



Melatonin Inhibits the Migration of Human Lung Adenocarcinoma A549 Cell Lines Involving JNK/MAPK Pathway

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Abstract

Objective: Melatonin, an indolamine produced and secreted predominately by the pineal gland, exhibits a variety of physiological functions, possesses antioxidant and antitumor properties. But, the mechanisms for the anti-cancer effects are unknown. The present study explored the effects of melatonin on the migration of human lung adenocarcinoma A549 cells and its mechanism.

Methods: MTT assay was employed to measure the viability of A549 cells treated with different concentrations of melatonin. The effect of melatonin on the migration of A549 cells was analyzed by wound healing assay. Occludin location was observed by immunofluorescence. The expression of occludin, osteopontin (OPN), myosin light chain kinase (MLCK) and phosphorylation of myosin light chain (MLC), JNK were detected by western blots.

Results: After A549 cells were treated with melatonin, the viability and migration of the cells were inhibited significantly. The relative migration rate of A549 cells treated with melatonin was only about 20% at 24 h. The expression level of OPN, MLCK and phosphorylation of MLC of A549 cells were reduced, while the expression of occludin was conversely elevated, and occludin located on the cell surface was obviously increased. The phosphorylation status of JNK in A549 cells was also reduced when cells were treated by melatonin.

Conclusions: Melatonin significantly inhibits the migration of A549 cells, and this may be associated with the down-regulation of the expression of OPN, MLCK, phosphorylation of MLC, and up-regulation of the expression of occludin involving JNK/MAPK pathway.

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Introduction

Lung cancer is the most common cancer and the leading cause of cancer deaths worldwide. The prognosis of patients can be improved through effective treatment, but the 5-year survival rate of the patients with advanced lung cancer is only about 16% [1]. Various novel therapeutic strategies currently under consideration as the clinical use of cytotoxic drugs is limited due to intrinsic or acquired resistant and toxicity [2]. The majority of patients with lung cancer presents with locally advanced inoperable or metastatic disease [3].

Cell migration is a biological process that contributes crucially to a variety of physiological, wound healing and the inflammatory reaction. Moreover, cell migration is also responsible for the malignance of cancer disease as it allows tumor cells to invade the surrounding tissues, thereby forming metastases [4].

Recent studies demonstrate that many proteins, such as myosin light-chain kinase (MLCK), osteopontin (OPN), play a critical role in non-muscle cell protrusion, contraction, and migration [5–6].

MLCK is a key Ca²⁺/Calmodulin (CaM)-dependent effector that is responsible for smooth muscle cell and non-muscle cell migration via phosphorylation of Ser19, Thr18 on myosin light chains (MLC), an event that facilitates myosin interaction with actin filaments [7]. MLCK expression reduction via antisense techniques is lead to rounding fibroblast cell, decreasing proliferation and attenuating chemoattractant-stimulated cell locomotion [8]. OPN undergoes extensive posttranslational modification, including phosphorylation, glycosylation and cleavage, resulting in molecular mass variants ranging from 25 to 75 kDa [9]. There is evidence suggesting that multiple signals may function in OPN-mediated tumor cell migration as inhibitors to phospholipase C/protein kinase C (PLC/PKC), mitogen activated protein kinase (MAPK), and PI3K could decrease OPN-induced migration [6].

Tumor cells, particularly in those cancers that manifest high metastatic potential, often exhibit loss of tight junctions (TJ). Occludin is a transmembrane protein of epithelial TJs, therefore its structure is relatively well characterized [10–12]. Down-regulation of specific TJ proteins has been shown to correlate

with the staging, invasiveness and metastasis potential of epithelial cancers [13–15]. MAPK signaling pathway is able to modulate TJ paracellular transport by up-or down-regulating the expression of several TJ proteins and hence altering the molecular composition within TJ complexes [16].

These observations directly implicate that MLCK, OPN, occludin in the signaling pathways controls the non-muscle cell motility. However, the exact mechanistics during cancer cell migration remain poorly understood.

MAPK pathways play pivotal roles in cell proliferation, differentiation, and survival [17]. The closely related MAPK pathways are regulated through a series of phosphorylation steps in a three-component module: MAPKs are activated by MAPK kinases (MAPKK) on dual residues of threonine and tyrosine, and MAPKKs are in turn phosphorylated by MAPKK kinases (MAPKKK) on dual residues of serine/threonine. MAPKs have been divided into three main groups: the extracellular-regulated kinases 1/2 (ERK1/2 or MAPK p44/42), MAP 38, and the c-jun-N-terminal kinases (JNK) [18]. It has been reported that JNK is constitutively activated in several tumor cell lines and that the transforming actions of several oncogenes have been reported to be JNK dependent (based on dominant-negative approaches) [19]. Recently, more and more evidences indicate that JNK substrates, especially the non-nuclear proteins, also have wide-ranging functional roles in cell migration, axonal guidance, neurite formation and outgrowth, brain development, dendritic architecture and regeneration of nerve fibers after injury [20].

Melatonin is an indole bioactivator mainly secreted by the pineal gland. It has a wide range of reported biologic effects including antioxidative [21–26], anti-inflammatory and antitumor activities [27–34] and has generated considerable interest as a pharmaceutical compound with a wide range of therapeutic activities. Melatonin has also been shown to possess chemotherapeutic potential in human cancers and to be capable of modulating several signal transduction pathways associated with cell survival, proliferation, apoptosis and invasion [35–39]. Recent studies have reported that melatonin can inhibit tumor invasion through increasing adhesion by elevating E-cadherin and β 1-integrin expression [40] or modulating microfilament [41–42], and decreasing matrix metalloproteinases (MMPs) production [43]. Anti-invasion effect of melatonin has been shown in human mammary epithelial cancer MCF-7 cells [40–41,43] and MDCK cells [42].

However, it is unknown whether melatonin affects the migration and invasion of A549 cells via OPN, occludin, as well as MLCK and through which pathway. Therefore, the present

study was undertaken to investigate the effect of melatonin on the migration and invasion of A549 cells. In addition, we also assess the expression of OPN, occludin, MLCK, and the function of JNK MAPK signal transduction pathway.

Materials and Methods

Cell culture

Human lung adenocarcinoma cell line (A549) was purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (TBD Science, Tianjin, China), 1 mmol/L glutamine, 100 U/mL penicillin and 10 mg/mL streptomycin (Ameresco, USA), at 37°C, 5% CO₂.

Reagents

Melatonin was provided by School of Pharmacy, Anhui Medical University (Anhui, China). Dimethyl Sulfoxide (DMSO) was obtained from sigma Chemical (USA). Dulbeccos modified Eagles medium (DMEM) was purchased from Gibco BRL life Technologies (USA). Phorbol-12-myristate-13-acetate (PMA) and SP600125 were obtained from Cayman Chemical (USA). Primary antibodies (anti-OPN, anti-occludin, anti-MLCK, anti-pMLC, anti-MLC, anti-pJNK, anti-JNK, anti- β -actin) were purchased from Santa Cruz Biotechnology (USA). All secondary antibodies were purchased from Millipore (USA). ECL reagent and BCA kit were purchased from quantitative Pierce Company.

Cell viability assay

Cell viability was measured using the MTT assay. A549 cells (4.5×10⁴ cells/well) were seeded into 96-well plates and cultured. The cells were treated with different concentrations of melatonin (0.1, 0.5, 0.75, 1.0, 2.5, 5.0 mmol/L), then incubated with MTT solution for 4 h. Finally, the cells were exposed to an MTT-formazan dissolving solution (DMSO) for 30 minutes. The optical density (OD) was measured using an absorbance microplate reader (Bio-Tek, ELX800) at a wavelength of 490 nm. The cell viability was expressed as a percentage of the OD value of the control cultures. For the A549 cells, the data were taken from one experiment with 4 replicates. IC₅₀ was determined using a sigmoidal equilibrium model regression using XLfit version 4.3.2 (ID Business Solutions Ltd.) and is defined as the concentration of melatonin required for a 50% reduction in growth/viability.

Wound healing assay

Migration of A549 cells was measured using the wound-healing assay in vitro. Cells were seeded into 12-well plates and grown to

Table 1. The effect of different concentrations of MLT on the viability of A549 cells in 3 day.

Group	OD570 nm ($\bar{x} \pm s$)	Inhibition rate (%)	IC50 (mmol/L)
DMSO	0.67±0.04	–	1.864
MLT (mmol/L)			
0.10	0.55±0.05	17.50*	
0.50	0.51±0.02	23.53*	
0.75	0.47±0.02	29.00*	
1.00	0.41±0.03	38.78*	
2.50	0.18±0.02	72.99*	
5.00	0.10±0.01	84.15*	

Compared with DMSO control group, *P<0.05.

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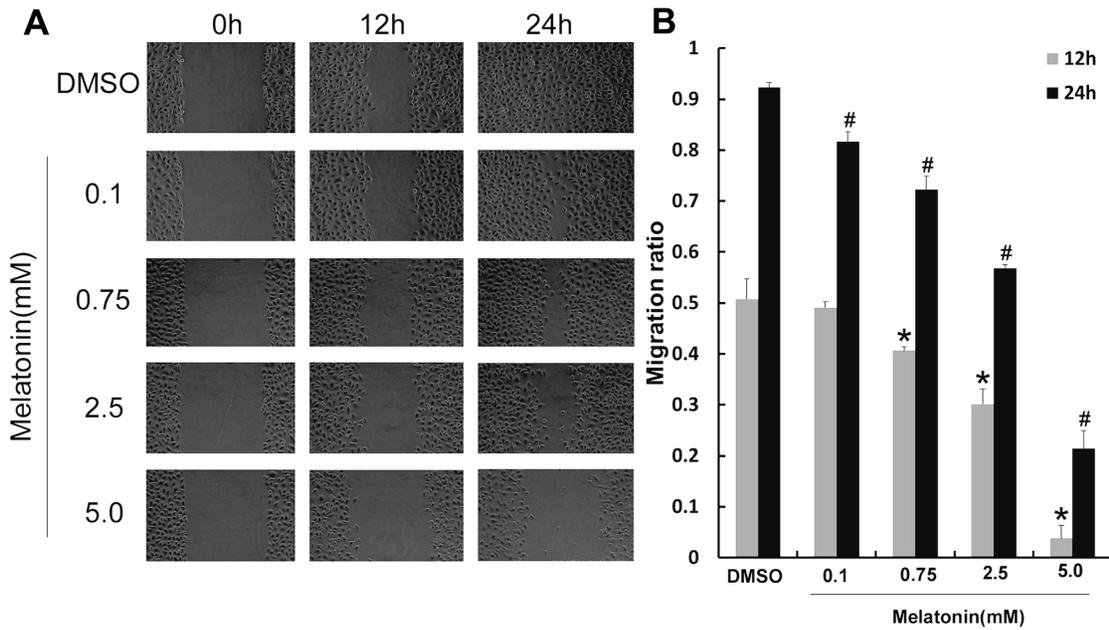


Figure 1. Melatonin inhibits the migration of A549 cells. (A) The migration of A549 cells at 0.1, 0.75, 2.5, 5.0 mmol/L melatonin groups respectively when A549 cells were treated for 0 h, 12 h and 24 h. (B) Analysis of migration rate (%), compared with control group (DMSO): *P<0.05, #P<0.05.

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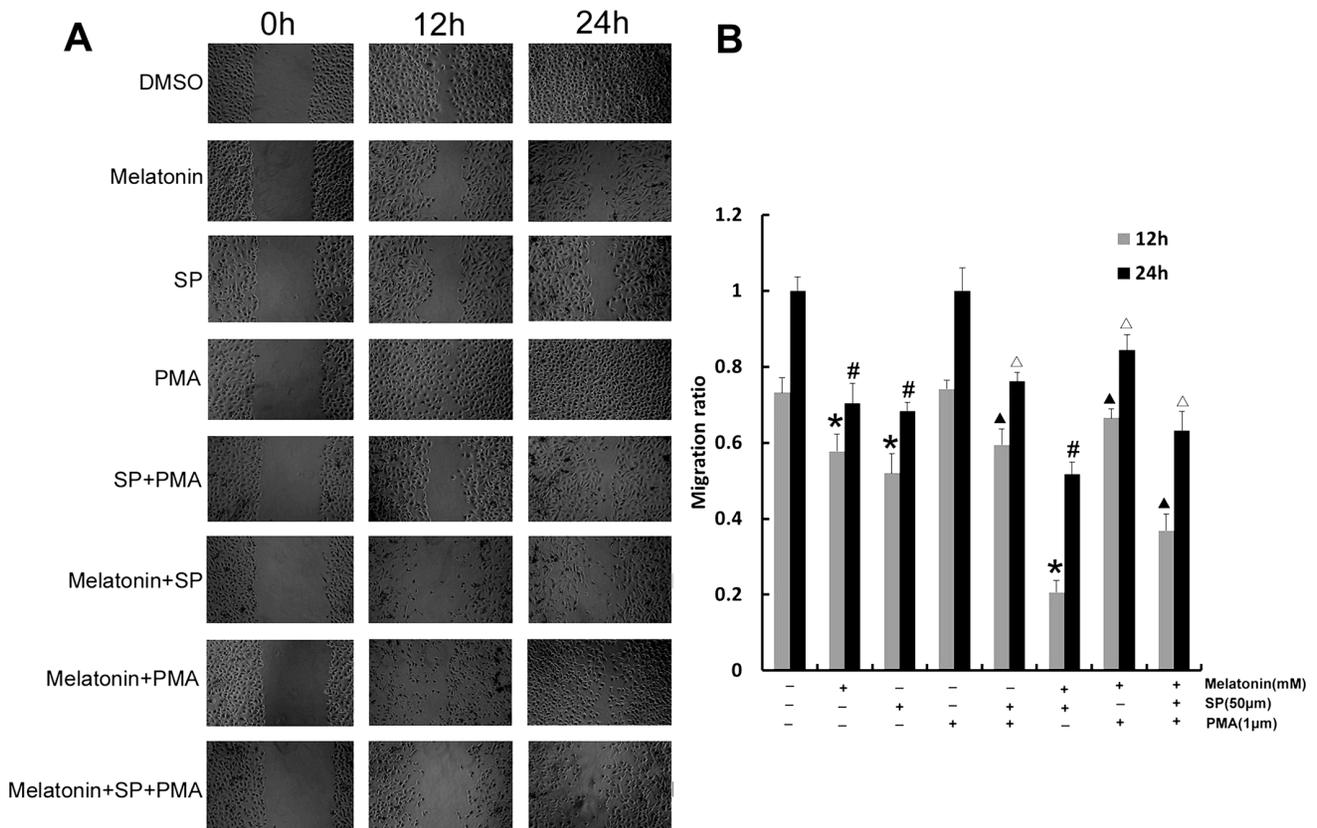


Figure 2. The effect of melatonin, SP600125 and PMA on migration of A549 cells. (A) The effect of melatonin, SP600125 and PMA on migration of A549 cells after 0 h, 12 h and 24 h. (B) Analysis of migration rate, compared with control group: *P<0.05, #P<0.05; compared with PMA group: [△]P<0.05, [△]P<0.05.

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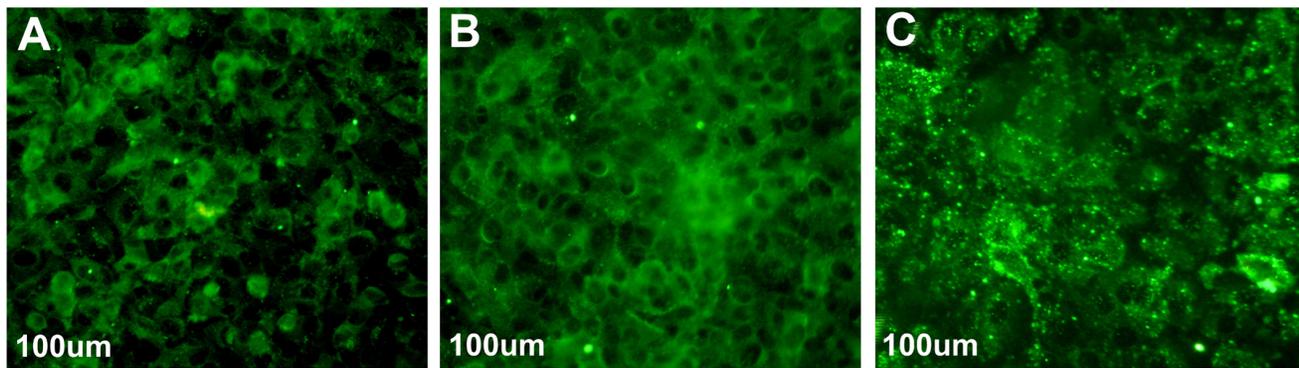


Figure 3. Immunofluorescence detection of tight junction correlated protein occludin in A549 cells. (A) DMSO control. (B) melatonin (0.1 mmol/L). (C) melatonin (2.0 mmol/L). doi:10.1371/journal.pone.0101132.g003

100% confluence. Wounds were created by scraping monolayer cells with a sterile pipette tip. At 0, 12, 24 h after the creation of wounds, cells were observed with 10× objective in an Olympus (Olympus Corporation, Tokyo, Japan) photomicroscope. Images were acquired with a Nikon (Tokyo, Japan) color digital camera. Wound distances were measured at each time point and expressed as the average percent of wound closure by comparing the zero time.

Immunofluorescence assay

A549 cells (0.5×10^4 cells/well) were seeded into 96-well plates with sterile aseptic cover glasses and cultured. Cells were treated with 0.1 mmol/L and 2.0 mmol/L concentration melatonin for 7 d. DMSO was added in the control group. The nutrient solution with melatonin was changed every day. After treatment, the cells were washed and fixed with 4% paraformaldehyde for 20 minutes at room temperature, then washed and blocked with blocking buffer (5% nonfat dry milk in PBS) for 2 h at room temperature. The cover glass with A549 cells was incubated with goat anti-human occludin (1:50) primary antibody overnight at 4°C. Cells were washed and incubated with donkey anti-goat IgG-FITC (1:200), then washed and mounted with aqueous-based anti-fade mounting medium. Images of stained cells were captured using fluorescence microscope.

Western blotting analysis

After treatment, cells were washed with PBS for 3 times and lysed in lysis buffer (1% TritonX-100, 0.015 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF, 10 µg/mL of each leupeptin and pepstatin A). The protein concentrations were measured with a BCA kit. The cell lysates were solubilized in SDS sample buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene fluoride (PVDF) membranes. The membrane was blocked with blocking buffer (5% nonfat dry milk) overnight at 4°C. After that, the membrane was incubated with the indicated primary antibody with OPN(1:500), occludin(1:250), MLCK(1:500), pMLC(1:500), MLC(1:1000), pJNK(1:500), JNK(1:1000), and β -actin(1:1000) respectively and followed by the appropriate horseradish peroxidase(HRP)-conjugated secondary antibody, and visualized with enhanced chemiluminescence using hydrogen peroxide and luminol as substrate with Kodak X-AR film. The images were scanned using a ScanPrisa 1240 OUT (Acer, China). Western blots data were quantified using Quantity One software.

Statistical analysis

Three or more separate experiments were performed for each experiment. Statistical analysis was performed by Student's t-test or ANOVA. Data are presented as means \pm standard deviation. Statistical significance was defined as *p* value less than 0.05.

Results

Effect of melatonin on viability in A549 cells

To determine the effect of melatonin on cell viability, A549 cells were treated with different concentrations of melatonin (0.1, 0.5, 0.75, 1.0, 2.5, 5.0 mmol/L) for 3 d. Viability was assessed using the MTT assay. The results showed that melatonin inhibited the proliferation of A549 cells in a concentration-dependent manner, compared with the control group. The inhibition rates were 17.50%, 23.53%, 29.00%, 38.78%, 72.99% and 84.15%, respectively (Table 1). The IC₅₀ of melatonin is 1.864 mmol/L.

Effect of melatonin on migration in A549 cells

To investigate the effect of melatonin on migration, A549 cells were treated with different concentrations of melatonin. Migration of A549 cells were inhibited by melatonin in a concentration-dependent manner (Fig. 1). We then used pharmacological inhibitors and activator to determine the role of JNK in the migration of A549 cells. The A549 cells were treated with SP600125 (JNK inhibitor) and PMA (MAPK activator) for 3 d. The results showed that SP600125 significantly inhibited the migration of A549 cells and PMA has no obvious effect on the migration of A549 cells compared with the control while PMA decreased the effects of Melatonin and SP600125 on migration of A549 cells ($p < 0.05$) (Fig. 2).

Melatonin up-regulates the expression of occludin and enhances occludin to locate on the cell surface, and down-regulates the expression of OPN, MLCK in A549 cells, which is partly through the JNK/MAPK signaling pathway

Tight junction correlated protein occludin was detected by immunofluorescence in A549 cells. The results showed that there was no occludin accumulation on the A549 cells surface, but occludin started to locate on the cell surface when A549 cells was treated with melatonin at the concentration of 0.1 mmol/L. Occludin located on the cell surface was obviously increased when treated with melatonin at the concentration of 2.0 mmol/L (Fig. 3). The effect of melatonin on the expression of proteins related with

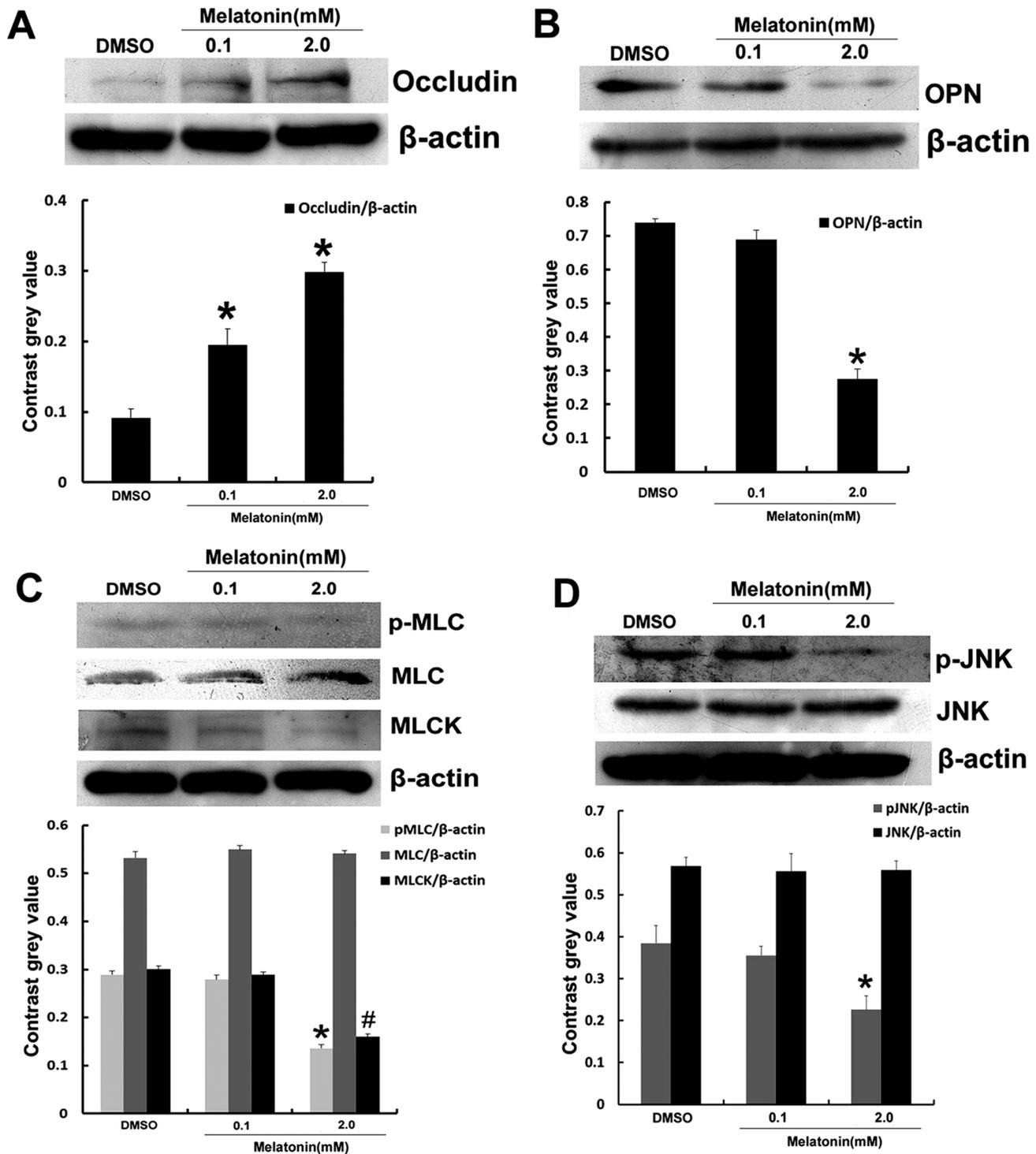


Figure 4. Effect of melatonin on the expression of occludin, OPN, MLCK and phosphorylation of MLC, JNK. (A) occludin, (B) OPN, (C) MLC and MLCK, (D) JNK. Results are presented as mean \pm SD of three independent experiments. * $P < 0.05$, in comparison to control group. doi:10.1371/journal.pone.0101132.g004

migration was determined using western blots analysis in A549 cells. After the cells were treated with different concentrations of melatonin for 3 d, the results revealed that melatonin (2.0 mmol/L) enhanced the expression of occludin ($p < 0.05$) (Fig. 4 A), reduced the expression of OPN (Fig. 4 B), MLCK and phosphorylation of MLC (Fig. 4 C), JNK (Fig. 4 D).

After that, we examined whether the expression of related proteins are associated with JNK/MAPK. In later western blots experiment, we then used pharmacological inhibitors and activator to determine the role of JNK in the expression of related proteins in A549 cells, the cells were treated with melatonin, SP600125, and PMA for 3 d. The phosphorylation status of JNK

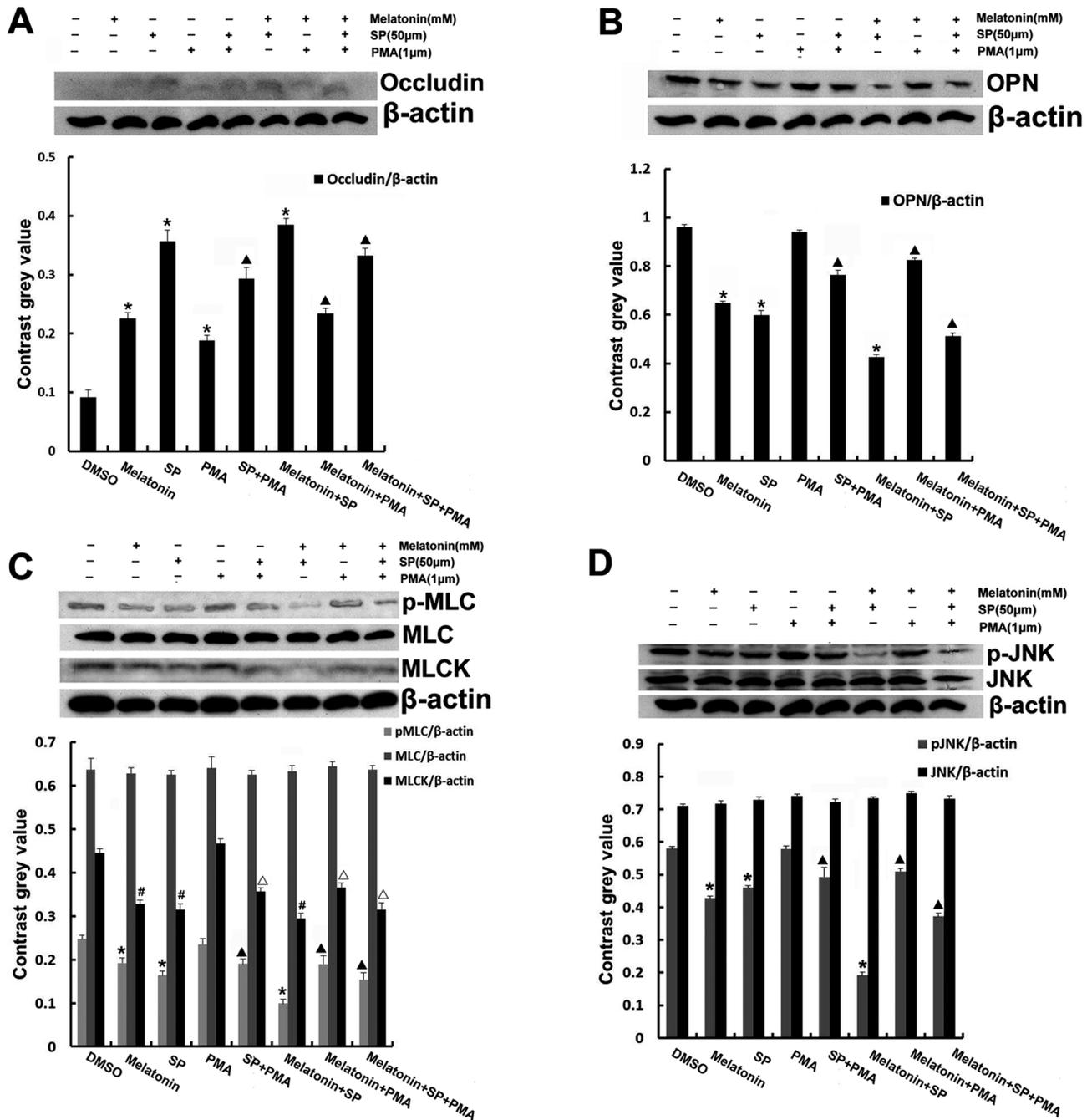


Figure 5. The Effect of melatonin, SP600125 and PMA on the expression of occludin, OPN, MLC and phosphorylation of MLC, JNK. (A) occludin, (B) OPN, (C) MLC and MLCK, (D) JNK. Results are presented as mean \pm SD of three independent experiments. * $P < 0.05$, # $P < 0.05$, in comparison to control; $\blacktriangle P < 0.05$, $\blacktriangle P < 0.05$, in comparison to PMA group. doi:10.1371/journal.pone.0101132.g005

was decreased. The expression level of OPN, MLCK, and phosphorylation of MLC were down-regulated, while the expression of occludin was up-regulated when cells were exposed to melatonin and SP600125 compared with control group ($p < 0.05$). The expression level of OPN, MLCK, phosphorylation of MLC, JNK were lower in melatonin plus SP600125 group while the expression level of occludin was increased ($p < 0.05$) (Fig. 5).

Discussion

Cell migration is a biological process that contributes crucially to a variety of physiological functions, such as wound healing and the inflammatory reaction. Moreover, cell migration is also responsible for the malignance of cancer as it allows tumor cells to invade the surrounding tissues, thereby forming metastases [4]. Melatonin can inhibit tumor cell proliferation, selectively blocking the signal transduction of tumor cells, and destroying its autonomous growth, which has become a research hot point

[32,44–45]. Recently, several studies have shown that melatonin can also regulate microtubule and microfilament structure formation and inhibit tumor cell invasion and metastasis process [46].

In our studies, we demonstrate, for the first time, the effect of melatonin on the migration of human lung adenocarcinoma A549 cells and observed an association between JNK/MAPK pathway and the expression of tight junction (TJ) related proteins occludin, OPN and MLCK. Some studies have found that melatonin affects cell cycle as it prolongs the cell cycle of cancer cells and delays tumor cells to enter mitosis, thus making cancer cell proliferation restrained. Melatonin inhibits cell proliferation via downstream cyclin E from cells entering S phase [47–48]. Our current data show that melatonin could inhibit the viability of A549 cells in a concentration-dependent manner, compared with control group. The melatonin may inhibit A549 cell proliferation and play an important role in the inhibition of tumor progression.

Recently, studies found that melatonin could convert cell subtype of microtubules and make the invasive cells into non-migrating cells with stationary microtubules subtypes in breast cancer MCF-7 cells [46]. Our results showed that migration distance shortened obviously when melatonin was used to treat A549 cells for 24 h and demonstrated that melatonin reduced the migration of A549 cell.

Occludin is the main functional regulatory protein in TJ and is thought to be the most sensitive and reliable sign of tight junction structure. The expression and dysfunction of occludin proteins is associated with the development and metastasis of tumor [49–50]. We observed that the expression of occludin was significantly increased in A549 cells treated with melatonin at the concentration of 0.1 mmol/L and 2.0 mmol/L. Immunofluorescence result revealed that melatonin promoted occludin to locate on the cell surface. The TJ leaks of human brain tumors Microvessels are responsible for cerebral edema in certain types of brain cancer, which is an excellent example of down-regulation of occludin in cancer [51–52]. We speculated that melatonin promoted occludin proteins to move and position on the cell surface and enhanced the formation of close connection between cells, which have the effect of inhibiting A549 cell metastasis.

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OPN has repeatedly been shown to be present at high levels in the circulation of patients with metastatic cancers [53–57] and increased metastatic potential [58–60], thus make it relevant in the context of studying its expression in the perspective of metastasis. Both activated myosin II and its activator MLCK are enriched in lamellar protrusive structures in several cell types during migration [61]. MLCK activation and expression have been found to be positively related with metastatic propensity [62–63]. Our results demonstrated that melatonin significantly suppressed the JNK/MAPK pathway in A549 cells. To determine the mechanism, we used PMA and SP600125 to respectively activate and inhibit MAPK/JNK signaling in A549 cells. Our results showed that SP600125 significantly inhibited the migration of A549 cells. Melatonin and SP600125 inhibited the relative migration rate of groups in PMA-stimulated groups. The expression of OPN and MLCK, the phosphorylated of MLC were down-regulated while the expression level of occludin was up-regulated when cells were exposed to SP600125. Melatonin and SP600125 inhibited the expression of MLCK, OPN and phosphorylated of JNK, MLC, and enhance the expression of occludin in PMA-stimulated groups. In accordance with our data, therefore, the anti-migration effect of melatonin is associated with its inhibition of JNK/MAPK pathway and regulation of the expression of occludin, OPN, and MLCK.

Our present results show that melatonin plays an important role in inhibiting the proliferation and migration of A549 cells. Occludin, OPN, and MLCK contribute to the migration of A549 cells involving JNK/MAPK pathway. Our findings support the potential application of melatonin in the treatment of lung cancer. Due to the migration of tumor cells and the formation of tumor metastasis is an extremely complex process which involves multiple steps and many factors, the comprehensive mechanism of melatonin inhibits tumor cell migration still needs further research.

Author Contributions

Conceived and designed the experiments: YW SG. Performed the experiments: Qiaoyun Zhou Qing Zhou YW SG. Analyzed the data: Qiaoyun Zhou Qing Zhou YW SG. Contributed reagents/materials/analysis tools: Qiaoyun Zhou Qing Zhou YW SG. Wrote the paper: Qiaoyun Zhou SG YW.

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