RESEARCH ARTICLE

Melatonin inhibits the Migration of Colon Cancer RKO cells by Down-regulating Myosin Light Chain Kinase Expression through Cross-talk with p38 MAPK

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Abstract

Background: Melatonin, which is mainly produced by the pineal gland, has a good inhibitory effect on cell growth of multiple cancer types. However, the underlying molecular mechanisms of anti-tumor activity for colon cancer have not been fully elucidated. In this study, we investigated the effects of melatonin on migration in human colon cancer RKO cells and the potential molecular mechanisms. Materials and Methods: The viability of RKO cells was investigated by MTT assay after treatment with melatonin, SB203580 (p38 inhibitor) and phorbol 12-myristate 13-acetate (PMA, MAPK activator) alone or in combination for 48h. The effects of melatonin, and ML-7, a selective inhibitor of myosin light chain kinase (MLCK), and SB203580, and PMA on the migration of RKO cells were analyzed by *in vitro* scratch-wound assay. The relative mRNA levels of MLCK was assessed by real-time quantitative RT-PCR. Western blotting analysis was performed to examine the expression of MLCK, phosphorylation of myosin light chain (pMLC) and p38 (pp38). Results: The proliferation and migration of human colon cancer RKO cells were inhibited significantly after treatment with melatonin. The expression levels of MLCK and phosphorylation of MLC of RKO cells were reduced, and real-time quantitative RT-PCR showed that melatonin had significant effects on suppressing the expression of MLCK. Furthermore, the phosphorylation level of p38, which showed the same trend, was also reduced when cells were treated by melatonin. In addition, ML-7 (25umol/l) could down-regulate the phosphorylation of p38. Conclusions: Melatonin could inhibit the proliferation and migration of RKO cells, and further experiments confirmed that p38 MAPK plays an important role in regulating melatonin-induced migration inhibition through down-regulating the expression and activity of MLCK.

Keywords: Melatonin - human colon cancer - MLCK - p38 MAPK - migration

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Introduction

Human colon cancer is the most frequent cancer worldwide as well as in china, and which is a malignant tumor arising from the inner wall of the large intestine has become the third global leading cause of death from different cancers and its metastasis at distant sites is the major cause of death (André et al., 2004). It's particularly worth mentioning here that the majority of individuals who were diagnosed with this disease are already at an advanced stage (Jemal et al., 2011). Although colorectal tumors that are detected prior to invasion and metastasis can be eliminated with surgery, approximately half of resected colon cancer patients still experience tumor recurrence, and metastatic colon cancer after treatment with chemotherapy ultimately fails due to development of drug resistance (Vellinga et al., 2015). In the meantime, considering the reduction of drugs' toxicity and the prolong survival of patients with colon cancer, novel therapeutic strategies are urgently needed.

Melatonin (N-acetyl-5-methoxytryptamine), a kind of endogenous substances, is entrained by the light/dark cycle (Claustrat et al., 2005; Blask, 2009), and predominantly produced by the pineal gland (Reiter, 1991), and partly by other organs like the eyes, bone marrow, gastrointestinal tract, skin and lymphocytes (Claustrat et al., 2005; Srinivasan et al., 2011). For the past few years, melatonin has been attracting more and more attention as a potential new therapeutic method. Studies have shown that melatonin plays key roles in the biologic regulation of circadian rhythms, sleep, antioxidant protection, possibly aging, immune, tumor growth, reproduction and bone physiology (Verma et al., 2014; Akbulut et al., 2015; Plaimee et al., 2014a). Undoubtedly, More recent studies have implied that melatonin has the potentially of being used as an effect therapeutic agent for treating breast

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cancer, lung cancer, colorectal cancer, melanoma and various other types of cancer (Martin et al., 2006; Mao et al., 2010; Zha et al., 2012; Wang et al., 2013c; Zhou et al., 2014a). Moreover, melatonin could enhance the anti-tumor effect of some agents through modulating multiply signaling pathways in some cancer cells (Wang et al., 2013c; Yi et al., 2014). The inhibitory action of growth by melatonin in colon cancer has been studied extensively both in vivo and vitro in the last ten years (Wenzel et al., 2005; Garcia-Navarro et al., 2007; Tanaka et al., 2009; Winczyk et al., 2009). In contrast, only a minimal amount of work has been done with regard to the role of melatonin in human colon cancer invasion and metastasis. Therefore, elucidation of the detailed molecular mechanisms involved in melatonin-mediated inhibitory effect of migration in colon cancer deserves further investigation.

Myosin Light Chain Kinase (MLCK, dependent increases in myosin ATPase activity), a key Ca^{2+}/Calmodulin (CaM)-dmependent effector, is a myosin regulator in the lamella and contractile ring, and could regulate nonmuscle myosin II activity via phosphorylation of Ser19, Thr18 on myosin light chains (MLC) (Chew et al., 2002), and assemble in protrusive during cell migration (Kolega, 2003). It has been reported that the activation and increased expression of MLCK are crucial to trigger non-muscle cell and smooth muscle cell motility (Even-Ram et al., 2007; Sun et al., 2011; Zhu et al., 2012). MLCK in cancer cell migration remains controversial. But, recently increasing studies have shown that increased expression levels of MLCK are crucial for migration activity in several types of human cancer (Kucharczak et al., 2001; Mills et al., 2011; Wang et al., 2013b; Fan et al., 2014). Furthermore, MLCK is not only required for myosin phosphorylation in a migrating cell, but also critical role of in cell migration involving regulating the cell membrane tension and protrusion necessary for migration (Chen et al., 2014). Mitogen-activated protein kinase (MAPK) signaling shows a cascade organization, in which activation of upstream kinases by receptors leads to sequential activation of a MAPK module (MAPKKK, MAPKK, MAPK), which play key roles in cell proliferation, differentiation, migration and cell survival (Joo et al., 2009; Mao et al., 2010; Liu et al., 2012). Recent researches and preliminary studies in our laboratory have shown that inhibiting tumor cells migration by down-regulating the expression of MLCK is associated with p38 signaling pathway (Wang et al., 2013a; 2013b), one of MAPKs pathway cascade. The MAPKs cascades include three classical signal pathways: extracellular signal-regulated protein kinases 1/2 (ERK1/2 or MAPK p44/42), C-Jun-N-terminal kinases (JNK) and p38 MAPK. The p38 activation is closely related to cell proliferation, migration, and cell survival in various colorectal cancer cells (Cumaoglu et al., 2014; Del et al., 2014; Grossi et al., 2014). However, little is known whether melatonin is able to inhibit colon cancer RKO cell migration by blocking MLCK, in which the signaling pathways involved in mediation by melatonin have not been completely elucidated.

In the present study, we aimed to investigate the potential effects of melatonin on the migration of RKO cell and assess the expression of MLCK and the function of p38/MAPK signal transduction pathway. Eventually, our studies showed that the anti-proliferation and anti-migration activity of melatonin in RKO cells were accompanied by inhibition of p38 MAPK pathways, and the inhibition of MLCK meditated by melatonin resulted in the depression of migration of colon cancer RKO cells through cross-talk between MLCK and down-regulated p38, in which both phosphorylated MLC serve as essential downstream effector. These results indicated that we have discovered a potent MLCK inhibitor that effectively blocks RKO cancer cell migration, and meanwhile a treatment might potentially become an effective way in colon cancer therapy.

Materials and Methods

Reagents and antibodies The human colon cancer cell lines RKO was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Melatonin was provided by school of Pharmacy, Anhui Medical University (Anhui province, China). A specific p38 MAPK inhibitor SB203580 and PKC activator Phorbol 12-myristate 13-acetate (PMA) were purchased from Cayman Chemical (USA). 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and Dimethyl Sulfoxide (DMSO) were obtained from sigma Chemical (USA). PMA, SB, and melatonin were dissolved in a small amount of DMSO before addition to the complete cell culture medium. Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco BRL life Technologies (USA). Fetal bovine serum was purchased from the Zhejiang Tianhang Biological Technology Co (China). Primary antibodies against MLCK, MLC, *p*-p38, p38, β-actin were purchased from Santa Cruz Biotechnology (USA). Antibody against *p*-MLC was purchased from Cell Signaling Technology. All secondary antibodies were purchased from Millipore (USA).

Cell culture Colorectal cancer RKO cells were cultured in DMEM high glucose culture media supplemented with 10% fetal bovine serum, 40 U/ml penicillin, and 100 U/ml streptomycin in an incubator with a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Cell viability assay Cells were seeded in 96-well plates at a density of 5x10^3 cells per well and were then continuously exposed to different combinations of melatonin, SB and PMA for 48 hr at 37°C. After that, MTT (5 mg/ml) dissolved in phosphate-buffered saline (PBS) was added to each well and incubated for 4 to 6 hr at 37°C. Then, the serum-free culture media containing MTT were discarded and DMSO was added to each well to dissolve the precipitate. The absorbance values were measured at 570 nm spectral wavelength using a Microplate reader after incubated and vibrated for 15 min at 37°C.

Effect of melatonin on cell morphology Cell were seeded in 6-well plates, and then the next day treated with melatonin at 0.5 mM, 1.5 mM, 2.5 mM for 48 hr. The cell morphology were photographed using a microscope (Leica DMI3000B).

Scratch-wound assays RKO cells were seeded in 12-well plates. After incubation when cell grow to 90%

confluence, each well was manually scratched with a sterile 200 μl pipette tip, washed with 1×PBS three times and incubated at 37°C with the melatonin or combination of ML-7, SB and PMA. After treated for12, 24 and 48 hr, the gap distance between two cell edges were measured using by Image-Pro Plus software.

Quantitative real time RT-PCR. Total RNA was extracted using the Trizol procedure (Invitrogen, USA). First-strand cDNA was synthesized from 4 μg RNA using the Reverse Transcription System (TAKARA). The resulting 4 μg cDNA was subjected to PCR amplification. Real-time PCRs were performed by using the SYBR Green QPCR master mixed with the following primer pairs: Human GAPDH (Fw: AGGTCGGAG TCAACGGATTTG; Rv:CCTGGAAGATGGTGATGGGAT). MLCK (Fw: GGACTTTCAGCCTTGTGATTC; Rv: CGCAAAACTTCCTTC TACTGTC). All primers were synthesized by the Sangon Biotech (Shanghai). Real-time PCR cycle conditions were as follows: an initial denaturation 95°C for 30s, followed by 40 cycles of a 5s extension step at 95°C, and annealing for 30s at 60°C.

Western Blot analysis After various treatments, the collected RKO cells was washed with PBS three times and lysed in RIPA lysis buffer (hepes 25 mM, 1.5% Triton X-100, 1% sodium deoxycholate, 0.1%SDS, NaCl 0.5 M, EDTA 5 mM, NaF 50 mM, sodium vanadate 0.1 mM, phenylmethylsulfonyl fluoride 1 mM, and leupeptin 0.1 g/l, pH 7.8) on ice for 30 min. The cell lysate was centrifuged at 14000 rpm for 30 min at 4°C and the protein concentration in the supernatant was determined by BCA assay. Loading buffer (Tris-HCl 42 mM, 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol and 0.025% bromophenol blue) was then added to the supernatant, which was subsequently boiled for 5 min and then electrophoresed on a 12.5% or 10% SDS-PAGE. Proteins were transferred to PVDF membrane and blocked the non-specific binding sites used PBST (PBS contained 0.05% Tween20) with 5% nonfat dry milk for 2 hr, After that, the membrane was incubated with primary antibody (antibodies against pp38, p38, pMLC, MLC, MLCK) over night at 4°C and then with peroxidase-conjugated secondary antibodies in the second reaction at room temperature for 2 hr. Detection was performed with enhanced chemiluminescence sequentially reagent.

Statistical analysis Data were analyzed by a one-way analysis of variance (ANOVA) followed by The Dunnett t-test or LSD- test using SPSS16.0 software. All the experiments were carried out three times, and results are represented as means ± standard deviation of the mean. Differences were determined to be significant with $P < 0.05$.

Results

Effect of melatonin on viability in human colon cancer RKO cells

In the previously work, our data established the growth inhibitory and pro-apoptotic effects of melatonin on RKO cells. Melatonin slightly attenuated the number of RKO cell at low concentration (less than 1mM) and significantly reduced the number at high concentration (more than 2mM) (data not shown). To ulteriorly explore the effect of melatonin on cell viability, RKO cells were treated with pharmacological inhibitor and activator to determine the role of p38 in the viability of RKO cells. The RKO cells were treated with SB203580, an inhibitor of p38, and PMA, an activator of PKC, for 48 hr. The results showed that SB203580 inhibited the viability of RKO cells and PMA had no obvious effect on the viability of RKO cells compared with the DMSO group, but interestly, PMA decreased the effects of melatonin and SB203580 on the viability of RKO cells ($p<0.05$) (Figure 1A). These data suggest that melatonin may inhibit the proliferation of RKO cells in vitro through p38 MAPK signaling pathway.

Effect of melatonin on RKO cells morphology and spread

At the same time, we also detected the changes in cell morphology and spread of colon cancer RKO cells

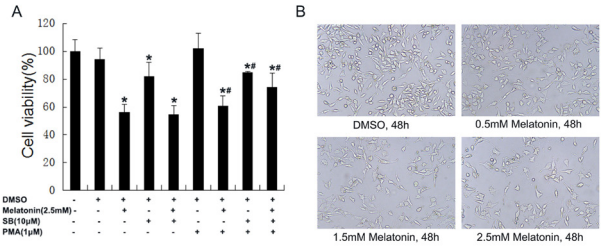

Figure 1. Effect of Melatonin, SB203580 and PMA on the Viability of RKO cells and morphology change. (A) The cells were treated with vehicle or melatonin (2.5mM), SB203580 (10μM) and PMA (1μM) alone, or in combination, for 48 hr, and cell viability was determined by a MTT assay. (B) The changes in cell morphology and spreading of RKO cells treated with different concentrations of melatonin for 48 hr were observed, and cells were photographed using Leica DMI3000B. *$P<0.05$ compared with the cell group; #$P<0.05$ compared with the PMA group

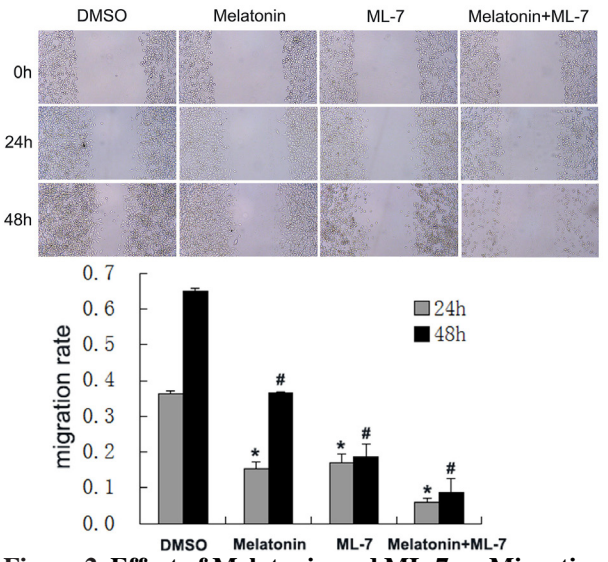

Figure 2. Effect of Melatonin and ML-7 on Migration of RKO Cells. After treating RKO cells with melatonin or ML-7 (25μM) alone, or in combination, for 48 hr, the treatment could block the migration of RKO cells. Calculating method: Relative migration ratio= (Start distance – End distance)/Start distance. *$P<0.05$, #$P<0.05$ compared with the DMSO group.

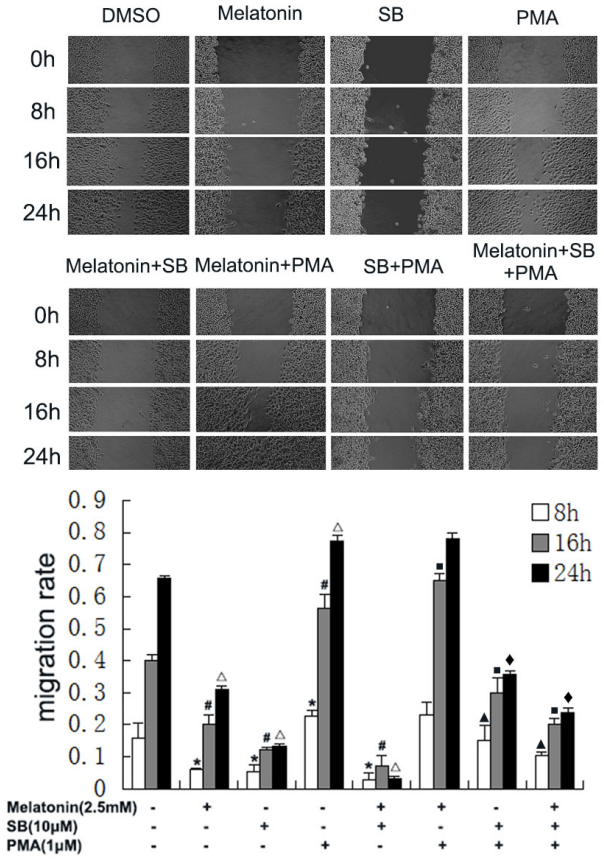

Figure 3. Effect of Melatonin, SB203580 and PMA on Migration of RKO Cells. The cells were treated with vehicle or melatonin (2.5mM), SB203580 (10μM) and PMA (1μM) alone, or in combination, for 24 hr.Melatonin and SB203580 significantly inhibited the migration of RKO cells, but PMA could partially prevented the decrease of melatonin and SB203580 on migration of RKO cells. *$P<0.05$, $^{\#}P<0.05$, $^{\Delta}P<0.05$ compared with DMSO group; $^{\blacktriangle}P<0.05$, $^{\blacksquare}P<0.05$, $^{\blacklozenge}P<0.05$ compared with PMA group

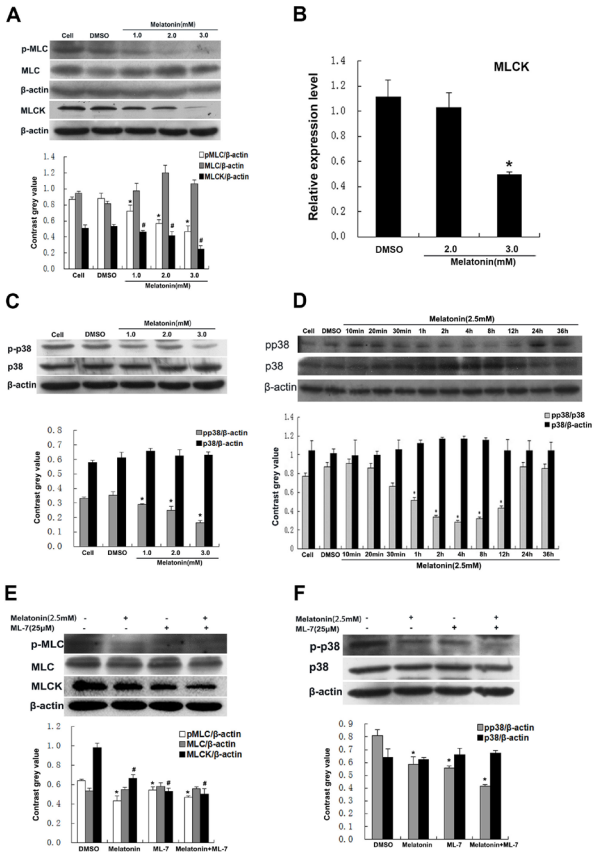

Figure 4. Effect of Melatonin, ML-7 on the Expression of MLCK and phosphorylation of MLC and p38. (A) After treating RKO cells with melatonin at different concentrations for 48 hr. The protein expression of MLCK and phosphorylation of MLC in RKO cells were decreased. (B) At the same time, the relative mRNA levels of MLCK was detected by real-time quantitative RT-PCR, which showed the same result. (C and D) Meanwhile, the phosphorylation of p38 was significantly decreased in a dose- and time-dependent manners. (E) ML-7 was used to treat RKO cells, and the expression of MLCK and phosphorylated of MLC were evidently decreased. (F) Furthermore, the phosphorylation of p38 was also decreased by ML-7. *P<0.05, #P<0.05 compared with DMSO group

treated with melatonin. As shown in figure 1B, the control cells were easy to congregate closely with neatness and fusiform. After treated with melatonin for 48 hr, the treatment exhibited highly reduced cell-to-cell contact and was mostly individualized as compared with DMSO control groups. Meanwhile, the cells formed a cell layer and multiple antennae, and the more spread and filopodia were observed. What's more, the cells became sparser with wider intercellular spaces and irregular arrangement as compared with vehicle (Figure 1B). These changes after treated with melatonin might be associated with cell migration.

Effect of melatonin on migration in RKO cells

As our previous studies suggested that migration of RKO cells was inhibited by melatonin in a concentration dependent manner. To the further verify the correlation between cell migration and MLCK, we used ML-7 to treat RKO cells and observed the wound healing of RKO cells. ML-7 is a specific inhibitor of MLCK, which can specifically inhibit MLCK phosphorylating MLC. After being treated with ML-7 (25μM) for 24 hr and 48 hr, the contrast migration rate of RKO cells was dramatically inhibited by ML-7 compared with the control group (Figure 2). We then used pharmacological inhibitor and activator to determine the role of p38 in the migration of RKO cells. Fig.3 did not only show the similar outcomes of melatonin groups, but also unveiled that the effect of melatonin combining SB203580 on inhibiting cell migration was more remarkable than that of melatonin alone. The results showed that SB203580 significantly inhibited the migration of RKO cells and PMA promoted the migration of RKO cells but not very obviously compared with the control, however, PMA partially prevented the decrease of melatonin and SB203580 on migration of RKO cells (Figure 3).

Melatonin decreased expression of MLCK and phosphorylation of p38 and MLC in RKO cells

After the cells were treated with different concentrations of melatonin for 48 hr, the results revealed that melatonin sharply reduced the expression of MLCK and phosphorylation of MLC, especially at the concentration of 3 mM (Figure 4A). Meanwhile, the relative mRNA

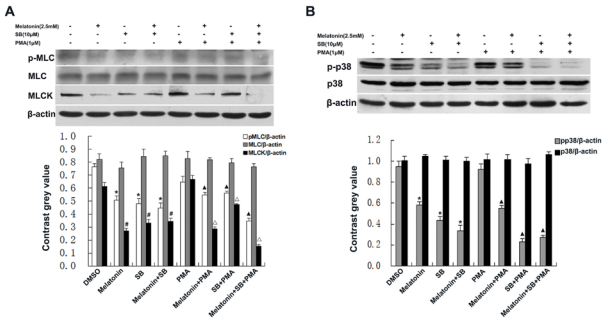

Figure 5. Effect of melatonin, SB203580 and PMA on the Expression of MLCK and Phosphorylation of MLC and p38. (A) The cells were treated with vehicle or melatonin, SB203580 and PMA alone, or in combination, for 48 hr. The expression of MLCK, the phosphorylated of MLC were down-regulated when cells were exposed to SB203580. Melatonin and SB203580 inhibited the expression of MLCK and phosphorylated of MLC in PMA-stimulated groups. (B) At the same time, the phosphorylation of p38 showed the same trend. *P<0.05, $^{\#}P$<0.05 compared with DMSO group; P<0.05, $^{\Delta}P$<0.05 compared with PMA group.

levels of MLCK was significantly decreased (Figure 4B). To evaluate the effect of melatonin on phosphorylation of p38, the level of phosphorylation of p38 in RKO cells treated by melatonin at various concentrations was measured by Western blot. Results showed that the level of phosphorylated p38 was decreased in a dose-dependent manner (Figure 4C). Furthermore, we also investigated the effect of melatonin (2.5mM) at different time points on the phosphorylation of p38 in RKO cells. Western blot showed that p38 phosphorylation, indicating p38 activation, was apparently decreased by melatonin-treated cells in a dose- and time-dependent manners (Figure 4 C and D). As shown in Figure 4E, after treated RKO cells with ML-7 for 48 hr, the expression of MLCK and the phosphorylation of MLC were significantly decreased (Figure 4E). And we have extended the study and found that the phosphorylation of p38 was decreased when the cells were treated with ML-7 (Figure 4F).

Melatonin down-regulates the expression of MLCK in RKO cells, which is partly through the p38/MAPK signaling pathway

After that, we conjectured that whether the expression and activity of MLCK is associated with p38/MAPK. To determine whether signaling pathway related to p38 was involved in melatonin-induced migration suppression, the effect of PKC activator (PMA) and p38 inhibitor (SB203580) on expression of MLCK was examined. Western blot analysis showed that melatonin inhibited the expression of MLCK and the phosphorylation of MLC as well as SB203580. Combination of melatonin and SB further reduced MLCK expression. Treatment with PMA partly prevented the decrease of the MLCK as compared to the melatonin-treated group (Figure 5A). At the same time, figure 5B showed that melatonin induced a significant reduction of p38 phosphorylation as compared to the control group. SB itself greatly reduced the p38 phosphorylation. Combination of melatonin and SB further reduced the phosphorylation of p38 to a very low level. What's more, the cells were coincubated with melatonin and PMA. We found that activation of PKC by PMA partially prevented the decrease of the p38 phosphorylation induced by melatonin (Figure 5B). Taken together, our findings indicated that melatonin induced the down-regulation of p38 MAPK signaling pathway in a dose- and time-dependent manner, suggesting that p38 MAPK may be specifically suppressed the migration of RKO cells by decreasing the expression and activity of MLCK.

Discussion

Cancer biological process is tightly associated with cell migration and invasion (Arafat et al., 2013; Pitchakarn et al., 2013). And the malignant process of metastasis has several components, including the cell proliferation, ability to invade through the acquisition of cell motility, degradation of basement membranes and extracellular matrices, and survival signaling. But, for current anticancer drugs mainly target tumour growth, finding a way to subdue cancer cell invasion, particularly in an adjuvant setting, molecularly-targeted inhibitors that blocked key invasion drivers would be expected to provide clinical benefit to a significant range of cancer patients with solid tumours at various stages. Melatonin is well-known indoles hormone mainly product secreted by the pineal gland in cyclical periods and a widely used antioxidant drug (Katzer et al., 2015; Shin et al., 2014; Borges et al., 2015). In addition, melatonin has been attracting more and more attention by exerting anti-proliferative, proapoptotic, and anti-angiogenic properties in multiple types of cancer cells (Lee et al., 2014; Plaimee et al., 2014b; Yi et al., 2014; Yun et al., 2014). And recently, an increasing number of studies have showed that melatonin could repress migration in various cancer cells (Wang et al., 2012d; Ordoñez et al., 2014; Zhou et al., 2014a). However, the effects and underlying molecular mechanisms of melatonin on colorectal cancer RKO cells migration inhibition are still not fully understood. Mechanically, we presented that melatonin might inhibit the proliferation and migration of RKO cells via down-regulating the phosphorylation of p38, and moreover, whether melatonin affected the migration of human colon cancer RKO cell via MLCK and through p38 MAPK pathway. To test this hypothesis, we first analyzed the effect of melatonin on tumor cell proliferation and migration in colon cancer RKO cells. Next, we analyzed the combined effects of SB203580, PMA, ML-7, and melatonin on some key proteins involved in cell proliferation and migration signaling pathways to uncover the molecular mechanisms of the natural products in colon cancer cells.

The morphology of tissue cells, acquire the ability for invasion mostly under special conditions, especially in the course of neoplastic evolution.The acquisition of cell locomotion, including migration and invasion, is closely associated with dynamic morphology of this cell and, especially with the dynamics of its cytoskeletal

and adhesive structures (Vasiliev et al., 2004; Vicente-Manzanares et al., 2007). And driving force for movement is mainly established by a dynamic structural balance between two kinds of cytoskeletal Arrangements: microfilaments and focal adhesions, whereas the structural balance of cancer cells is lost. In this paper, we have briefly discuss the influence of morphological changes in migration.After RKO cells were treated with melatonin for 48 hr, we found that the melatonin inhibited ruffle formation and increased cell anchorage possibly by augmenting the number and thickness of stress fibers, as well as focal adhesions (Figure 1B). The results supported that the cytoskeleton may be a therapeutic target for melatonin to block invasiveness and metastasis formation in cancer cells. In particular, some studies also confirmed the same results (Vasiliev et al., 2004; Ortiz-Lopez et al., 2009). Increasing evidences indicated that myosin light chain kinase (MLCK) and mitogen-activated protein kinase signaling, in addition to their roles in the control of proliferation and cell death, are also implicated in the regulation of cell motility (Viala et al., 2004; Zhou et al., 2008b). It is known to us that MLCK is subsequently activated by the increase of the intracellular Ca2+.Recent studies have also suggested that MLCK inhibitors could be useful as anti-cancer agents. Because MLC phosphorylation, which is activated by MLCK, is essential to trigger actin-myosin interaction (Lai et al., 2003). This interaction in smooth muscle and non-muscle cells is regulated by MLC phosphorylation. Consequently, changes in MLC phosphorylation should affect the ability of cells to divide, but also result in cell migration (Wilson et al., 1991; Klemke et al., 1997). In our study, we investigated the signal pathways involved in the inhibition of proliferation and migration of colon cancer cells mediated by melatonin using RKO cell line as a model. we found that the treatment with melatonin could potently inhibit the proliferation and migration of RKO cells by cell viability assay and vitro scratch- wound assay (Figure 1A and 2), and western blotting assay indicated that the expression of MLCK and phosphorylation of MLC were markedly decreased compared with control group (Figure 4). Studies have been reported that treatment with ML-7, a specific inhibitor of MLCK, was able to inhibit the proliferation and migration of tumor cells through blocking MLCK (Kaneko et al., 2002). Moreover, ML-7 was used to treat RKO cells and the similar result that ML-7 could more notably suppress RKO cells migration revealed that melatonin suppressed RKO cells migration by down-regulating the expression of MLCK and phosphorylation of MLC. Furthermore, recently, several studies have shown that MLCK can also retard the proliferation of tumor cells (Gu et al., 2006; Zhou et al., 2008b). Our studies also discovered that the proliferation of RKO cells was inhibited, the phosphorylation of p38 was decreased by ML-7, which showed the same trend as MLCK, suggesting that MLCK may also contribute to the proliferation of colon cancer RKO cells through cross-talk with inhibited p38/MAPK. p38, one of MAPKs signal cascade, is associated with the proliferation and migration properties of tumor cells as a prominent signal pathway (Wang et al., 2013a; Arechederra et al., 2015; Yan et al., 2015). But, the detailed mechanisms of p38/MAPK together with MLCK in colon cancer RKO cell migration have not been clarified. To determine the mechanism, we used PMA and SB203580 to respectively activate and inhibit MAPK/p38 signaling pathway in RKO cells. The results we did as shown in Figure 3, the migratory ability of colon cancer RKO cells was inhibited by melatonin and SB, but the decrease was prevented by PMA (Figure 3). Meanwhile, The expression of MLCK and the phosphorylated of MLC were down-regulated when cells were exposed to SB203580. Melatonin and SB203580 inhibited the expression of MLCK and phosphorylated of p38, MLC in PMA-stimulated groups (Figure 5). Therefore, in accordance with our data, the anti-migration effect of melatonin is associated with its inhibition of p38 MAPK pathway and regulation of the expression of MLCK.

Taken altogether, our present results strongly implied that p38 MAPK played an important role in inhibiting the proliferation and migration of RKO cells by melatonin. Down-regulation of pp38 levels by melatonin may contributed to RKO cell migration through decreasing the expression of MLCK and the activation of MLC, and inhibiting myosin light chain kinase possibly retards the growth of colon cancer RKO cells.Inhibition of p38 MAPK may be an important therapeutic target aimed at preventing the migration of colon cancer cell, suggesting that a treatment might potentially become an effective way in colon cancer therapy. Further researches are still needed to uncover the comprehensive molecular mechanisms of the natural products in tumor cells migration, because of the complex process which involves many factors and multiple steps.

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