

Research Article

## Melatonin down-regulates microRNA-10a and decreases invasion and migration of triple-negative breast cancer cells

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### ABSTRACT

Breast cancer metastasis is one of the main factors associated with high mortality rates among patients. MicroRNAs (miRNAs) play an important role in gene expression regulation, and are associated with the metastatic process in breast cancer. Melatonin, a hormone secreted mainly in the pineal gland, has several oncostatic effects. The aim of this study was to investigate the action of melatonin in the modulation of miRNA-10a-5p and its association with metastatic mechanisms. We have evaluated the effects of melatonin on cell viability in MDA-MB-468 cell line after 24 hours of treatment. MDA-MB-468 and MDA-MB-231 cells were either transfected with inhibitor of miR-10a, or received a scrambled miRNA sequence as a negative control, then these cells were treated with or without melatonin. Gene expression of miR-10a was verified by real-time PCR. Invasion and migration assay using matrigel inserts were performed. The protein expression was analyzed by western blotting to quantify the epithelial-mesenchymal transition (EMT) markers (E-cadherin, claudin 7, and vimentin) and proliferation marker (PIK3CA). Our results showed that melatonin (1 mM) significantly decreased cell viability, and also affected miR-10a expression, which suppressed cell invasion and migration. Melatonin reduced vimentin and claudin 7 protein expressions, and increased E-cadherin. In contrast, inhibition of miR-10a reduced vimentin and did not modulate claudin 7 and E-cadherin. In conclusion, we demonstrated the effectiveness of melatonin in decreasing miR-10a, affecting invasion and migration, and proteins involved with the EMT process, which supports its potential role in the regulation of metastasis.

**Keywords:** melatonin, microRNAs, epithelial-mesenchymal transition, metastasis, pineal gland, breast neoplasms.

## 1. INTRODUCTION

Breast cancer is one of the major malignancies that affect women. About 1.7 million new cases are diagnosed annually worldwide (1). High mortality rates of this disease are reported, mainly due to complications of the metastasis (2, 3). Early diagnosis, the introduction of more effective treatments, and a better quality of life for patients can lead to the decline of cancer-related deaths (4).

Metastasis is a complex process capable of allowing the colonization of tumor cells and the formation of tumors in new sites, distant from the primary tumor (5). The process involves a series of biological events, such as angiogenesis, cell migration, invasion of the basement membrane and extracellular matrix, penetration of cells into the bloodstream, and finally fixation and tumor growth in another organ (6). One of the crucial processes for metastatic development is epithelial-mesenchymal transition (EMT), which consists in transition of cellular phenotype with dissociation of the tight-junction, adherents junction, and desmosomes, as well as loss of the apical polarity decreasing the cell-cell contact (7, 8).

In association with the tumorigenic process, microRNAs (miRNAs) can regulate the expression of genes related to metastasis (9, 10). MiRNAs are small non-coding RNA molecules, containing 18-24 nucleotides, which play an important role in the regulation of gene expression (11). In breast cancer, oncomicroRNAs (oncomiRs) such as miR-21, miR-155 and miR-125b are deregulated and promote tumor progression (12–15). Other important miRNAs, such as miR-10a and miR-10b, have oncostatic activity and are related to the metastatic process (16, 17). Both miRNAs are encoded by the *Hox* cluster genes, and differ in only one nucleotide. However, their targets and mechanisms of action may be different (18, 19).

A large number of studies have demonstrated the efficiency of melatonin in controlling growth and metastasis in lung cancer, glioma and breast cancer (20). Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine, secreted mainly by the pineal gland according to circadian rhythm. Physiologically, this molecule is involved in the modulation of neuroendocrine axis, lipids and carbohydrates metabolism, circadian rhythm, and oxidative status, among others (21). In breast cancer, melatonin promotes apoptosis, decreases tumor growth *in vitro* and *in vivo*, attenuates cell proliferation, angiogenesis and, above all, controls metastasis (22–25). In addition, recent studies have investigated the ability of melatonin to modulate microRNAs (26–32).

In this study, we investigated the role of melatonin in the modulation of miR-10a-5p in triple-negative breast cancer (TNBC) cell lines. In addition, we verified the relation of melatonin with tumor progression through the analysis of cell proliferation-associated proteins and EMT proteins, which indicate the first steps in the development of metastasis in breast cancer. Our results suggest that melatonin has the capacity to regulate the metastatic process by down-regulating miR-10a, invasion/migration and EMT-related proteins.

## 2. MATERIAL AND METHODS

### 2.1. Cell culture.

This study was performed using TNBC cell lines MDA-MB-468 (ATCC Cat# HTB-132, RRID:CVCL\_0419) and MDA-MB-231 (ATCC Cat# HTB-26MET, RRID:CVCL\_VR67). In addition, MCF-7, luminal A breast cancer cells were used

(ATCC Cat# HTB-22, RRID:CVCL\_0031). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/L), supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution containing penicillin and streptomycin (LGC Biotechnology). Cells were maintained in culture at 37°C in atmosphere containing 5% CO<sub>2</sub> and 95% air.

## 2.2. MTT assay of cell viability.

Cell viability was measured using the Vibrant MTT Cell Proliferation Assay Kit 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Invitrogen, Life Technologies, Cat# V13154). Individual wells (96-well plate) were inoculated with 100 µL of culture medium containing 5×10<sup>4</sup> cells. Cells were incubated in medium supplemented with 2% FBS and different concentrations of melatonin (Sigma-Aldrich, Cat# M5250) 0.0001, 0.001, 0.01, 0.1 and 1 mM. The control group received the vehicle solution (ethanol 100% : PBS). The treatment was maintained for 24 hours, and then 10 µL of MTT solution was added in each well followed by incubation for 1 hour. Subsequently, for solubilization of crystals formed from the metabolism of MTT, 100 µL of the dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Cat# W387509) was added and maintained for 10 minutes. The absorbance was measured on ELISA reader at 570 nm by ThermoPlate TP-Reader equipment (ThermoPlate). The percentage of cell viability (%) was calculated for all groups compared to the control sample. The assays were performed in triplicate.

## 2.3. Transient modification of tumor cells.

Transient modification was performed using the Ambion MirVana™ miRNA Inhibitor 10a-5p (ThermoFisher Scientific, Cat# 4427975), consisting of small molecules of single-stranded RNA, which are designed to bind and inhibit the activity of endogenous miRNA. The scrambled miRNA sequence was used as a negative control. Cells were plated (6-well) and transfected with HiPerfect Transfection Reagent (Qiagen, Cat# 301704).

## 2.4. RNA extraction, reverse transcription, and quantitative PCR real-time (RT-qPCR).

Quantitative real-time PCR was performed to evaluate the gene expression of miR-10a. The cells were plated (6-well) (10 cm<sup>2</sup> each) and considered 4 groups for each cell line including: Control; Melatonin treatment; Negative control; Inhibitor miR-10a. The treatments were performed in triplicate and maintained for 24 hours.

Total RNA samples were extracted from all groups using miRNeasy Mini Kit (Qiagen, Cat# 217004) following the manufacturer's recommendations. Quantification and RNA quality were evaluated using NanoDrop 2000C Spectrophotometer (ThermoFisher Scientific). For reverse transcription, TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems, Cat# 4366596) was used as recommended by the manufacturer. qPCR real-time was evaluated by StepOnePlus™ (Applied Biosystems, RRID:SCR\_015805). Reactions for expression analysis were performed with TaqMan™ Universal PCR Master Mix (Applied Biosystems, Cat# 4304437), TaqMan Assay (Applied Biosystems) and 10 ng cDNA. To normalize miRNA expression, the endogenous U6 was used as housekeeping. The levels of relative expression between samples were calculated using relative quantification method ( $2^{-\Delta\Delta Cq}$ ) (33).

## 2.5. Cell invasion and migration assay.

To evaluate the invasion and migration capability of cells after treatments, inserts, having pores approximately 8  $\mu\text{m}$  in diameter with matrigel, were used (BD Biosciences, Cat# 54480). Cells ( $0.8 \times 10^5$ ) were resuspended in 300  $\mu\text{L}$  incomplete culture medium, and arranged in the compartment of each insert. In the lower compartment of plates (24-well), 500  $\mu\text{L}$  of complete culture medium (10% FBS) was added, followed by incubation for 24 hours. Then, the inserts were fixed with paraformaldehyde 4%, and stained with violet crystal. The migratory cells contained in the lower compartment of the insert were counted using Nikon Eclipse E200<sup>®</sup> microscope. The assays were performed in triplicate for each group.

## 2.6. Protein extraction and western blotting.

Protein extraction from tumor cells was performed with lysis buffer containing NP40, protease and PMSF (Sigma-Aldrich, Cat# 10837091001) and quantified with BCA Protein Assay Kit (ThermoFisher Scientific, Cat# 23227). Equal amounts of protein (50  $\mu\text{g}$ ) were electrophoretically separated on 10% SDS-PAGE gel and transferred to PVDF membranes (BioRad, Cat# 1620177). Membranes were blocked for 1 hour in TBS-T + 5% BSA. Then, membrane was incubated with primary antibodies vimentin (1:100 - Millipore Cat# AB1620, RRID:AB\_90774); Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA) (1:500 - Abcam, Cat# ab135958); claudin 7 (1:100 - Abcam Cat# ab27487, RRID:AB\_470990); E-cadherin (1:1000 - Cell Signaling Technology Cat# 3195, RRID:AB\_2291471) and  $\beta$ -actin (1:5000 - Sigma-Aldrich Cat# A5316, RRID:AB\_476743) overnight at 4°C. Specific secondary antibody was added, incubated for 1 hour at room temperature, and visualized by enhanced chemiluminescence Clarity Western ECL substrate (Bio-Rad, Cat# 1705061) for visualization on Fusion Capt Advance apparatus. Finally, quantification was verified using IMAGE J<sup>®</sup> software (RRID:SCR\_003070).

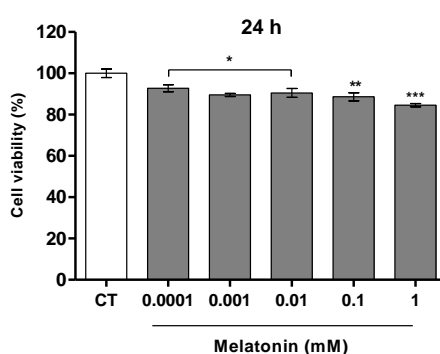
## 2.7. Statistical analyses.

The results have been previously submitted to descriptive analysis to determine normality. For samples with normal distribution, Student's t-test (two samples) or Analysis of Variance (ANOVA), followed by the Bonferroni's test (more than two samples). Data express the mean  $\pm$  Standard Error of the Mean (SEM). Values of  $p \leq 0.05$  were considered significant, and analyses were verified using GraphPad Prism 5 software (GraphPad Software, RRID:SCR\_002798).

## 3. RESULTS

### 3.1. Melatonin decreases cell viability.

In order to determine the most effective melatonin concentration for MDA-MB-468 cells, the MTT colorimetric assay was performed. As shown in figure 1, all concentrations of melatonin decreased cell viability. However, the pharmacological concentration of 1 mM showed the best statistical values compared to control cells.

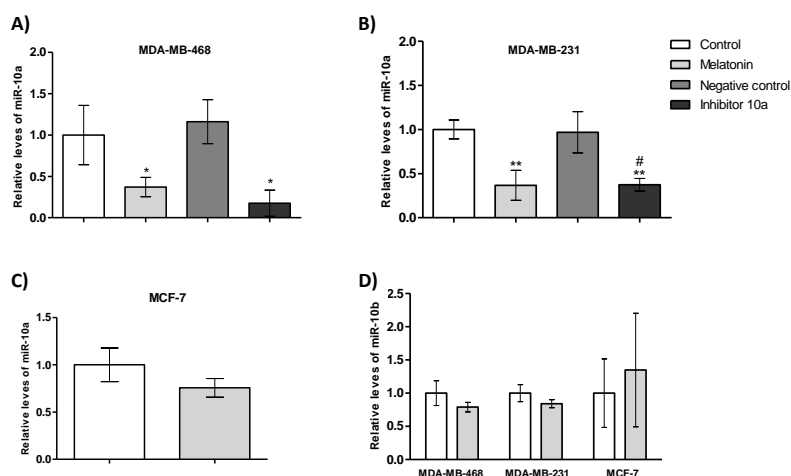


**Fig. 1. Melatonin affects MDA-MB-468 cell viability.**

The cells were treated for 24 hours with the different concentrations of melatonin or vehicle. MTT assay was performed, and all concentrations decreased cell viability when compared to the control group. The 1 mM concentration presented the best statistical values. Data are shown as mean  $\pm$  SEM of triplicate (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus CT: control group) (one-way ANOVA and post hoc Bonferroni).

### 3.2. Melatonin down-regulates miR-10a in triple-negative breast cancer cell lines.

To investigate the actions of melatonin on miR-10a in breast cancer tumor cells, we evaluated its expression by RT-qPCR. In both triple-negative cell lines MDA-MB-468 and MDA-MB-231, miR-10a was significantly down-regulated after melatonin treatment (Figure 2A and 2B). In MCF-7 cells, it was possible to observe a modest decrease of miR-10a on the same concentration, but it was not significant statistically (Figure 2 C). In additional, one of the miR-10 family members, the miR-10b, was also analyzed in order to observe if melatonin could regulate its expression. The analysis showed no difference in miR-10b expression among the groups with or without melatonin treatments (Figure 2D). Based on the results, only miR-10a was selected for further investigation. Then, both TNBC cells were transiently transfected with Inhibitor miR-10a, and all groups were used for comparisons.



**Fig. 2. Melatonin decreases miR-10a expression in triple-negative cell lines.**

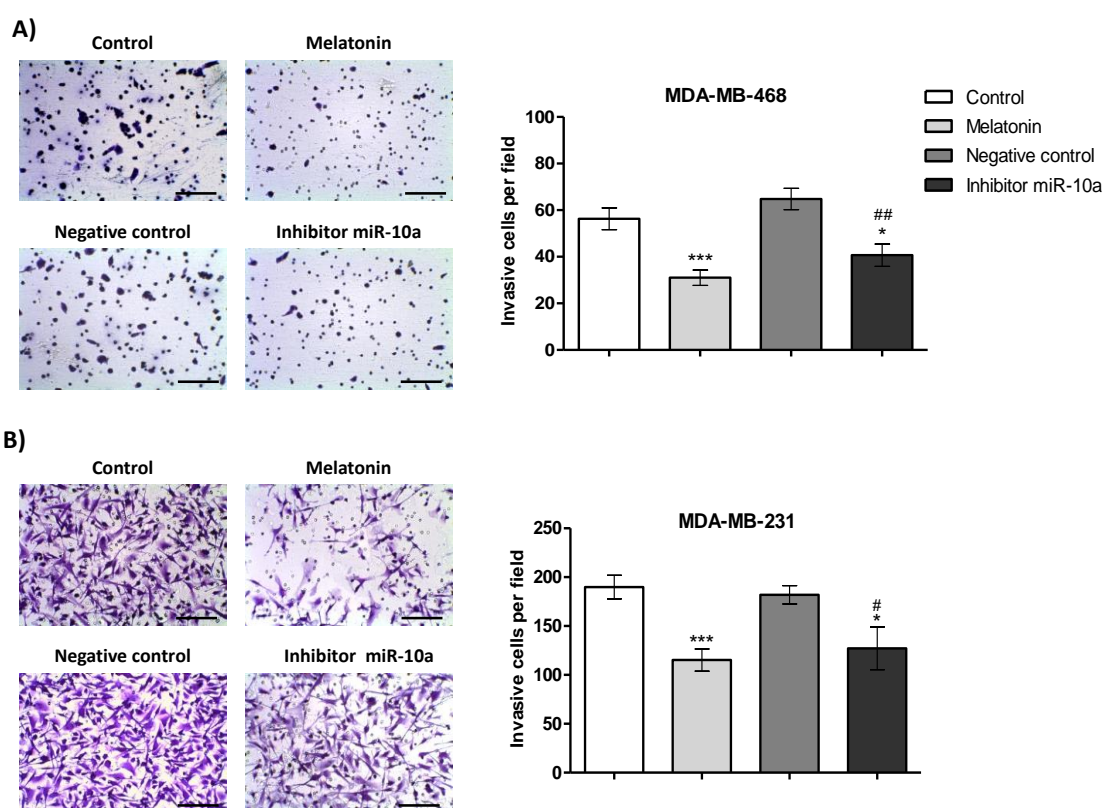
All breast cancer cell lines were treated with melatonin (1 mM). TNBC cells were transfected with Inhibitor miR-10a or Negative control. Relative levels of miR-10a and miR-10b expression were verified by RT-qPCR. The treatment reveals that melatonin decreased miR-10a in TNBC cells (A) and (B). (C) A statistically non-significant decrease



of miR-10a was observed in MCF-7 cells. (D) No difference in miR-10b expression in breast cancer cell lines. The data were determined on a log2 scale, since fold change varies with mean of Ct  $\pm$  SEM in triplicate (\* $p < 0.05$ , \*\* $p < 0.01$  versus control group; # $p < 0.05$  versus negative control) (Student's *t*-test).

### 3.3. Melatonin and miR-10a suppression impair invasion and migration.

To investigate the potential role of melatonin and miR-10a on TNBC cell lines, invasion and migration assays were performed. The data showed that melatonin affected cell invasion and migration in both MDA-MB-468 and MDA-MB-231 cell lines. In addition, as expected, low levels of miR-10a impaired invasion and migration of cells, when compared with the control group (Figure 3A and 3B).



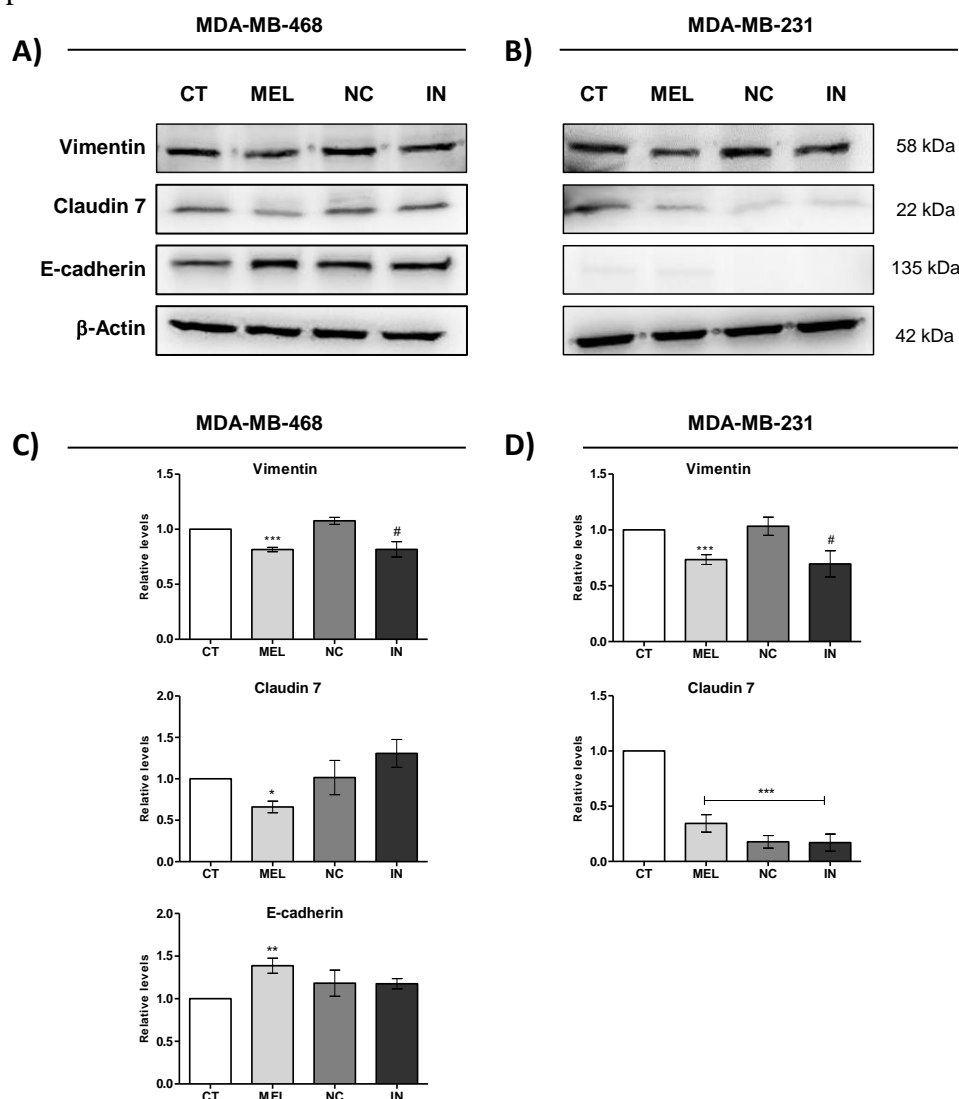
**Fig. 3. Melatonin and Inhibitor of miR-10a reduces invasion and migration.**

MDA-MB-468 (A) and MDA-MB-231 (B) cells were treated with melatonin or transfected with Inhibitor of miR-10 for 24 h and subjected to matrigel invasion assays. Histograms represent cell invasion/migration rate. Data are shown as mean  $\pm$  SEM in triplicate (\* $p < 0.05$ , \*\*\* $p < 0.001$  versus control; ## $p < 0.01$  versus negative control) (Student's *t*-test). Scale bars: 50 $\mu$ m.

### 3.4. Melatonin and Inhibitor of miR-10a modulate EMT-related proteins.

To investigate the influence of melatonin and miR-10a, we evaluated protein expression related to epithelial-mesenchymal transition by western blotting. The protein levels of vimentin, E-cadherin, and claudin 7 were verified in MDA-MB-468 and MDA-MB-231 cell lines. The relative quantification of proteins showed a decrease of vimentin

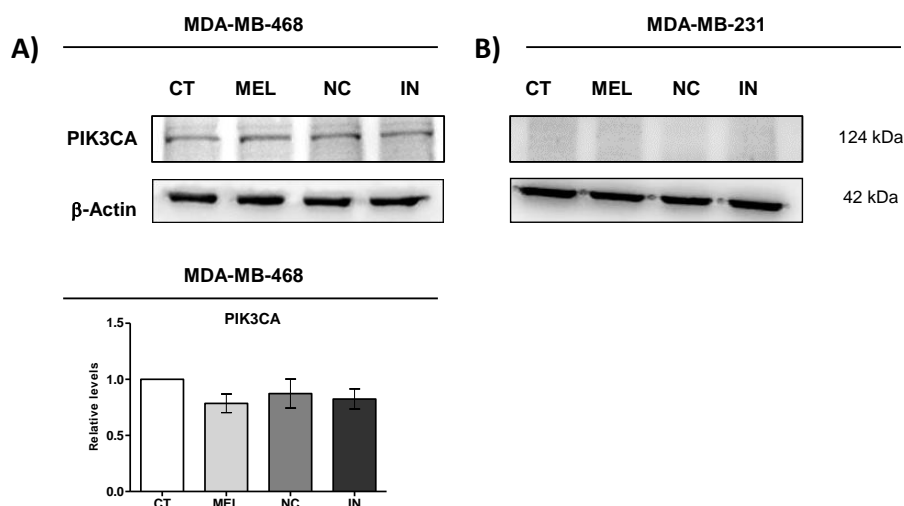
and claudin 7, but an increase of E-cadherin after melatonin treatment in MDA-MB-468 cells. Regarding cells showing inhibition miR-10a, it was possible to observe a decrease of vimentin expression, but not modulation of claudin 7 and E-cadherin when compared with control groups (Figure 4A and 4C). In MDA-MB-231 cell line (Figure 4B and 4D), the melatonin and Inhibitor of miR-10a also decreased the protein vimentin and claudin 7. The protein levels of E-cadherin could not be measured because of the low expression of this protein in cells.



**Fig. 4. Melatonin and Inhibitor of miR-10a modulate EMT-related proteins.**

MDA-MB-468 and MDA-MB-231 cells were treated with melatonin for 24 h or transfected with Inhibitor of miR-10a. The extracted proteins were subjected to western blotting with the indicated antibodies. Protein expression was quantified by Image J program, calculated relative to controls and normalized with the endogenous  $\beta$ -Actin. Blots and bar graphs show protein expression of vimentin, claudin 7 and E-cadherin in MDA-MB-468 (A, B) and MDA-MB-231 cells (C, D). Data represent the mean  $\pm$  SEM of three independent experiments \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001, versus control; # $p$ <0.05 versus negative control) (one-way ANOVA and post hoc Bonferroni). CT: Control, MEL: Melatonin, NC: Negative Control, IN: Inhibitor of miR-10a.

In addition, we evaluated protein expression of PIK3CA, considered a potential target of miR-10a, and which correlates with survival and proliferation. The data demonstrated a slight, but not significant, decrease of PIK3CA protein expression on MDA-MB-468 cells treated with melatonin (Figure 5A). The same was observed on cells with Inhibitor of miR-10a. Regarding MDA-MB-231 cells, it was not possible to verify the presence of PIK3CA expression (Figure 5B).



**Fig. 5. Melatonin slightly decreased PIK3CA expression.**

MDA-MB-468 and MDA-MB-231 cells were treated with melatonin for 24 h, or transfected with Inhibitor of miR-10a. The extracted proteins were subjected to western blotting with the indicated antibody. Protein expression was quantified by Image J software and normalized with the endogenous  $\beta$ -Actin. Blots and bar graphs show protein expression of PIK3CA in MDA-MB-468 (A) and MDA-MB-231 cells (B). Data represent the mean  $\pm$  SEM of three independent experiments. (One-way ANOVA and post hoc Bonferroni). CT: Control, MEL: Melatonin, NC: Negative Control, IN: Inhibitor of miR-10a.

#### 4. DISCUSSION

The potential therapeutic use of melatonin as an antitumor agent has been demonstrated by several studies (24, 34, 35). The actions of this hormone reveal regulation in controlling the growth of some human breast tumors (36). Our results demonstrated the ability of melatonin to control the metastatic process by regulating miR-10a activity, decreasing invasion and cell migration, and modulating EMT-related proteins in TNBC cell lines. Melatonin down-regulates miR-10a, which may be involved in the tumor invasion process. In addition, we observed the action of melatonin in decreasing vimentin and claudin 7 proteins, and increasing E-cadherin. According to the literature, this molecule regulates the proliferation of mammary tumor cells, angiogenesis-promoting proteins, and reduces breast cancer tumors in xenographic model (37–39). Melatonin also acts in metastatic control (22) and decreases the ability of tumor cells to invade and migrate (40).

Melatonin acts in cancer cells mainly through their membrane receptors MT1 and MT2 (41). Both ER-positive and ER-negative cells express these receptors. ER-positive cells are characterized by high levels of MT1 and several studies have demonstrated melatonin



action in these cells being through the classical pathway of membrane receptors (34, 36). In ER-negative cells, the action of melatonin has been associated with its lipophilic nature, which allows its passage through cell membrane and interaction with several cytoplasmic molecules and transcription factors present in the nucleus of cells (34, 42).

Relevantly, we observed that melatonin at a concentration of 1 mM decreased the viability of ER-negative MDA-MB-468 cells during 24 hours of treatment. Our results are consistent with, Mao and collaborators (43). They studied the activity of melatonin in ER-negative cell line SK-BR-3 and verified a reduction in cell viability. Our group has also demonstrated that this indoleamine affects cell viability of ER-positive MCF-7, and triple negative MDA-MB-231 and MDA-MB-468 cells after 24 and 48 hours of treatment, respectively (28, 37, 44). Similar studies have explained this action on the promotion of autophagy. As a consequence, the apoptosis ability of the tumor cells is induced (45).

Recently, our group demonstrated thirteen melatonin-modulated miRNAs in breast cancer cell line of MDA-MB-468 (28). Among them, we observed a significant decrease of miR-10a after melatonin treatment. High expression of this miRNA is described in metastatic TNBC cell lines compared with non-tumoral cells (46). Its action may be related to tumor progression, invasion and metastasis (47). There are no studies demonstrating the action of melatonin on the regulation of miR-10a yet. Thus, in order to investigate its role in breast cancer, we suppressed miR-10a expression by transient transfection in triple-negative cell lines. Our results revealed that melatonin and inhibition of miR-10a decreased invasion and migration of tumor cells.

In other types of tumor, it has already been observed that miR-10a may act on adhesion molecules located on the cell surface, directly impacting the invasiveness and metastatic behavior (48, 49). In breast cancer, contradictory functions of miR-10a are reported (47, 50). As a suppressor, low levels of this miRNA have been reported in patients with breast cancer, and high levels were related to longer relapse-free survival (50–52). On the other hand, as an oncomiR, this miRNA was found to be highly expressed in cells resistant to chemotherapy (53), and this may induce colony formation, and invasion and migration of these cells (47, 54). We also investigated the miR-10b, which is deregulated in some cancers, including metastatic breast cancer (55–59). However, our results showed that melatonin did not significantly modulate the expression of miR-10b in breast cancer cells.

To explore the potential mechanisms regarding that melatonin treatment reduced invasive and migratory capacity in both triple-negative cells MDA-MB-468 and MDA-MB-231, we verified that the inhibitor of miR-10a produced the same results as melatonin, showing low levels of miR-10a may affect the cell dissemination process. Similarly, Mao *et al.* (60) and Borin *et al.* (22) both reported melatonin activity in the inhibition of the invasion and migration process in MDA-MB-231, SK-BR-3 (HER2 positive) and MCF-7 (HER2-Modified) by decreasing protein kinases.

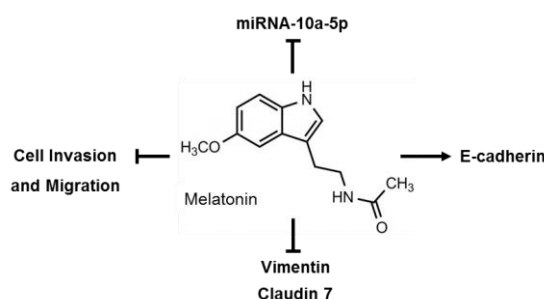
In addition to anti-invasive actions, a suppressive role of melatonin in EMT process was identified. A decrease in vimentin and claudin 7 protein expressions and an increase of E-cadherin in MDA-MB-468 line were observed after melatonin treatment, suggesting its protective role in EMT. Melatonin inhibits the EMT process by interfering with NF- $\kappa$ B signaling (61, 62). The increase of this molecule allows suppression of tumor cells epithelial phenotype, contributing to an increase in vimentin cytoskeleton protein, which permits transition to mesenchymal profile (61). Moreover, it was observed that low levels of miR-10a also decreased expression of vimentin.

In this study, the action of melatonin to increase E-cadherin protein expression in MDA-MB-468 cells was observed. The elevated level of E-cadherin supports the protective role of melatonin in metastatic process. E-cadherin loss causes several changes in cellular junction, allowing cells transition for mesenchymal phenotype, the initial stage for metastasis (63). Although the increase of E-cadherin in the MDA-MB-468 cells was observed, the same was not verified in MDA-MB-231. The E-cadherin protein expressions are low in this metastatic cell line, a fact noted by other authors too (64). Additionally, miR-10a investigation showed no difference in E-cadherin levels after miRNA inhibition.

The levels of claudin 7 were reduced with melatonin treatment in both TNBC cell lines. In cancer, claudins appear to be linked to worse prognosis (65, 66). Based on the results found in the migration and invasion events, we suggest that the low expression of claudin 7 would imply a better scenario of cancer prognosis. A recent study associated the high expression of this protein with malignancy of breast cancer, contributing to the disease progression (67).

PIK3CA is a component molecule of the signaling pathways that regulates survival and proliferation of cells, and is also associated with several malignancies, acting mainly in promoting tumorigenesis (68, 69). The association between PIK3CA and miR-10a was studied by Ke & Lou (46), who observed that down-regulation of PIK3CA by miR-10a resulted in inhibition of cell proliferation. However, our results did not show modulation in PIK3CA levels after inhibition of miR-10a.

In summary, we identified the action of melatonin on the modulation of miR-10a and EMT-related proteins, as well as suppression of invasion and cell migration. The potential associations among them were illustrated in the figure 6.



**Fig. 6. The illustrations of the possible effects of melatonin in EMT.**

Our data supports the hypothesis that melatonin acts in the metastatic process by targeting some proteins involved in the EMT process. The relation between miR-10a and this process needs to be further studied in order to be better understood.

Collectively, melatonin decreases the miR-10a and EMT-proteins such as vimentin and claudin 7 and increases cellular adhesion protein E-cadherin suggesting the potential role of melatonin reducing the invasion and migration of the tumor cells.

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The funding body had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## AUTHORSHIP

Jessica Gisleine de Oliveira was responsible for the study design, performed all the experiments and analyzed the data obtained. Jéssica Helena de Mora Marques performed the culture of cells, contributed to the analysis the data and to the writing of the manuscript. Jéssica Zani Lacerda and Lívia Carvalho Ferreira assist the experiments the study, contributed to analysis the data and to the writing of the manuscript. Marcelo Mafra Campos Coelho contributed to data analysis and writing of the manuscript. Debora Aparecida Pires de Campos Zuccari was responsible for the study design, supervised all.

## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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