechanisms, including cellular

differentiation and apoptotic cell death [39,48–51]. In agreement with our results, di Bella et al. [11] and Sanchez-Hidalgo et al. [52] showed that the direct anti-tumor effect of melatonin occurs *via* caspase activation, and Rodriguez et al. [53] observed that both the intrinsic and extrinsic apoptosis pathways can be activated by melatonin in cancer cells, but not in normal cells. Induction of apoptosis by IL-25 and/or siIL-17B in MCF-7 and MDA-MB-231 cells, as observed here, was also previously reported [26,27,46,54].

In agreement with the observed pro-apoptotic activity of melatonin in metastatic MDA-MB-231 cells, our pilot assessment of the effect of melatonin on apoptosis mediators showed an increase of proteins involved in the extrinsic apoptosis pathway, e.g. TNF-RII and DR6, and in the intrinsic apoptosis pathway, such as CYTO-C. The latter has a crucial role in apoptosis, as upon release into the cytosol it binds apoptotic protease activating factor-1 (APAF-1) and pro-caspase-9, to promote apoptosome formation [55,56]. Wang et al. [57] demonstrated that melatonin induces the expression of APAF-1 and stimulates the release of CYTO-C, causing the activation of caspases and inducing apoptosis in MDA-MB-361 breast cancer cells.

Growth factors such as IGF-1, IGF-1R and IGFBP-3, –5 and –6 also showed increases after melatonin treatment, in agreement with our previous results [29]. Butt et al. [58] showed that high IGFBP-5 levels correlate with enhanced transcription of pro-apoptotic BAX and a decrease in anti-apoptotic BCL-2, resulting in MDA-MB-231 apoptosis. Interestingly,

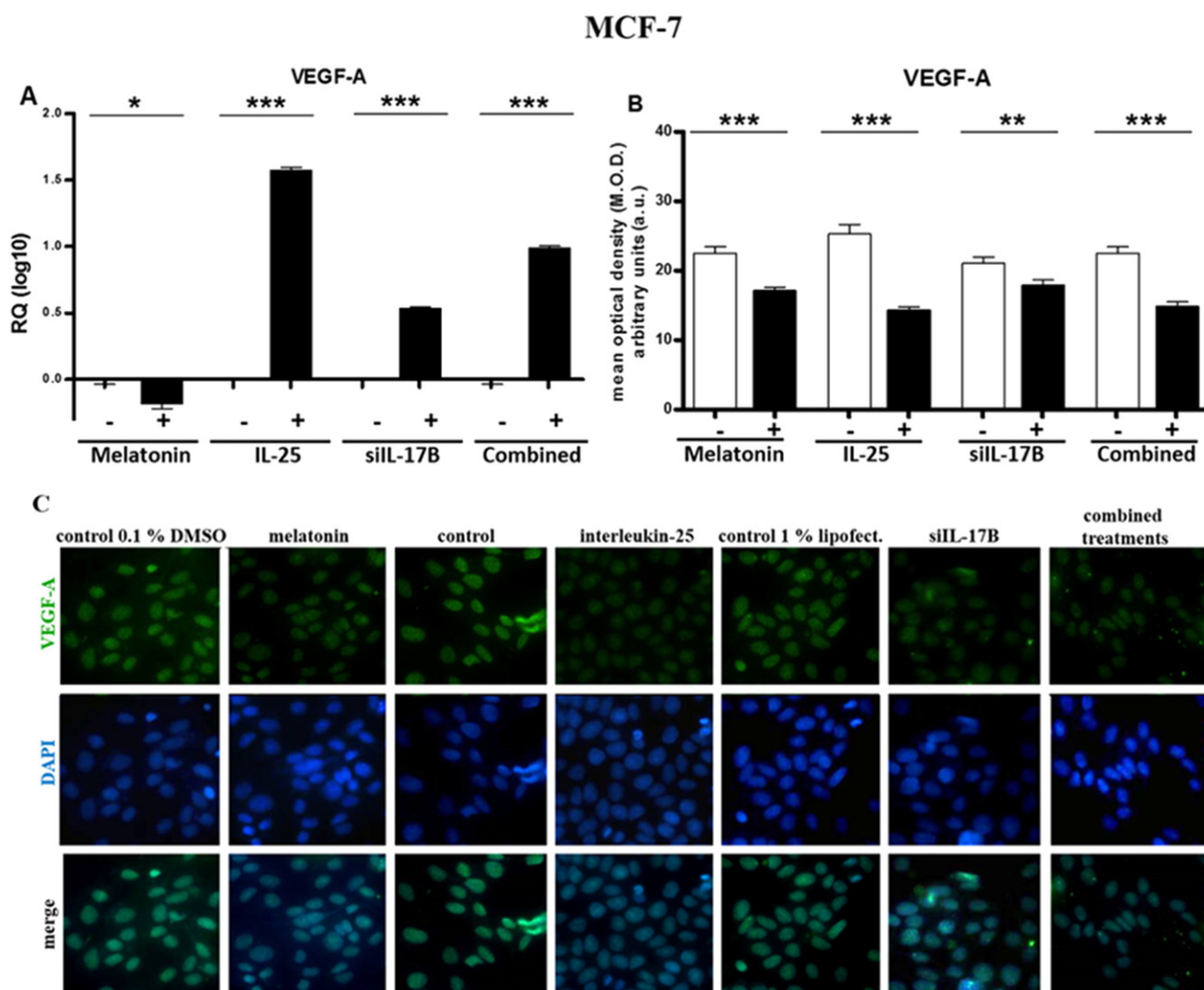


Fig. 8. VEGF-A expression in MCF-7. A) Decrease VEGF-A gene expression after treatments compared to control groups. B) Quantification of VEGF-A protein expression showing decreased cytoplasm labeling after treatments, compared to control groups for each treatment. C) Photomicrographs of immunofluorescence staining for VEGF-A in MCF-7 cells. Magnification = 100 \times . VEGF-A (green) and nuclei DAPI (blue). Each column represents the mean \pm standard error of triplicate experiments ($*p \leq 0.05$). Significance was determined by Student's *t*-test. $*p < 0.01$ $**p < 0.001$ $***p < 0.0001$.

the anti-apoptotic proteins Livin, XIAP and hTRA were enhanced by melatonin treatment. One possibility is that an increase in survival-promoting molecules occurs as a compensatory mechanism to the major apoptotic stimuli of melatonin. Further studies are necessary to address these possibilities as well as to verify, *via* Western blotting, the changes in apoptosis mediators observed in this study. The membrane array employed here was meant as a pilot screen to provide a general idea of the apoptosis mediators potentially responsible for melatonin-induced apoptosis, and thus lays the foundation for future mechanistic studies.

Another interesting finding was the observed increase in caspase-3 (CASP3) mRNA in response to all treatments in both MCF7 and MDA-MB-231 cells. Modulation of caspase-3 mRNA was previously reported in normal hippocampal neurons of rats treated with melatonin prior to irradiation, where the neuroprotective effect of melatonin associated with a reduction in both caspase-3 mRNA and caspase-3 protein cleavage [59]. To the best of our knowledge, ours is the first study to assess the effect of melatonin on caspase-3 mRNA in cancer cells, as most studies examined levels of cleaved caspase-3 protein. The increase in caspase-3 mRNA could potentially be a compensatory response to caspase-3 cleavage induced by diverse pro-apoptotic stimuli, and will be important to address in future studies. Similar discrepancies were found for caspase-3 protein expression, where melatonin treatment resulted in reduced

expression in the membrane array, while it enhanced the expression of its cleaved form (active), as shown by immunofluorescence. A logic explanation for this is the post-translational regulation of the caspase pathway, wherein caspase-3 is converted into its cleaved form by melatonin treatment, resulting in an increase of the cleaved form at the expense of a reduction of its inactive, full-length form.

Given that melatonin can suppress angiogenesis directly or indirectly [60–62], in both *in vivo* and *in vitro* models [63,64], we also looked at the effect of treatment on VEGF-A expression. VEGF-A is the most active endogenous pro-angiogenic factor, and it is a specific endothelial cell mitogen, also promoting microvascular permeability [65]. In agreement with a study by Dai et al. using MCF-7 cells [61], we observed VEGF-A protein reduction after melatonin treatment in both MCF-7 and MDA-MB-231 cells. Carbajo-Pescador et al. [60] proposed that melatonin effects on VEGF expression occur at a post-transcriptional level, which could explain the high, possibly compensatory, VEGF-A mRNA expression after melatonin treatment in MDA-MB-231 cells, as opposed to the reduced VEGF-A protein expression caused by the same treatment in our study. In addition to reduced VEGF protein expression, we previously found [29] lower expression of VEGFR2 and VEGFR3 in melatonin-treated compared to vehicle-treated breast cancer xenografts, which associated with significantly reduced microvascular density. Another study [66]

showed a decrease in a number of pro-angiogenic proteins, including EGF, ENA-78, bFGF, IL-8, Leptin, MCP-1 and PDGF-BB, in MDA-MB-231 cells treated with melatonin.

Complementary to the aforementioned findings, melatonin was also found to suppress HIF-1 α transcriptional activity during hypoxia [67]. In this regard, it will be interesting to address whether the G13D *K-ras* mutation that exists in MDA-MB-231 cells [68] could be responsible for the different effect of melatonin of VEGF mRNA expression in MCF-7 versus MDA-MB-231 cells, as mutant over-active RAS is a potent inducer on VEGF-A transcription in epithelial cells, in part by inducing HIF-1 α protein expression [69]. In our study, IL-25 and siIL-17B treatments also reduced VEGF-A protein expression in breast cancer cells, although addition of these treatments to melatonin did not significantly enhance the effect of the latter. Studies looking specifically at IL-25 effect on angiogenesis mediators in cancer cells are scarce. Several studies reported correlations between IL-17 family expression and microvessel density in human ovarian cancer [70], hepatocellular carcinoma [71], and non-small-cell lung carcinoma [72]. According to Liu et al. [73] and Maniati and Hagemann [74], IL-17 (a.k.a. IL-17A) can modulate angiogenesis directly (through IL-17R binding on endothelial cells), or indirectly, by stimulating cancer cells to produce angiogenic factors, including VEGF.

Finally, it is noteworthy that we did not observe a significant enhancement of pro-apoptotic or VEGF-reducing effects by combined engagement of IL-25/IL-17RB signaling with melatonin. Possible explanations for this could be an overlapping mechanism of action of the two approaches, or inadequate timing of combination of the different targeting modalities. A better understanding of the mechanism of action of IL-25 will facilitate the design of single or combined agent strategies for effective tumor targeting in the future.

5. Conclusions

Our study demonstrated the independent efficacy of melatonin and IL-25 in reducing cell viability of ER-positive and triple negative breast cancer cell lines with minimal effect on non-tumorigenic cells. In addition, melatonin treatment or the modulation of interleukins 25/17E and 17B promoted apoptosis in both monolayer and three-dimensional cultures, and reduced VEGF-A protein expression. Although the combined treatments did not significantly enhance the individual effects of the tested approaches, our results independently confirm the effectiveness of melatonin and IL-25/siIL-17B as individual agents with a dual capacity to enhance apoptosis and potentially inhibit angiogenesis. Future combination approaches should aim at enhancing these capacities by targeting possible compensatory pathways.

Conflict of interest statement

The authors declare that they have no competing interests.

Funding

This research was funded by the Fundacao de Amparo a Pesquisa do Estado de Sao Paulo – FAPESP (grants # 2012/06098-0 and 2012/02128-1) and Fundacao de Apoio a Pesquisa e Extensao de Sao Jose do Rio Preto – FAPERP (grant # 175/2014). The Laboratory of Molecular Research in Cancer (LIMC, FAMERP, Brazil) and the Laboratory for Integrated Study of the Mechanisms of Breast Cancer Invasion and Metastasis (University of Guelph, Canada) provided the infrastructure to carry out this project. The latter was funded by a Canadian Foundation for Innovation (CFI; project # 26742) and Ministry of Research Infrastructure (MRI, Ontario; grant # 460342) grant to A.V.P.

Authors' contributions

GBG designed the study, carried out all experiments assessing cell response to treatments, including qRT-PCR, immunofluorescence

staining, and statistical analysis, and drafted the manuscript. TFB participated in experimental design, gene silencing and qRT-PCR experiments, and revised the manuscript. LBM carried out the membrane array assay and analyzed the results. MGM and BVJ carried out the cell viability assay, contributed to the interpretation of results and revised the manuscript. GBC and MCF helped with monolayer and tridimensional cell cultures and with the interpretation of results. AMVP supervised the research, helped with the immunofluorescence staining, analysis and interpretation of results, and drafted the manuscript. DAPCZ conceived and designed the study, coordinated the research, helped with result interpretation and drafted the manuscript. All authors read and approved all aspects of the final manuscript.

Acknowledgements

We thank Livia Carvalho Ferreira, Camila Leonel and Gustavo Rodrigues Martins for their help with manuscript writing and editing, and Dr. Patricia Simone Leite Vilamaior for helping in capture and processing of confocal 3D images (ZEISS, model LSM 710, software ZEN 2010, Thornwood, NY, USA).

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