

The evaluation of Melatonin and EGF interaction on breast cancer metastasis

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Research article

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Abstract

Background

Breast cancer is currently one of the most common types of cancer in women, and metastasis is the first cause of death in breast cancer patients. The epidermal growth factor (EGF) increases the invasion, growth, and migration of cancer cells. In the present study, melatonin, as a natural hormone, in EGF-induced tumor metastasis, was investigated.

Methods

First, MDA-MB-231 and MCF7 cells were cultured, and then the effects of melatonin on cell viability were determined by MTT assay. Transwell invasion assay was employed to identify the invasiveness of these breast cancer cell lines. Real-time RT-PCR then investigated the expression of MMP9 and MMP2. Cell proliferation was also determined under EGF and melatonin treatment using Ki67 assessment by flow cytometry.

Results

The rate of invasion and migration of EGF-treated cells increased in both groups, in which melatonin caused increased invasion by EGF in MCF7 cells. MMP9 and MMP2 expression increased significantly in both cell lines under EGF treatment, and melatonin increased these genes' expression in both cell lines ($p < 0.05$). EGF increased the MMP9 and MMP2 gene expression, and melatonin increases EGF-induced expression ($p < 0.05$). The EGF reduced the expression of the Ki67 protein in the MCF7 cell line, which was negatively affected by Melatonin and EGF. In contrast, along with Melatonin, EGF did not affect the proliferation of the MDA-MB-231 cell line.

Conclusions

Our results show that melatonin, as a natural compound, can increase the effects of EGF in the proliferation, migration, and invasion of cancer cells at low dosages.

Background

The epidermal growth factor receptor (EGFR) family and its associated ligands are involved in proliferation, angiogenesis, metastasis, and tumor cell invasion (1, 2). EGF-related signaling pathways, including phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) and mitogen-activated protein kinases (MAPKs), have a central role in the function of various cancer cells(3, 4). However, depending on the cellular context, different cancer cells demonstrate paradoxical responses to EGFR signaling. In this regard, it has been shown that EGF-treatment promotes survival signals in some cancer cells, while others

induce cell death (5). Moreover, activation of EGFR by EGF could activate MMPs, which consequently contribute to cancer cell motility and invasion (2, 3). In particular, overexpression of MMP-2 and MMP-9 and its association with a high metastatic rate has been seen in breast cancer (6, 7).

Breast cancer is one of the most common malignancies among women that, despite various therapeutic advances, it has a high mortality rate in the world (8). Several studies have shown that overexpression of EGFR has a significant role in breast cancer pathogenesis and progression. Furthermore, EGFR has been suggested as a favorable prognostic factor for this cancer (9).

Melatonin (N-acetyl-5-methoxytryptamine) - a hormone secreted mainly through the pineal gland - has different functions in controlling circadian rhythms, seasonal reproduction, and immune system modulation(9, 10). It has recently been reported that melatonin also has an anti-tumor activity by inhibiting proliferation and angiogenesis and even inducing apoptosis in different cancers such as ovarian and breast cancers (9-11). It has shown that melatonin exhibits its effects via receptor-dependent (MT1 and MT2) or -independent processes (12). Furthermore, some different evidence points pointed towards underlying mechanisms by which melatonin exerts its oncogenic actions (13, 14). For example, the interaction between melatonin and estrogen-responsive pathway of MCF-7 tumor cells might be responsible for the oncostatic properties of melatonin (15). Many studies have been carried out to explain the underlying molecular mechanisms of the anti-tumor effects of melatonin. Interestingly, melatonin exhibits crosstalk with the EGFR signaling pathway, especially in cancer cells (15-17). Given the critical role of EGF in breast cancer development and the presence of an association between Melatonin and EGF, in the present study, we investigated the effects of Melatonin on EGF-induced metastasis in breast cancer cells, as well as the underlying molecular mechanisms.

Methods

Cell culture

MDA-MB-231, MCF-7 (breast carcinoma cancer) cell lines were obtained from the National Cell Bank of Iran (NCBI) affiliated to the Pasteur Institute of Iran (Tehran, Iran). The cells were cultured in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) 100 IU/ml penicillin and 100 mg/ml streptomycin (Gibco) under a humidified atmosphere (95 %) at 37 °C with 5 % CO₂. All the cells used in the current experiment were at passage 3–6. Melatonin (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO, Merck, Germany) to prepare 1 M as our stock solution. After that, the stock solution was diluted with DMEM/LG (Gibco, USA) to achieve a final concentration of 0.1 mM as an effective concentration. The maximum concentration of DMSO for each experimental condition was less than 0.01%.

Cell viability assay

MTT assay was applied to evaluate IC₅₀ of Melatonin. First, the cells (10⁴ cells/well) were plated in 96-well culture plates. After incubation of the cells with different concentrations of melatonin, the media was replaced with fresh media containing 2 mg/ml MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) (Roche Molecular Biochemicals, Indianapolis, IN) solution and incubated for an additional four h at 37 °C. Then, the solution was replaced with a 200µl DMSO solution. Finally, the absorbance value was measured at 570 nm using a Micro-plate reader system (BioTek, USA). IC₅₀ was determined for each agent by calculating the slope and intercept of different concentrations. The experiment was performed in triplicate and repeated three times. After determining the IC₅₀ values for melatonin, all experiments were performed in 6 groups, including control groups of MCF-7 and MDA-MB-231 cell lines and melatonin treated groups(0.625mM) absence or presence of EGF (100 ng/mL) for 48h.

Flow cytometry analysis

According to the manufacturer's instructions, a flow cytometry assay was performed to evaluate cancer stem cells' surface markers. Briefly, 24 h post-treatment, cells were washed twice with PBS solution and diluted into 100µl of flow cytometry buffer (PBS solution/BSA 0.5%, pH 7.2). Then the suspension was incubated with 10µl of both PE-CD44 (Miltenyi Biotec, Germany) and FITC-CD24 (Miltenyi Biotec, Germany) antibodies for 10 minutes at 4 °C. Finally, the cells were centrifuged and immersed in the buffer to remove the unconjugated antibodies. Samples were analyzed by the FACSCalibur™ (BD Bioscience, NJ, USA) and processed by FlowJo ver. 7.6.1 software. Appropriate isotype-matched antibodies identified nonspecific protein labeling.

Cell proliferation assay

After harvesting and counting 48-hour-treated cells, 100µl flow cytometry buffer (PBS solution supplemented with 0.5% BSA, pH 7.2) was added into the cell pellet (1×10⁶ cells). The cells were treated with 0.1% Triton X100 for 1 minute to make the cell membrane relatively permeable. After centrifugation (at 300×g for 10 minutes), the supernatant was discarded, and the cell pellet was incubated (at 4 °C for 20 minutes) in 100 µl flow cytometry buffer containing 5µl anti-Ki-67 FITC antibody (Miltenyi Biotec, Germany). Then, 1-2 ml of buffer was added to the suspension, and the unconjugated antibodies were removed through centrifugation. Finally, the cell pellet was resuspended into the 500µl buffer and proceeded to flow cytometry analysis. Samples were analyzed by the FACSCalibur™ (BD Bioscience, NJ, USA) and processed by FlowJo ver. 7.6.1 software.

Cell apoptosis assay

According to the manufacturer's protocol, the cell's apoptosis rate is determined by a commercial Annexin V/PI Apoptosis Detection Assay kit (EXBIO). Briefly, After 24h incubation with the desired concentration of melatonin in the presence and absence of EGF, the cells were cultured. They washed with PBS, stained with Annexin V–FITC/PI, and analyzed for apoptotic cells using FACSCalibur™ (BD Bioscience, NJ, USA) FlowJo (7.6.1) software for acquisition and analysis as described before.

RNA isolation and real-time RT PCR

According to the manufacturer's procedure, to analyze the expression level of genes involved in EMT and invasion, total RNA was extracted using Total RNA isolation solution RiboEx (GeneAll). The extracted RNA was reverse-transcribed into cDNA by GeneAll Kit, and Real-time RT PCR performed using particular primers (listed in Table 1). Real-time RT PCR was performed using QuantiTect SYBR Green dye (TaKaRa, Japan) and Rosch system. The expression levels of each gene were analyzed by Pfaffl methods with normalization to the housekeeping gene, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). All experiments were carried out in triplicate.

Table 1: Sequences of primers used for real-time PCR analysis.

Gene	Forward primer	Reverse primer
ZEB1	5'-CTGGAGAAAAGCCCTATCAATGT	5'-CTGTCTTCATCCTCTTCCCTTGT
ZEB2	5'-CAGCCATTACCCAGTTAAGA	5'-CCCGTGTGTAGCCATAAGA
Snail	5'-GAGTTTACCTTCCAGCAGCC	5'-CAGAGTCCCAGATGAGCATT
Vimentin	5'-AGATGCGTGAAATGGAAGAGAA	5'-TAGGTGGCAATCTCAATGTCAA
E-cadherin	5'-TGCCCAGAAAATGAAAAAGG	5'-GTGTATGTGGCAATGCGTTC
B-actin	5'-CAAGATCATCACCAATGCCT	5'-CCCATCACGCCACAGTTTCC
MMP2	5'-CACATAGTGATGGTTCCCCTGTT	5'-CGGCCACTCAGTAGGTGTCTTT
MMP9	5'-ATTTCTGCCAGGACCGCTTCTAC	5'-ATCCGGCAAACCTGGCTCCTTC

Western blot analysis

The western blot analysis was performed as previously described (9). Cells (1.5×10^6) lysed in a protein extraction buffer (25 mm HEPES, 1% Triton X-100, 2 mm EDTA, 0.1 m NaCl, 25 mm NaF, 1 mm Sodium Orthovanadate) containing protease cocktail inhibitor (Roche, Basel, Switzerland) for 30 min on ice. Cell lysates were centrifuged at 12000 g for 20 min. The supernatant was then collected, and concentration of protein was determined with picodrop (Picodrop LTD, Cambridge, UK). The equal amount of protein (~100 µg) loaded at 12% SDS- Acrylamide gel followed by transferring to the PVDF membrane. Skim milk (5%) in PBS was used to block the membrane and then incubated with phospho ERK1/2, phospho P38, ERK1/2, P38, and β -actin antibodies (Santa Cruz Biotechnology, Santa Cruz, USA) overnight at four °C. The HRP-conjugated antibody was applied as a secondary antibody (Santa Cruz Biotechnology, Santa Cruz, USA) for 1 hour at room temperature. Finally, protein detection was performed with an ECL reagent. The density of each band was determined using ImageJ software.

Transwell invasion assay

The effect of melatonin on the invasion of EGF-stimulated MCF-7 and MDA-MB-231 cells evaluated using trans-well chambers. The chamber's upper surface was coated with 30-50µL of matrigel and then incubated at 37 °C for 15-30 minutes. MCF-7 and MDA-MB-231 cells (1×10^6 cells/ml) were seeded in serum-free RPMI 1640 in the chambers and exposed to EGF and melatonin treatment (600 µM). After 24 h of incubation at 37 °C, the cells on the upper surface of the chamber were removed, and the invasive cells on the other side of the chamber were fixed with ethanol 70%. The fixed cells were stained with 0.1% crystal violet and counted using an inverted microscope (Olympus IX71, Japan) at 20 × magnification from at least five random fields. The relative cell invasion calculated using the following equation:

$$\text{Relative cell invasion} = \frac{\text{number of invaded cells in the treated sample}}{\text{number of invaded cells in control}}$$

Statistical analysis

All experiments were performed in triplicate. Results were expressed as mean \pm standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA using GraphPad Prism (ver: 7.0). P <0.05 was considered statistically significant.

Results

Different effects of melatonin on the viability, proliferation, and stemness of MCF-7 and MDA-MB-231 cells

As shown in Figure 1, EGF treatment significantly decreased the cell viability of MCF-7 cells ($P<0.05$). In contrast, melatonin could neutralize the EGF effect and so increased the viability of the cells ($P<0.05$) (Figure 1A). However, treatment of MDA-MB-231 cell line with EGF alone or in combination with melatonin had not a significant effect on the viability of the cells ($P>0.05$) (Figure 1B).

Ki67 as a nuclear protein is an approved marker for cell proliferation, so we did the cell proliferation assay evaluating the protein expression of Ki67. For this purpose, MCF-7 and MDA-MB-231 were treated with EGF alone or with melatonin for 48 h. Flow cytometry results indicated that the expression of Ki67 in MCF-7 cells treated with EGF was reduced compared to the untreated group ($P<0.05$) (Figure 2A). Moreover, in this cell line, treatment with melatonin could significantly increase the levels of Ki67 protein ($P<0.05$) (Figure 2B).

Interestingly, no meaningful change was observed in the concentration of Ki67 among MDA-MB-231 cells without treatment or treated with EGF alone or with melatonin ($P>0.05$) (Figure 2C).

Flow cytometric analysis revealed that after treatment with EGF, the number of cancer stem cells decreased in MCF-7 and MDA-MB-231. Moreover, melatonin significantly increased CD44/CD24 levels in MCF-7 cells. However, melatonin treatment caused only a negligible increase in the level of CD44/CD24 in MDA-MB-231 cells (Figure 3).

The interaction of Melatonin and EGF on apoptosis of MCF-7 and MDA-MB-231

Representative results of flow cytometry showed that in MCF-7 cells, EGF treatment increased apoptotic cell population up to 32.5%. Meanwhile, melatonin treatment decreased this population to 12.3% in EGF-treated MCF-7 cells. In MDA-MB-231 cells, also EGF increased the portion of the apoptotic cell up to 22.6%, and melatonin attenuated apoptosis and reached the apoptotic cell population to 3.22% (Figure 4).

The role of Melatonin on EGF-induced invasion in MCF-7 and MDA-MB-231

As shown in Figure 5, the transwell invasion assay demonstrated that the EGF-induced invasion was modulated by melatonin in both EGF-treated MCF-7 and MDA-MB-231 cells.

Real-time RT-PCR results represented no significant difference in expression levels of MMP2 and MMP9 between EGF-treated and untreated MCF-7 cells. However, EGF could increase the expression levels of these genes in MDA-MB-231 cells. Moreover, the mRNA expression levels of MMP2 and MMP9 in EGF-treated MCF-7 and MDA-MB-231 cells were significantly increased by Melatonin ($P<0.05$, Figure 5A, B)

Different impacts of Melatonin on EGF-induced phosphorylation of ERK1/2 and P38 MAPK

The western blotting analysis demonstrated that phosphorylated and active P38 and ERK1/2 were not considerably present in MCF-7 and MDA-MB-231 cells. However, EGF increased its phosphorylation,

particularly in MCF-7 cells. As shown in Figure 6, melatonin attenuated phosphorylation of ERK1/2, especially in MDA-MB-231 cells. However, melatonin treatment did not alter the phosphorylation of P38.

Discussion

Studies have shown the crucial role of EGF in the regulation of cancer development and progress. Overexpression of EGFR and its signaling pathways are valuable prognostic factors for breast cancer patients (2). Therefore, identifying these processes and using appropriate inhibitors may exhibit the way for the control and treatment of breast cancer. A line of evidence highlighted the anti-tumoral properties of melatonin in various cancers, including breast cancer. However, there is no particular study investigating melatonin regulatory impacts on EGF-induced pathways involved in proliferation, invasion, and migration in breast cancer cells. The purpose of this study is to investigate the modulatory effects of melatonin and underlying molecular mechanism on EGF-induced proliferation and metastasis in breast cancer. Studies have shown that various concentrations of EGF may exert different responses in cancer cell proliferation. Some studies suggest that EGF leads to the downregulation of proliferation, while others indicate that EGF increases cell growth and proliferation. This difference is dependent on the dose of EGF, type of cancer cell, and other cellular and molecular factors(5, 18). In A431 cells, EGF caused cell death, and this inhibitory effect was augmented by transfecting the HER2 gene.

Moreover, in HEK293T cells overexpressing EGFR and HER2, EGF treatment led to cell death and apoptosis (18). Although overexpression of EGFR is associated with apoptosis, there is no consensus about the underlying mechanisms. A study by Song et al. (2009) demonstrated that EGF could activate STAT1, which results in activating P21, caspase1 expression, and induce apoptosis. Additionally, in EGFR-overexpressed cancer cells, EGF activates the P38/MAPK pathway and leads to apoptosis(19).

Consistent with this data, our results showed that EGF treatment decreased cell proliferation and induced cell death and apoptosis in the MCF-7 cell line rather than inducing tumor cell proliferation. Though, proliferation rate and cell death in MDA-MB-231 had not significantly affected by EGF treatment. Moreover, EGF treatment upregulated the P38/MAPK pathway in MCF-7 and MDA-MB-231, particularly in MCF-7 cells. It concluded that the P38/MAPK signaling pathway is involved in the induction of apoptosis and reducing cell proliferation. A growing number of works leads to investigate the inhibitory effects of melatonin on cancer cell proliferation. Accordingly, the effective dose of melatonin varies among different cancer cells, including colon, liver, and ovarian cancer cells; however, studies have estimated the effective treatment of melatonin on breast cancer cells within the low nanomolar concentration range (9, 19). We observed that melatonin could reverse apoptosis induction and proliferation inhibitory effect of EGF. Similar to our results, another study showed that melatonin exerted a stimulatory effect on normal and cancer cell growth(20). Invasion and migration are the main characteristics of metastatic cancer cells. It has demonstrated that melittin-a natural peptide- inhibited EGF-stimulated invasion via phosphorylation of FAK and expression of MMP9(3). Another compound, SIP-SII, suppressed p38/MAPK and PI3K/Akt/mTOR signaling pathways to inhibit migration and invasion of EGF-induced SKOV-3 cells(21). Also, the Cannabidiol inhibitory effect has been studied on EGF-induced breast cancer cells, and the

involvement of ERK and AKT in this process has been confirmed(22). However, brain-derived neurotrophic factor (BDNF) increased proliferation and migration of neural stem/progenitor cells via the PI3K/Akt pathway(23). The EGF-augmented invasion has been reported in MDA-MB-231 and MCF-7 cells(3, 24). Our findings correspond well with the mentioned studies showing that EGF enhances invasion and migration in MDA-MB-231 and MCF-7 cells. The transwell invasion assay results indicate the additive effect of EGF on invasion in MDA-MB-231 and MCF-7 cells. However, MCF-7 cells did not demonstrate a significant increase. This effect is probably due to low levels of EGFR in MCF-7 compared to MDA-MB-231 cells. Matrix metalloproteinases (MMPs) have substantial roles in the growth, metastasis, angiogenesis, and invasion of tumor cells(25–27). Overexpression of MMP9 led to decreased expression of E-cadherin in ovarian cancer cells(28).

In our study, Real-time RT-PCR results indicate that EGF increased MMP9 and MMP2 in MCF-7 and MDA-MB-231 cells. However, this increase was not significant in the MCF-7 cell line. Similar to our results, EGF-induced overexpression of MMP9 and MMP2 in MCF-7 has been reported in Majumder A et al. research(29).

Conclusions

In the present study, melatonin's modulatory effects on EGF-induced proliferation, apoptosis, and invasion in breast cancer cell lines were investigated. EGF treatment could reduce the proliferation rate and increase apoptosis by activating the P38/MAPK signaling pathway. Moreover, EGF enhanced invasion by upregulation of MMP2, MMP9. As a result, melatonin strengthened EGF-enhanced breast cancer properties; in this regard, melatonin failed to counteract the EGF-induced signaling pathway through EGFR.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: MA, DV and NR: Conceptualization; Supervision; Methodology, Resources. MA and AF: Data curation. MA, MFM and SB: Formal analysis; Writing - original draft. MA, PG, APT and AF:

Investigation, Project administration; Validation. MA, NM, YF and MT: Writing - review & editing. All authors read and approved the final manuscript.

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Figures

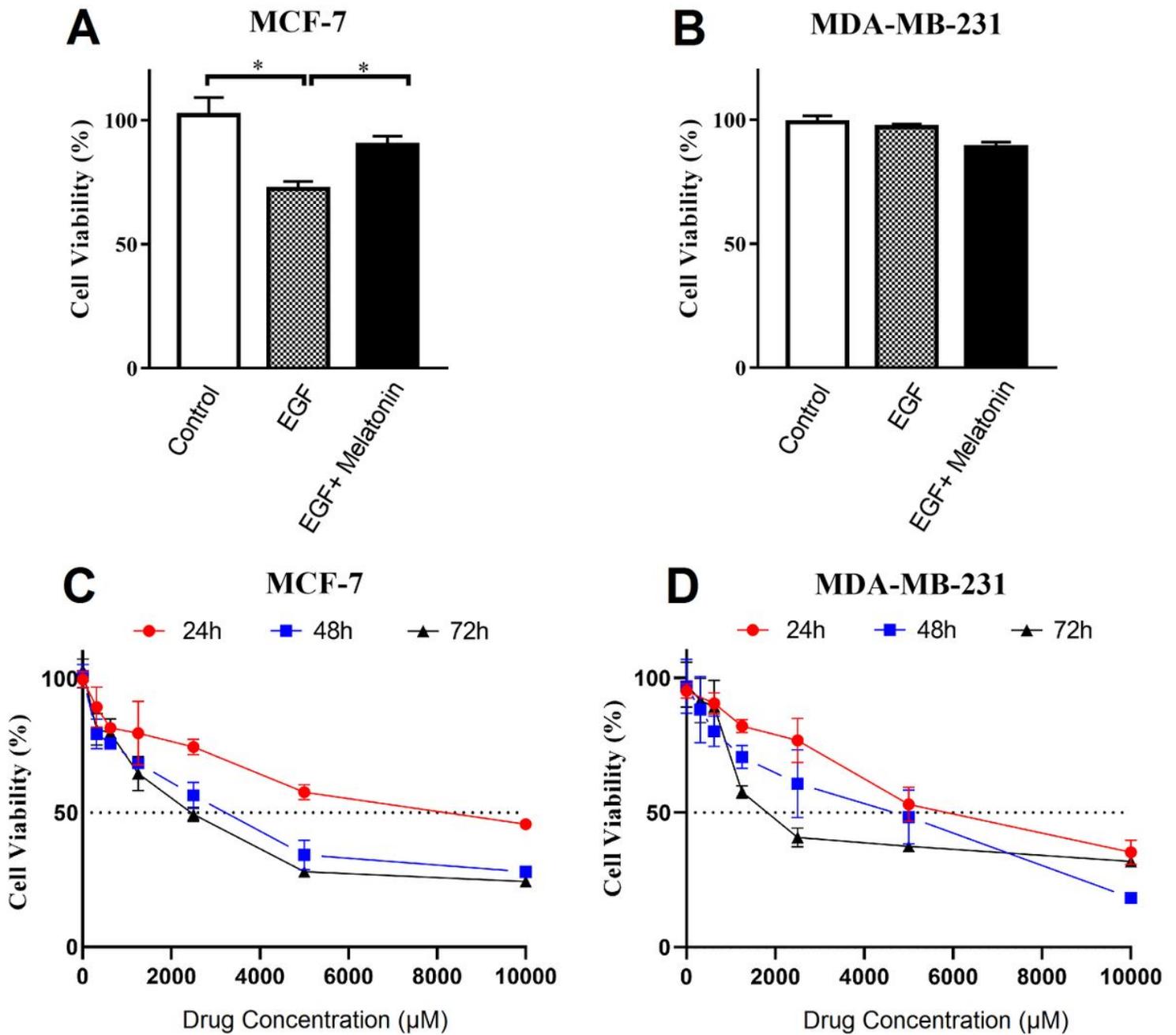


Figure 1

Effects of Melatonin on the cell viability of MCF-7 and MDA-MB-231231 treated with EGF. EGF treatment significantly decreased the cell viability of MCF-7 cells ($P < 0.05$), whereas melatonin could neutralize the EGF effect and so increased the viability of the cells ($P < 0.05$). Treatment of MDA-MB-231 cell line with EGF alone or in combination with melatonin had not a significant effect on the viability of the cells ($P > 0.05$).

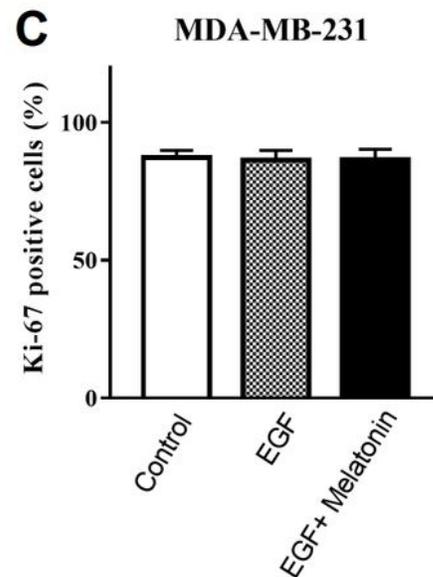
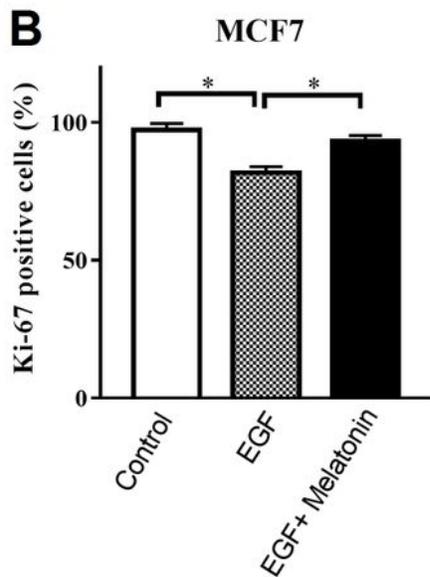
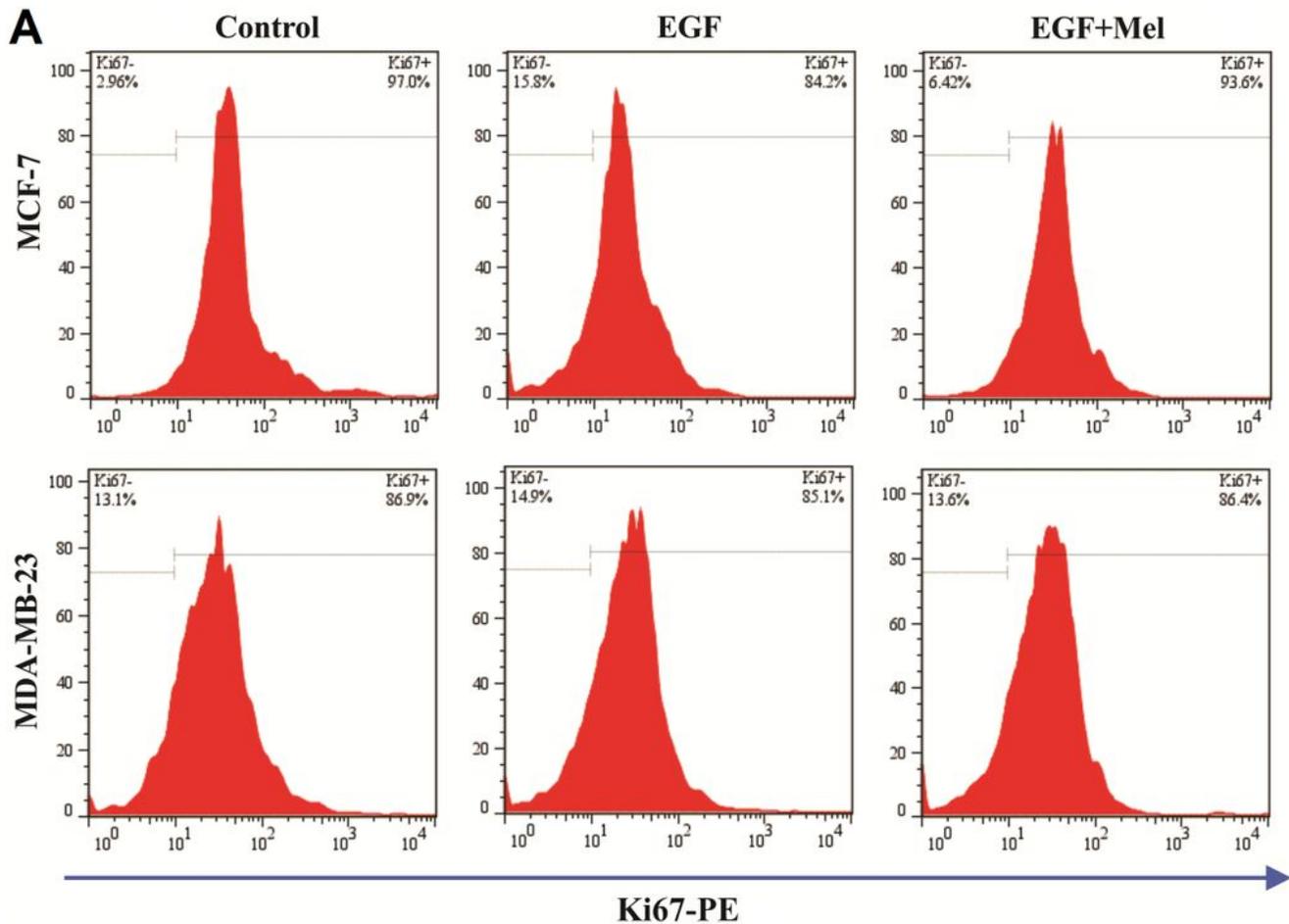


Figure 2

Effects of Melatonin on the cell proliferation of MCF-7 and MDA-MB-231 treated with EGF. Expression of Ki67 in MCF-7 cells treated with EGF reduced compared to the untreated group ($P < 0.05$). Moreover, in this cell line, treatment with melatonin could significantly increase the levels of Ki67 protein ($P < 0.05$). No significant change was observed in the concentration of Ki67 among MDA-MB-231 cells without treatment or treated with EGF alone or with melatonin ($P > 0.05$) (Figure 2B).

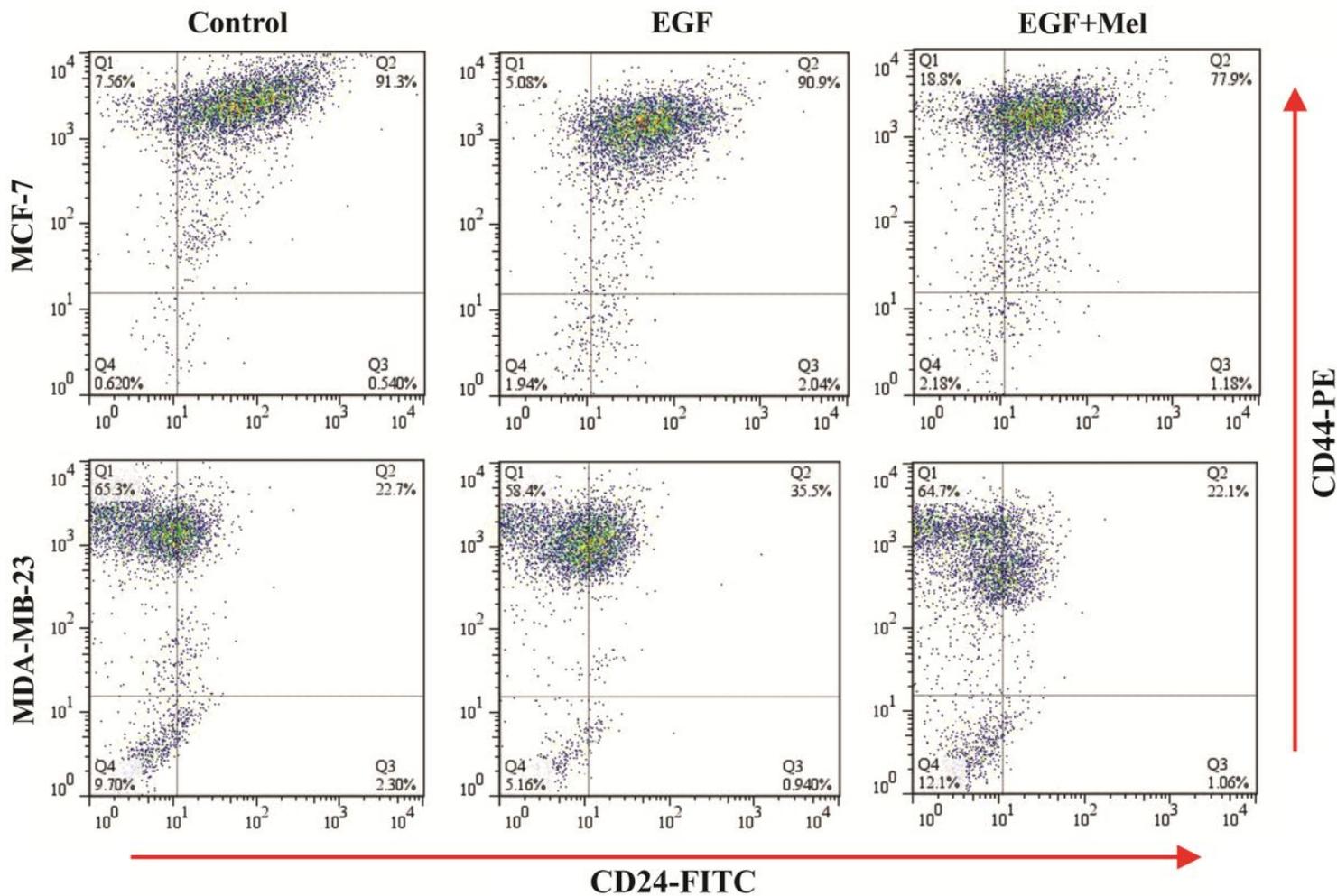


Figure 3

Effects of Melatonin on the stemness marker of MCF-7 and MDA-MB-231 treated with EGF. EGF treatment led reduction in the number of cancer stem cells in both MCF-7 and MDA-MB-231. Moreover, melatonin significantly increased CD44+/CD24- levels in MCF-7 cells. However, melatonin treatment caused only a negligible increase in the level of CD44+/CD24- in MDA-MB-231 cells.

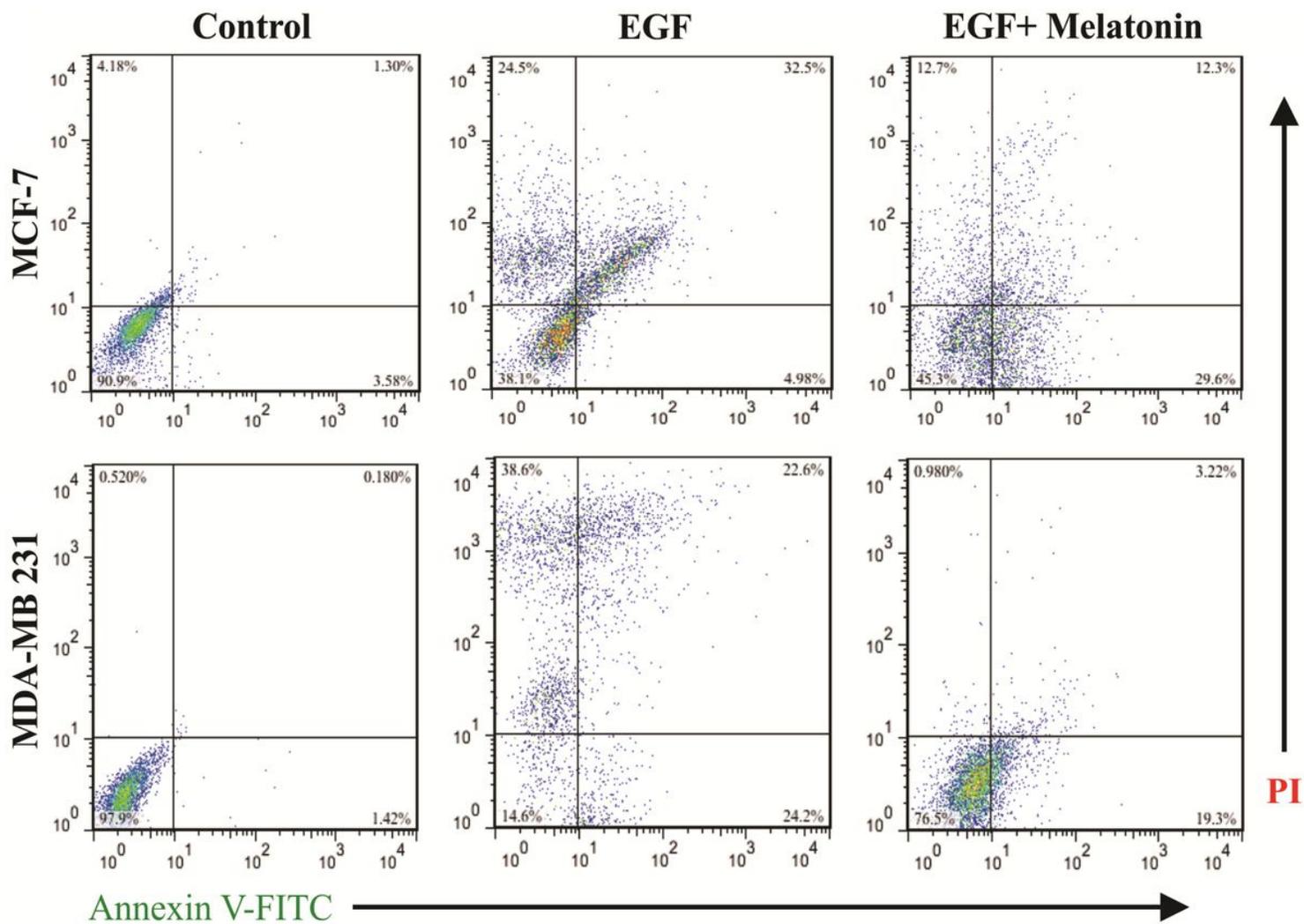


Figure 4

Effects of Melatonin on the apoptosis of MCF-7 and MDA-MB-231 treated with EGF. In MCF-7 cells, EGF treatment increased apoptotic cell population up to 32.5%. Meanwhile, melatonin treatment decreased this population to 12.3% in EGF-treated MCF-7 cells. In MDA-MB-231 cells, also EGF increased the portion of the apoptotic cell up to 22.6%, and melatonin attenuated apoptosis and reached the apoptotic cell population to 3.22%.

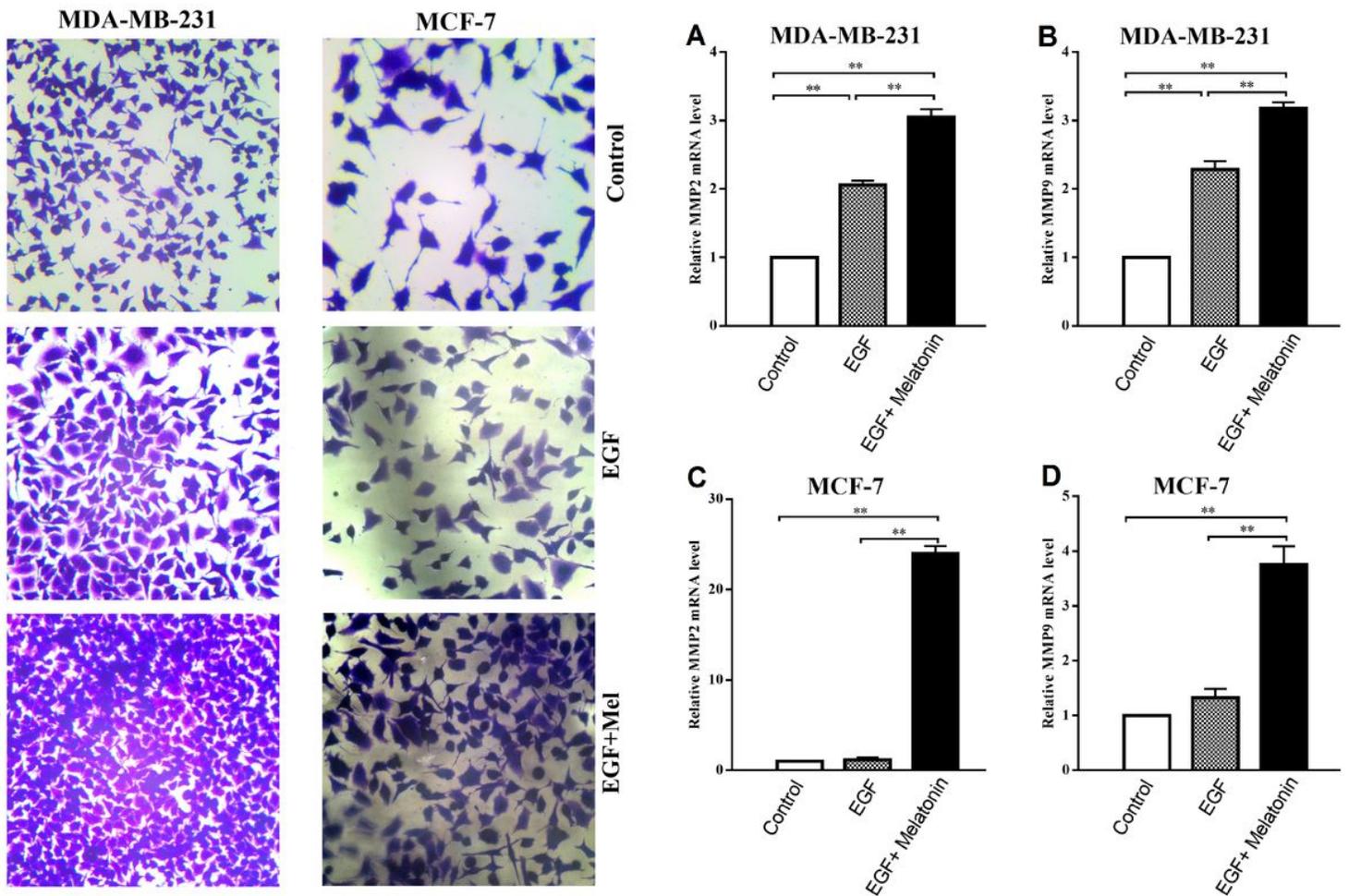


Figure 5

The role of Melatonin on EGF-induced invasion in MCF-7 and MDA-MB-231. The transwell invasion assay demonstrated that the EGF-induced invasion was modulated by melatonin in both EGF-treated MCF-7 and MDA-MB-231 cells. The expression levels difference of MMP2 and MMP9 between EGF-treated and untreated MCF-7 cells was not significant. EGF could increase the expression levels of these genes in MDA-MB-231 cells. Moreover, the mRNA expression levels of MMP2 and MMP9 in EGF-treated MCF-7 and MDA-MB-231 cells were significantly increased by Melatonin ($P < 0.05$, Figure 5A, B)

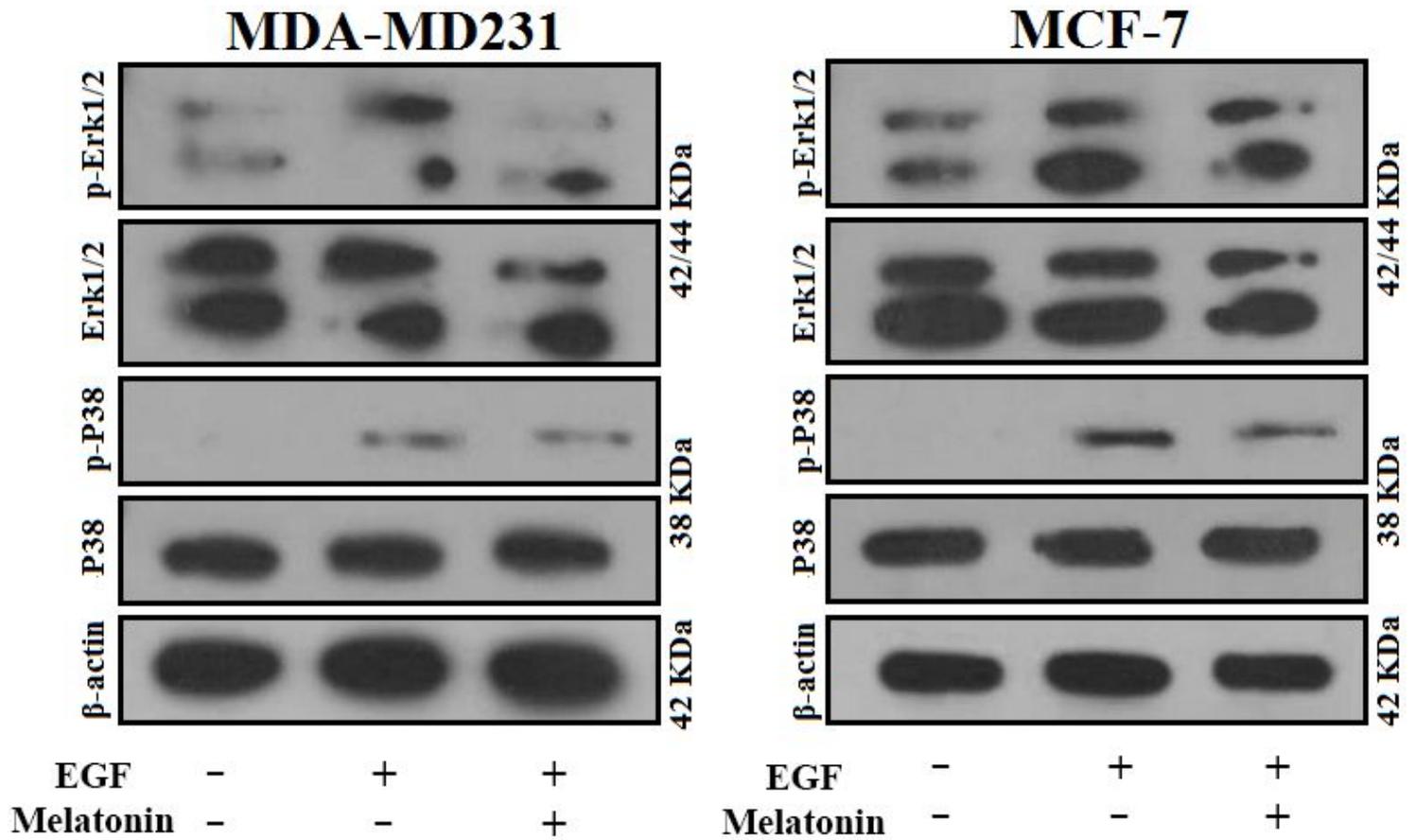


Figure 6

Effects of Melatonin on EGF-induced phosphorylation of ERK1/2 and P38 MAPK. The western blotting analysis demonstrated that phosphorylated and active P38 and ERK1/2 were not considerably present in MCF-7 and MDA-MB-231 cells. However, EGF increased its phosphorylation, particularly in MCF-7 cells. As shown, melatonin attenuated phosphorylation of ERK1/2, especially in MDA-MB-231 cells. Melatonin treatment did not alter the phosphorylation of P38.