Life Sciences 277 (2021) 119497

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Melatonin indirectly decreases gastric cancer cell proliferation and invasion via effects on cancer-associated fibroblasts

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ARTICLE INFO

Keywords:
Gastric cancer
Melatonin
Cancer-associated fibroblasts
NF-κB pathways

ABSTRACT

Aims: Gastric cancer is a malignant tumor with a poor prognosis, and the interaction between tumor cells and cancer-associated fibroblasts (CAFs) further contributes to progression and treatment failure. Recent studies have revealed the potential value of melatonin in cancer therapy, but its role in gastric cancer and CAFs requires further exploration.

Main methods: CAFs were isolated using the tissue block method. Cell Counting Kit-8 and cell cycle assays were used to determine the cell proliferation ability, while the cell metastatic capacity was detected by a wound healing assay and Transwell migration/invasion assay. Furthermore, the expression levels of proteins involved were examined using quantitative real-time PCR (qRT-PCR) and western blotting.

Key findings: Melatonin not only inhibits cell proliferation and metastasis by reducing the production of reactive oxygen species (ROS) in gastric cancer cells but also inhibits CAFs-induced gastric cancer cell progression by reducing the production of metalloproteinase 2 (MMP2) and metalloproteinase 2 (MMP9) in CAFs. The direct and indirect inhibitory effects of melatonin on gastric cancer cells are involved in the NF-kB signaling pathways.

Significance: This study provides insights into the role of melatonin in the tumor microenvironment, further deepens available knowledge regarding the mechanism of action of melatonin in gastric cancer and suggests the potential value of melatonin in gastric cancer treatment.

1. Introduction

Gastric cancer is one of the most fatal cancers and the third leading cause of cancer deaths worldwide. Despite efforts to develop effective therapies for cancer treatment, the 5-year overall survival rate of patients with gastric cancer remains low [1]. Tumors not only contain cancer cells but also the extracellular matrix (ECM) and various types of stromal cells, such as cancer-associated fibroblasts (CAFs), inflammatory cells, mesenchymal stem cells, and macrophages, which are known as the tumor microenvironment (TME), plays important and diverse roles in tumor progression [2]. The complex interactions between tumor cells and the TME provide new insights for improved understanding of cancer, and strategies for tumor treatment targeting the TME are also being explored [3]. Therefore, further exploration of the role of the TME in gastric cancer could provide a comprehensive understanding of the mechanism of gastric cancer development and help screen more appropriate drugs for gastric cancer treatment.

CAFs are major components of the TME and promote tumor progression through direct cell–cell interactions, cytokines, or as exosomes. Moreover, CAFs are associated with tumor resistance to chemoradiotherapy, and tumor recurrence can affect the prognosis of patients [4,5]. CAFs can influence the progression of gastric cancer cells through cytokines, miRNAs, and other associated factors produced by CAFs, and may induce therapeutic resistance [6–11]. Tumor treatments targeting CAFs have long been a research hotspot. These treatments include reducing the generation of CAFs, reversing the CAF phenotype, and interfering with the upstream and downstream signaling pathways of CAFs. However, owing to the heterogeneity and non-specificity of the CAFs within and among tumors, further screening for more specific and effective drugs is needed [12].

Melatonin is a hormone secreted by the pineal gland and other gastrointestinal tract-associated organs that exhibit pleiotropic effects [13,14]. Low levels of melatonin in the body are associated with a high risk of tumorigenesis in breast, prostate, and endometrial cancers

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https://doi.org/10.1016/j.lfs.2021.119497
Received 28 November 2020; Received in revised form 13 February 2021; Accepted 3 April 2021
Available online 18 April 2021

[15–17]. A growing number of in vitro and in vivo experiments are exploring the effect of melatonin on various cancer cell types, and most results suggest that melatonin suppresses cancer cell progression [18–20]. Therefore, some clinical trials have explored the function of melatonin supplementation in terms of survival, side effects of radiation and chemotherapy, and quality of life in patients with cancer, further suggesting the value of additional melatonin use in these patients [21–26]. In gastric cancer, melatonin can inhibit cell proliferation and metastasis by inducing apoptosis, autophagy, and inhibiting epithelial–mesenchymal transition [27–30]. Because of the considerable potential applications of melatonin in cancer therapy and the complex crosstalk between the TME and tumor cells, the role of melatonin in the regulation of the TME, especially CAFs, which is less often reported, requires further in-depth investigation, particularly in gastric cancer.

We hypothesized that melatonin not only affects the proliferation and metastasis of gastric cancer cells directly but also indirectly through its effect on CAFs. This study further deepens the understanding of the mechanism of action of melatonin in gastric cancer and suggests the potential value of melatonin in gastric cancer treatment.

2. Materials and methods

2.1. Cell culture and reagents

The human gastric cancer cell lines MGC-803 and SGC-7901 were obtained from the Shanghai Institute of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 medium or conditioned medium (CM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic with 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified incubator at 37 °C with 5% CO_2. All cell culture reagents were obtained from Gibco (Invitrogen, Carlsbad, CA, USA). Primary antibodies against phospho P65 (P-P65), P65, cyclin D1, metalloproteinase 2 (MMP2), MMP9, β-actin, and secondary antibodies were obtained from CST (Cell Signaling Technology, MA, USA).

A stock solution of melatonin (Sigma-Aldrich, China) was prepared by dissolving it in vehicle (anhydrous ethanol), for cell treatment, stock solutions were dissolved in the culture media, final concentrations of vehicle did not exceed 0.25% (*v/v*).

2.2. CAFs isolation and CM collection

For the isolation of CAFs and normal fibroblasts (NFs), samples derived from patients with gastric cancer were minced into 1 mm^3 pieces, washed in RPMI 1640, plated in Petri dishes at a distance of 5 mm apart, and cultured in RPMI 1640 containing 10% FBS. The fibroblasts were then allowed to grow from the tumor fragments for 2–3 weeks. α-SAM was used to identify CAFs, and cells ranging from passage 2 to 10 were used for subsequent experiments.

CAFs were cultured in complete media with or without 3 mM of melatonin for 48 h. Then, the cells were cultured in medium with RPMI 1640 for 48 h. CM was collected and centrifuged at 1000 ×*g* at 4 °C for 15 min. Subsequently, CAFs CM and melatonin-treated CAFs CM (MLT-CAFs CM) were obtained, and RPMI 1640 medium was used as a negative control (NC).

2.3. Cell viability and colony formation assays

A Cell Counting Kit-8 assay (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to test the cell viability after treatment with melatonin or CM. The gastric cancer cells MGC-803 or SGC-7901 or CAFs (8000/well) were seeded onto a 96-well plate and allowed to adhere to the walls. The medium was changed to CM, and the cells were incubated at 37 °C for 0–72 h. Each treatment group was placed in three parallel wells. Thereafter, 10 μL of the CCK-8 solution was added to each well and cultured for 2 h. Finally, the absorbance value was read at 450 nm using a SpectraMax i3 (Molecular Devices, USA). The experiment was repeated three times.

MGC-803 cells (200 cells/well) were seeded onto six-well plates. After adhering to the walls, 3 mM of melatonin was added to each well and cultured for 7 days. The cells were then washed three times with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min before staining with 0.5% crystal violet for 5 min. After washing with water, the wells were examined using a microscope (Nikon Corporation, Tokyo, Japan).

2.4. Microscopic observation of cell morphology

Cells (1×10^6 cells/well) were seeded onto six-well plates. At 70% confluence, 3 mM of melatonin was added to the cells and incubated for 48 h. Subsequently, the cells were washed three times with PBS and fixed with 4% paraformaldehyde for 10 min. Thereafter, the cells were stained with 0.5% crystal violet for 5 min. After washing with water, cell morphology was visualized with a TE2000 inverted fluorescence microscope (Nikon Corporation, Tokyo, Japan).

2.5. Wound healing assay and Transwell migration/invasion assay

The cells (1×10^6 cells/well) were seeded into six-well plates and cultured in a complete medium at 37 °C. After the formation of a monolayer of cells, 200 μL pipette tips were used to form a scratch on the surface of the media. The debris was washed away with PBS and the gastric cancer cells were cultured in RPMI 1640 containing various concentrations of melatonin (vehicle, 3 mM) or CM (CAFs CM and MLT-CAFs CM). Images were captured between 0 and 48 h using a microscope, and the percentage of wound closure was analyzed and calculated with ImageJ software. Each experiment was conducted three times.

Next, 24-well Transwell chambers with 8 μM polycarbonate nucleopore filters with or without 10 μg/well Matrigel in the upper chamber were used to perform the invasion and migration assays, respectively. The cells were first treated with melatonin or CM for 48 h then were seeded (at a density of 3×10^4 cells/well for migration, 4×10^4 cells/well for invasion) onto the upper chamber with serum-free medium. The lower chamber was filled with medium containing 10% FBS. After 48 h, the cells from the upper chamber were fixed with 4% paraformaldehyde for 10 min then stained with 0.5% crystal violet for 5 min. After washing, a cotton swab was used to wipe the upper surface of the membrane, and the lower surface of the membrane was imaged using a phase-contrast microscope. ImageJ software was used to count the number of cells in each picture. Each experiment was conducted three times.

2.6. Cell cycle assay

The cells were seeded onto six-well plates and allowed to adhere to the walls. They were then incubated with CM for 48 h. After trypsin digestion, cells were washed twice with PBS and fixed in 75% ethanol at 4 °C for 4 h, followed by propidium iodide staining for 30 min in the dark at 25 °C. We tested the cell cycles using BD FACS Canto II (BD Biosciences, USA) flow cytometry, and the results were analyzed by Modifit.

2.7. ROS analysis

Gastric cancer cells were seeded onto 6-well plates and treated with melatonin for 1 h. Afterwards, the cells were incubated with 2 2′,7′–dichlorofluorescein diacetate (DCFDA) for 30 min at 37 °C. A fluorescence picture was taken immediately with the inverted fluorescence microscope and the results were analyzed with Image J software. The image was analyzed in terms of the percentage of the intensity of fluorescence versus control. The fluorescence signal was recorded by Flow cytometry; and the mean fluorescence intensity was analyzed by

FlowJo.

2.8. Gelatin zymography

Equal amount of raw protein (40 μg) was loaded into 10% SDS-polyacrylamide gels containing 1% gelatin (Sigma-Aldrich, USA). After electrophoresis, 2.5% Triton-X100 solution was used to wash the SDS from the gel for three times, and then added the gels in the zymography buffer (40 mM Tris-HCl, pH 7.4, 0.2 M NaCl and 10 mM $CaCl_2$) for 48 h at a temperature of 37 °C. Afterwards, the gel was stained with 0.1% Coomassie brilliant blue (Sigma-Aldrich, USA) and destained with 10% acetic acid/30% methanol for 0.5 h, 10% acetic acid/20% methanol for 1 h, and 5% acetic acid/10% methanol for 2 h. The clear regions on the gels reflected the gelatinolytic activity and was measured by Image J.

2.9. ELISA assay

CAFs were cultured in complete media with or without 3 mM of melatonin for 48 h. Then, the cells were cultured in medium with RPMI 1640 for 48 h. After centrifugation, cell culture supernatants were tested using Human MMP2 and MMP9 ELISA Kits (Abcam, Cambridge, CA, USA) based on the manufacturer's instructions.

2.10. Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from gastric cancer cells using Trizol reagent (Sangon, Shanghai, China). Then, total RNA was reverse transcribed into cDNA using an M-MuLV First Strand cDNA Synthesis Kit (Sangon, Shanghai, China). Quantitative real-time PCR was performed using a 7500 Realtime PCR System (Applied Biosystems, Carlsbad, CA, USA) with 2× SG Fast qPCR Master Mix (Sangon, Shanghai, China). The specific primers were designed as following: MMP2 forward, 5′-ATTGTATTTGATGGCATCGCTC-3′ and MMP2 reverse, 5′-ATTCATTCCCTGCAAAGAACAC-3′; MMP9 forward, 5′-CAGTACCGAGAGAAAGCCTATT-3′ and MMP9 reverse, 5′-CAGGATGTCATAGGTCACGTAG-3′; cyclin D1 forward, 5′-GTCCTACTTCAAATGTGTGCAG-3′ and cyclin D1 reverse, 5′-GGGATGGTCTCCTTCATCTTAG-3′; β-actin forward, 5′-GCATCGTCACCAACTGGGAC-3′ and β-actin reverse, 5′-ACCTGGCCGTCAGGCAGCTC-3′; β-actin was used as an internal control and results are shown as relative expressions calculated by the $2^{-\Delta\Delta CT}$ method.

2.11. Western blot analysis

After culturing with melatonin or CM, RIPA was used to lyse cells to obtain total proteins. The protein concentration was determined using the BCA method. Equal amounts of protein (40 μg) were added to 10% SDS-acrylamide gel then separated by electrophoresis. The proteins were transferred to nitrocellulose membrane filters. Subsequently, the membranes were blocked with 5% skim milk, followed by incubation with the following primary antibodies: P—P65, P65, Akt, cyclin D1, MMP2, MMP9, and β-actin overnight at 4 °C. The membranes were then washed and incubated with HRP-conjugated secondary antibody for 1 h. After washing six times for 30 min, the protein bands were detected with ECL reagent. Images were obtained using an AI 600 imaging system.

2.12. Statistical analysis

Data are presented as the mean ± standard deviation (SD) from the three independent experiments. Statistical analysis of data was carried out by *t*-test or by one-way ANOVA in multiple comparisons of means with GraphPad Prism software version 7.0. A *p*-value <0.05 was considered as statistically significant.

3. Results

3.1. Melatonin suppresses gastric cancer cell proliferation, migration, and invasion

The CCK-8 assay was performed to estimate the effects of different concentrations of melatonin on MGC-803 cells at different time points. The results showed that the effects of melatonin exhibited a certain concentration-dependent and time-dependent (Fig. 1A). For identifying the suitable treatment time and concentration, the dose response curve was applied, results showed that the IC_{50} with treatment time point of 24 h, 48 h and 72 h was 4.783 mM, 3.198 mM and 2.69 mM respectively (Fig. 1B). For convenience, we selected the treatment time of 48 h with 3 mM concentration for subsequent experiments. In terms of cell morphology, melatonin stimulation for 48 h produced cells with narrow and irregular edges and gaps compared with those of the vehicle (Fig. 1C). Colony formation assays were used to confirm the effect of melatonin on the proliferation of gastric cancer cells. The results revealed that the proliferation of MGC-803 cells was markedly reduced after 7 days of treatment with 3 mM of melatonin ($p < 0.001$) (Fig. 1D).

We then performed a wound healing assay to explore the effect of melatonin on cell migration. After 48 h of 3 mM melatonin treatment, the percentage of wound closure was nearly 40% in the vehicle group but 10% in the treated group ($p < 0.001$) (Fig. 1E). The Transwell migration and invasion assay was used to further evaluate the influence of melatonin on cell migration and invasion. The number of cells at the bottom of the membrane was determined. Melatonin (3 mM) remarkably reduced MGC-803 cell migration ($p < 0.001$) and invasion ($p < 0.05$) compared to those in the vehicle (Fig. 1F).

3.2. Melatonin suppresses gastric cancer cell migration and invasion via reactive oxygen species (ROS)-induced inhibition of the NF-κB signal pathway

To investigate the antitumor role of melatonin with respect to its antioxidant effects, we determined the production of ROS. ROS production was relatively decreased by 20% after treatment with 3 mM of melatonin for 30 min ($p < 0.001$) (Fig. 2A). Flow cytometry was used to measure the mean fluorescence intensity between the control and treatment groups (1574 ± 54.93 vs. 1194 ± 32.66, $t = 5.95$, $p < 0.01$) (Fig. 2B). NF-κB is a downstream transcription factor that is targeted by ROS. We further explored whether melatonin could elicit anti-metastasis effects by influencing the ROS-mediated regulation of the NF-κB signaling pathway. The activation of P—P65 after treatment with 3 mM of melatonin for 30 min was determined. The expression of P—P65 decreased in the treatment group. The expression of cyclin D1 and MMP2, downstream targets of the NF-κB signaling pathway that play a role in cancer proliferation and metastasis, was also reduced (Fig. 2C). Furthermore, SDS-PAGE gelatin zymography revealed that MMP2 activity decreased in a concentration-dependent manner following treatment with melatonin (Fig. 2D).

3.3. Establishment of gastric CAFs

To establish gastric CAFs, normal or cancer gastric tissues were cultured using the tissue explant method. After screening, the residual cells stained with crystal violet displayed spindle-like cell morphology. Gastric CAFs appeared with more cells and a more rounded appearance than NFs. Interestingly, after melatonin application, the CAFs atrophied and decreased in number and had similar morphology as that of NFs (Fig. 3A). A hallmark of CAFs is the high expression of α-SMA, results of qRT-PCR showed that α-SMA expression was higher in CAFs than in NFs (Fig. 3B). These results demonstrated that we successfully cultured CAFs.

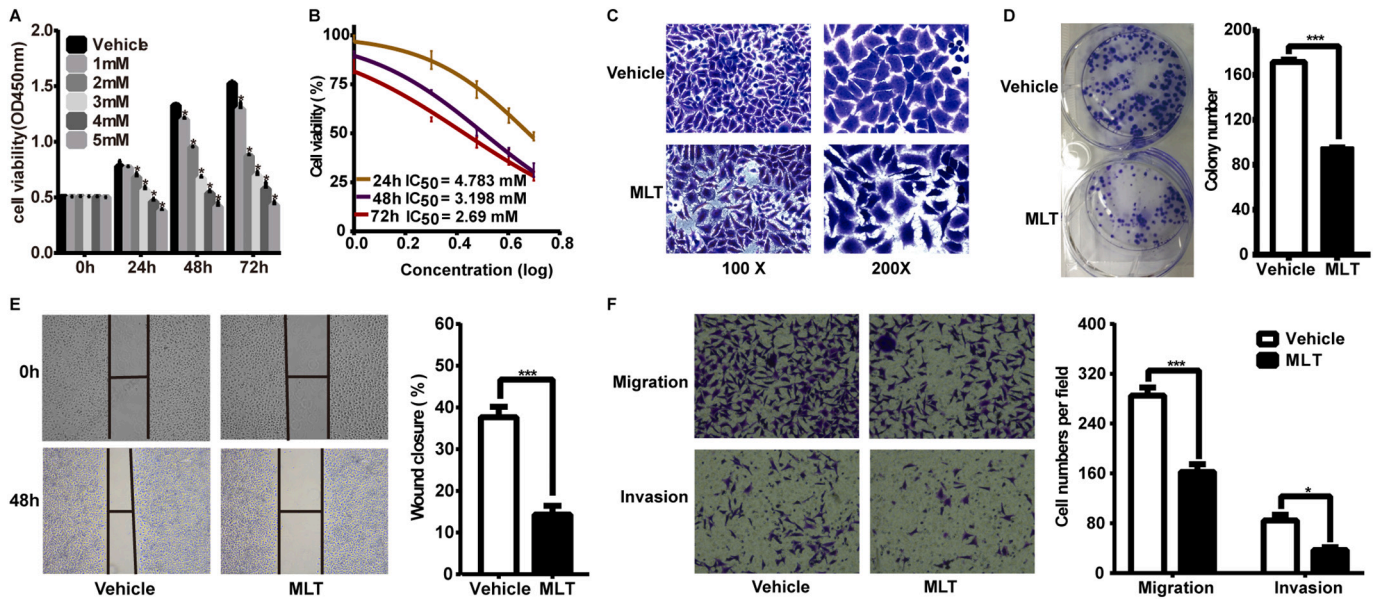

Fig. 1. Effects of melatonin on gastric cancer cell proliferation, morphology, migration, and invasion. (A) CCK-8 cell viability assay at different concentrations and time points. (B) Dose-response curve and IC_{50} of melatonin in different time points. (C) Effects of melatonin (3 mM) on gastric cancer cell morphology (magnification, ×100 and ×200). (D) Colony formation assays after treatment with 3 mM of melatonin for 7 days. (E) Wound healing assay. (F) Migratory ability and invasiveness of the cells assessed by Transwell respectively. All experiments were performed in triplicate and data are expressed as mean ± SD. $^{*}p < 0.05$, $^{***}p < 0.001$.

3.4. Melatonin treatment of CAFs suppresses gastric cancer cell proliferation

CAFs are important components of the TME and can promote tumor cell proliferation. To demonstrate this phenotype and further investigate the effect of melatonin on this process, we obtained the CM of CAF supernatant (CAFs CM), the CM of CAF supernatant after melatonin (3 mM) treatment for 48 h (MLT-CAFs CM), and RPMI 1640 medium was used as a NC. The CCK-8 assay showed that CAFs CM elevated the viability of gastric cancer cells of MGC-803 and SGC-7901 compared to that of the NC. In contrast, MLT-CAFs CM suppressed the effect of CAF CM (Fig. 4A). Next, cell cycle analysis revealed that CAFs CM could assist cancer proliferation by promoting S phase entry; however, the MLT-CAFs CM may reverse the trend by arresting gastric cancer cells in the G0/G1 phase (Fig. 4B). These results indicate that melatonin treatment of CAFs suppresses gastric cancer cell proliferation, which formerly elevated by CAFs, and can interfere with the cell cycle of gastric cancer cells.

3.5. Melatonin treatment of CAFs suppresses gastric cancer cell migration and invasion

To investigate whether CAFs play an important role in the metastasis of gastric cancer and the function of melatonin in this phenomenon, we cultured gastric cancer cells for 48 h under different conditions. Our results showed that the extent of wound closure and number of migrating and invading cells were significantly greater in the CAFs CM group than in the NC group. By contrast, the MLT-CAFs CM group could partly or overly reverse these trends when compared with that in the CAFs CM group in both MGC-803 and SGC-7901 gastric cancer cells (Fig. 5). These results showed that CAFs can prompt gastric cancer cell migration and invasion and that these trends may be interrupted by melatonin treatment of CAFs.

3.6. Melatonin suppresses the proliferation of CAFs and decreases the expression of MMP2 and MMP9 in CAFs

Our results demonstrated that melatonin can influence the function of CAFs then inhibit the growth of gastric cancer cells. Therefore, we next focused on the effect of melatonin on CAFs. Our results showed that melatonin repressed the viability of CAFs (Fig. 6A). Cell cycle analysis revealed that after melatonin exposure for 48 h, the cell cycle of CAFs was arrested in the G0/G1 phase (Fig. 6B). Further investigation showed that the expression of MMP2 and MMP9 decreased in CAFs after melatonin treatment (Fig. 6C). Moreover, we investigated the concentration of MMP2 and MMP9 in the supernatant of CAFs. The results were consistent with those of the western blot, the secretion of MMP2 and MMP9 were both decreased after melatonin treatment (Fig. 6D).

3.7. Melatonin treatment of CAFs suppresses the NF-κB signaling pathways in gastric cancer cells

To illustrate the mechanism of CAFs and melatonin-treated CAFs on gastric cancer proliferation, migration, and invasion, we first detected the expression of MMP2, MMP9, and cyclin D1. The qRT-PCR results showed elevated expression of MMP2, MMP9, and cyclin D1 in CAFs CM group compared to that in the NC group. However, the treatment of CAFs with melatonin reversed this expression (Fig. 7A). Likewise, western blot analysis indicated that P—P65, MMP2, MMP9, and cyclin D1 levels were lower in the MLT-CAFs CM group than in the CAFs CM group, in which these were highly expressed compared with NC group (Fig. 7B). Therefore, we inferred that melatonin treatment of CAFs can suppress the NF-κB signaling pathways activated by CAF CM in gastric cancer cells.

4. Discussion

The treatment of gastric cancer faces considerable challenges, especially as advanced gastric cancer cannot be effectively treated by either surgery or chemotherapy and is a malignant tumor with a poor prognosis [31]. Meanwhile, the interaction between tumor cells and CAFs during the development of gastric cancer further contributes to progression and treatment failure. Intervening in the crosstalk between cells may be a more feasible direction for cancer therapy [32]. Melatonin is an endocrine hormone mainly secreted by the pineal gland with a high lipophilicity and moderate hydrophilicity and is widely distributed in tissues and cells. In addition, the gastrointestinal tract is a major site of melatonin secretion, playing an important role in maintaining the

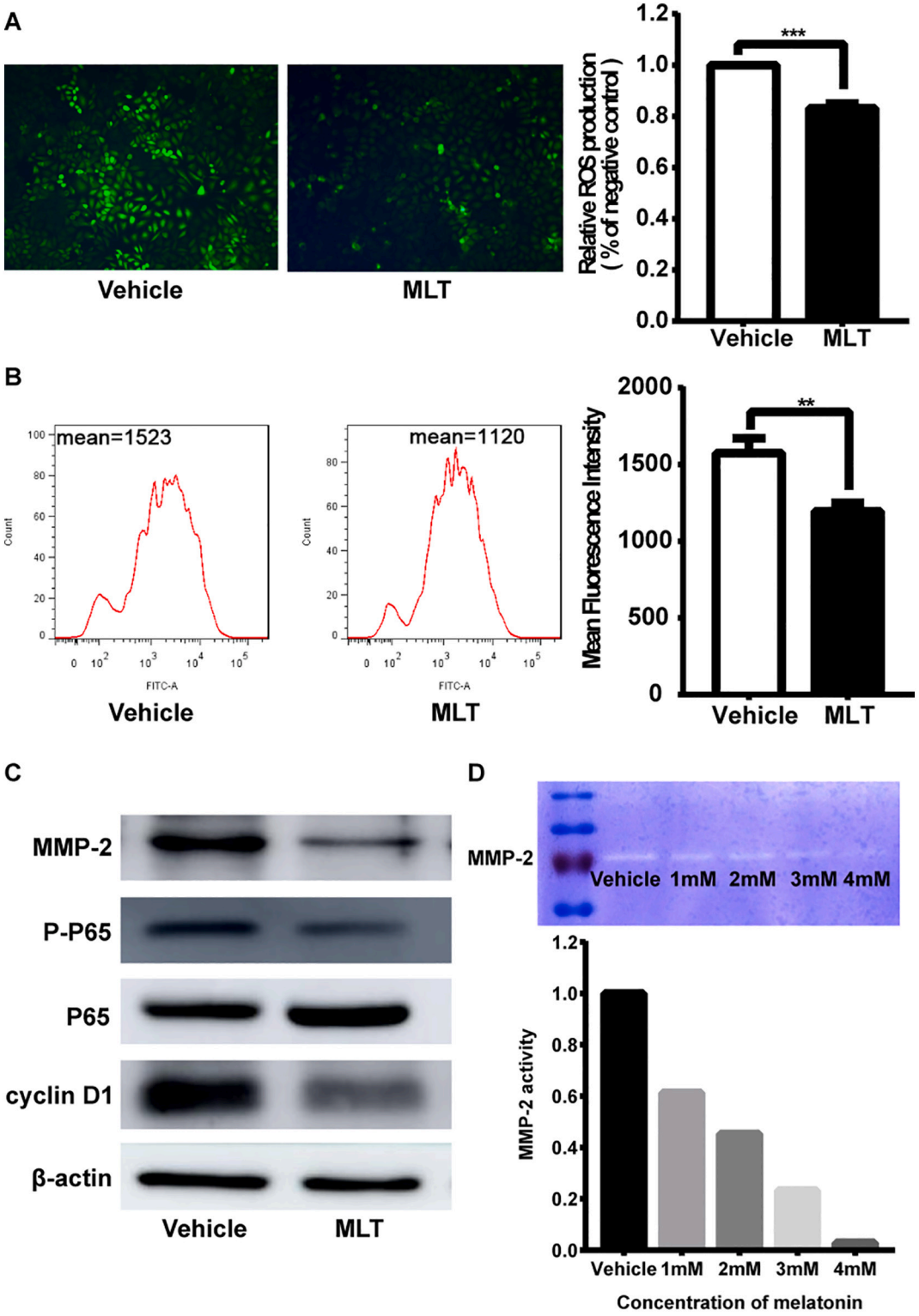

Fig. 2. Melatonin inhibits gastric cancer cells via the reactive oxygen species (ROS)-regulated NF-κB signaling pathway. (A, B) Melatonin affects the production of ROS in gastric cancer cells as determined using a fluorescence microscope or flow cytometry. (C) Effects of melatonin on the expression of P—P65, MMP2, and cyclin D1 in gastric cancer cells. P65 and β-actin were used as the controls. (D) SDS-PAGE gelatin zymography of MMP2 at different concentrations of melatonin. All experiments were performed in triplicate and data are expressed as mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$.

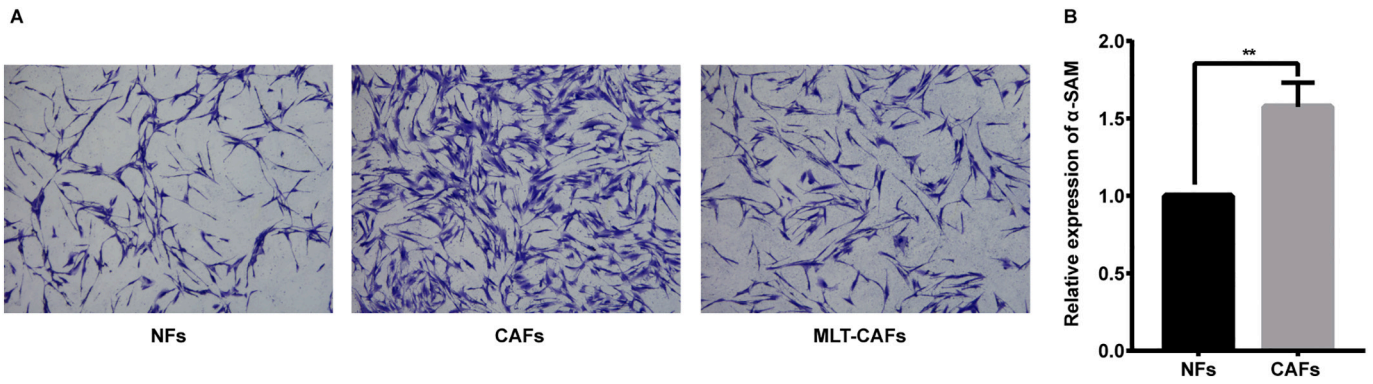

Fig. 3. Identification of gastric cancer-associated fibroblasts (CAFs). (A) Morphology of normal fibroblasts (NFs), gastric CAFs, and melatonin-treated CAFs (MLT-CAFs). (B) α-SAM mRNA expression was determined by Quantitative real-time PCR (qRT-PCR). All experiments were performed in triplicate and data are expressed as mean ± SD. $^{**}p < 0.01$.

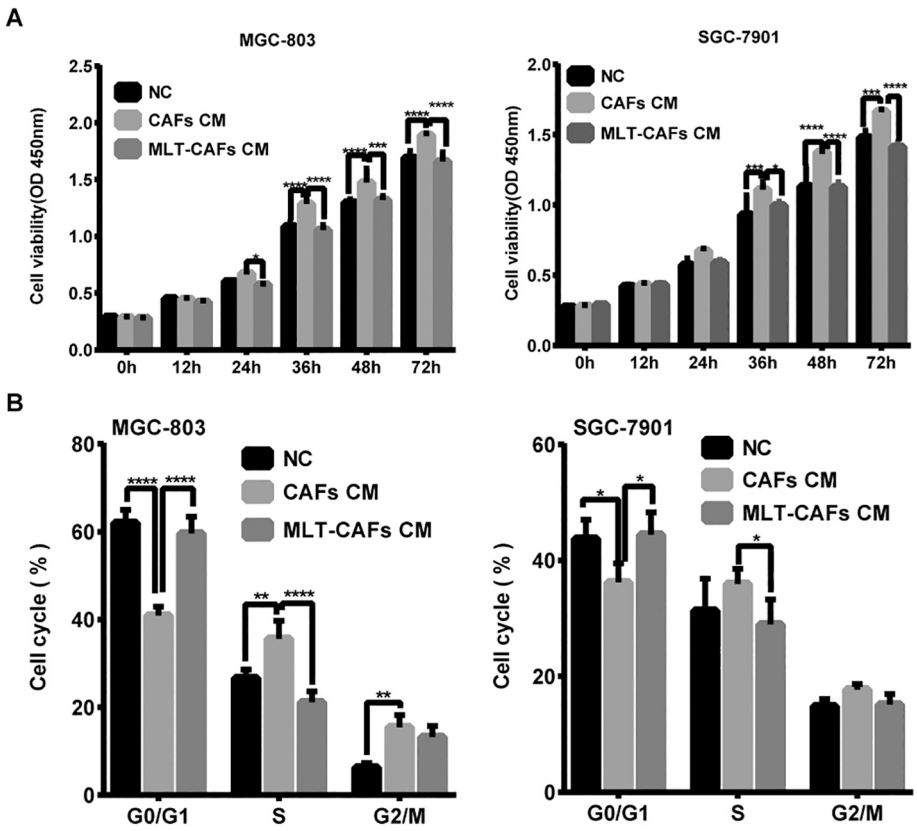

Fig. 4. Melatonin treatment of CAFs suppresses gastric cancer cell proliferation. (A) CCK-8 cell viability assay of gastric cancer cells MGC-803 and SGC-7901 in different media and at different time points. (B) Cell cycle analysis of different media for gastric cancer cells MGC-803 and SGC-7901. NC, negative control. CAFs CM, gastric CAFs conditioned medium. MLT-CAFs CM, melatonin-treated gastric CAFs conditioned medium. All experiments were performed in triplicate and data are expressed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, $^{****}p < 0.0001$.

normal function of the gastrointestinal tract, and under some conditions, melatonin disruption is related to gastrointestinal cancers [14]. Recent studies have further revealed the potential value of melatonin in cancer therapy. In colorectal cancer, it can play a potential therapeutic role by increasing apoptotic and autophagy, interfering with cell proliferation and metastasis, and improving tumor cell resistance to chemo-radiotherapy [33,34]. However, the role of melatonin in gastric cancer and CAFs requires further investigation. Our study revealed that melatonin directly inhibits the proliferation and metastasis of gastric cancer cells and can further affect the action of CAFs to reverse the progression of gastric cancer caused by CAFs.

Excessive generation of ROS leads to oxidative stress and various diseases, including malignant neoplasms. Therefore, antioxidant agents may counteract these effects and play a role in cancer treatment [35]. Melatonin is an endogenous compound with antioxidant properties. In a study of thyroid cancer, melatonin suppressed cell growth via the reduction of ROS-induced P65 phosphorylation [36]. In another study, melatonin inhibited the ROS-driven proliferation of oral cancer cells [37]. Here, we found that direct melatonin treatment in gastric cancer cells decreased the level of ROS. These results suggest that melatonin can affect tumor proliferation and metastasis by directly inhibiting the production of ROS in gastric cancer cells, which further illustrates the potential value of antioxidant effects in gastric cancer treatment.

The TME plays a significant role in tumor progression, and interventions in the TME are an indispensable part of cancer treatment [3]. CAFs are the main components of the TME and can promote tumor progression, a study in hepatocellular carcinoma have shown that the secretion of CCL2 and IL6 by tumor cells can further promote the secretion of these factors in CAFs, which can then promote the proliferation of tumor cells through positive feedback [38]. Our study also

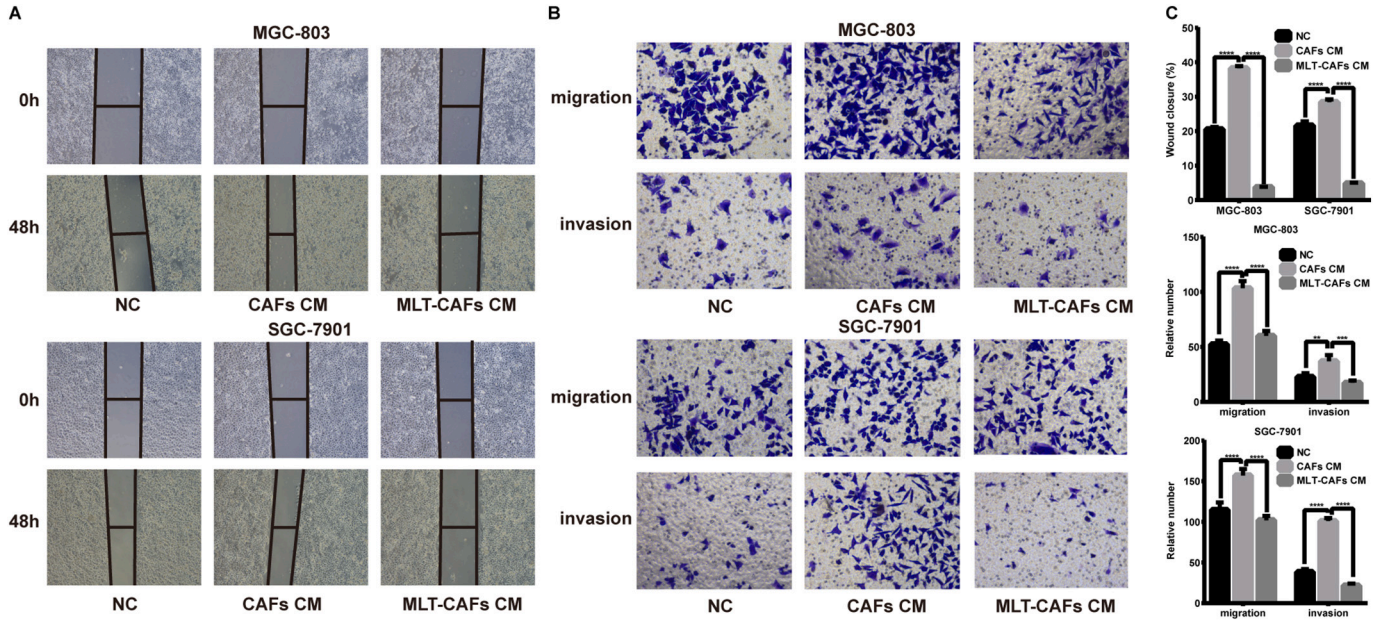

Fig. 5. Melatonin treatment of CAFs suppresses gastric cancer cell migration and invasion. (A) Wound healing assay. (B) Migratory ability and invasiveness of the cells assessed by Transwell chambers with non-coated or Matrigel-coated membranes, respectively. (C) Quantitative analysis of percent wound closure and cell numbers between groups. CAFs CM, gastric CAFs conditioned medium. MLT-CAFs CM, melatonin treated gastric CAFs conditioned medium. All experiments were performed in triplicate and data are expressed as mean ± SD. **p < 0.01, ***p < 0.001, ****p < 0.0001.

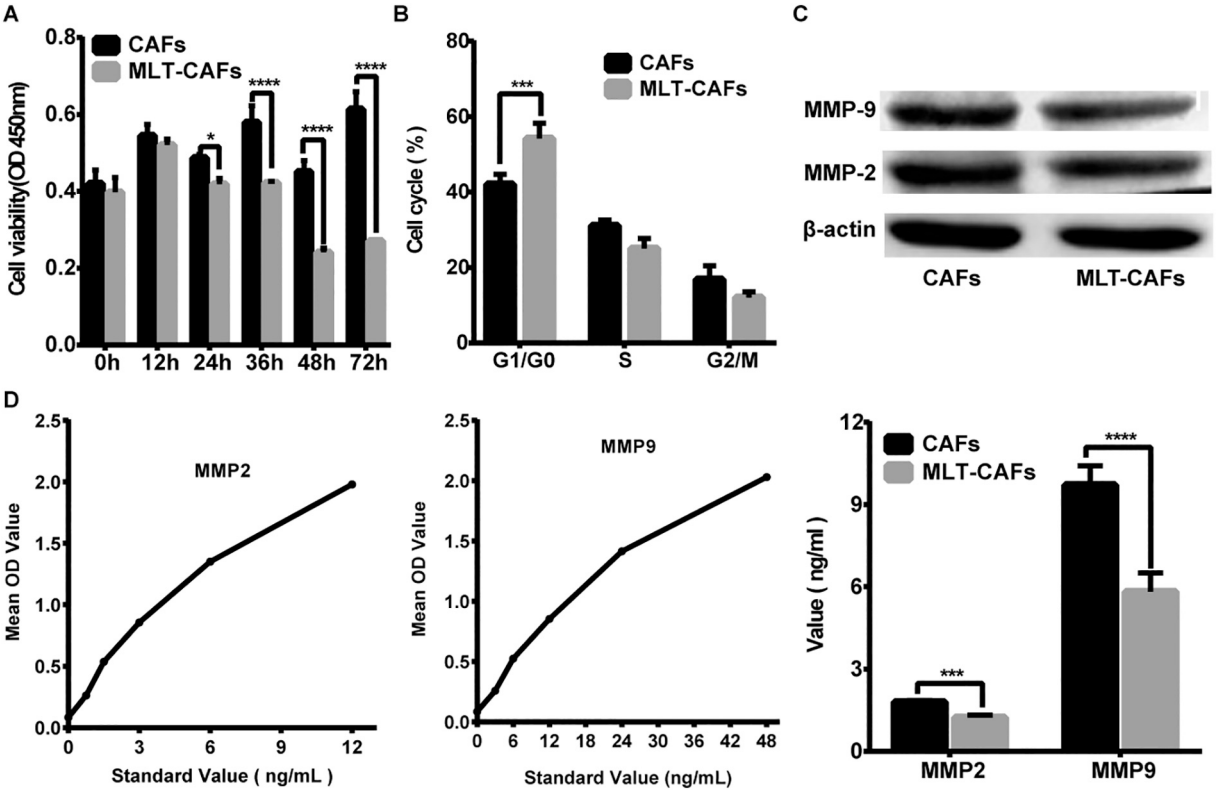

Fig. 6. Melatonin suppresses the proliferation of CAFs and decreases the expression of MMP2 and MMP9 in CAFs. (A) CCK-8 cell viability assay of melatonin-treated CAFs (MLT-CAFs). (B) Cell cycle analysis of MLT-CAFs. (C) Effects of melatonin on the expression of MMP2 and MMP9 in CAFs. β-actin was used as the control. (D) ELISA results of melatonin on CAFs. All experiments were performed in triplicate and data are expressed as mean ± SD. *p < 0.05, ***p < 0.001, ****p < 0.0001.

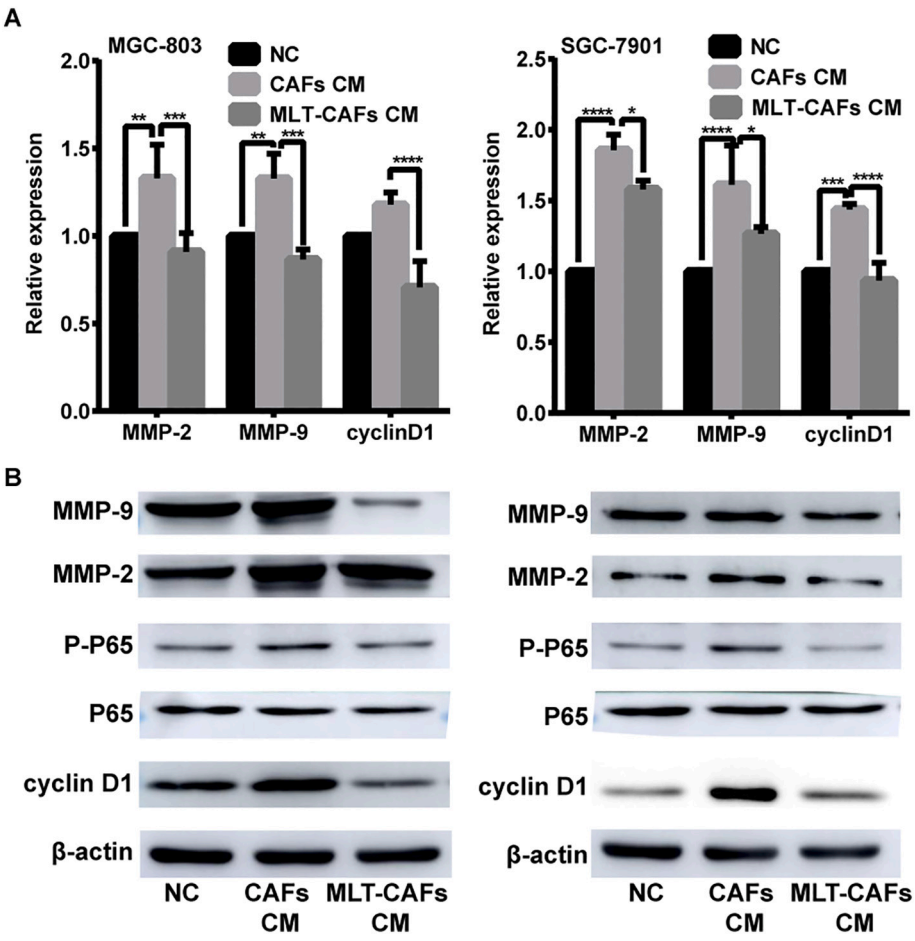

Fig. 7. Melatonin treatment of CAFs suppresses the NF-κB signaling pathways in gastric cancer cells. (A) Quantitative real-time PCR (qRT-PCR) to detect the expressions of MMP2, MMP9, and cyclin D1 in the gastric cancer cells MGC-803 and SGC-7901. (B) Western blot to detect the expressions of p-p65, MMP2, MMP9, and cyclin D1 in the gastric cancer cells MGC-803 and SGC-7901. All experiments were performed in triplicate and data are expressed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

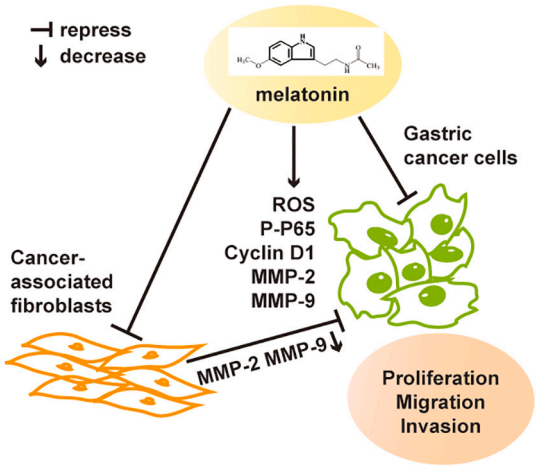

Fig. 8. Schematic diagram of the mechanism of melatonin suppressing the proliferation, migration, and invasion of gastric cancer cells.

demonstrates that the supernatant of CAFs can significantly enhance gastric cancer cell proliferation and metastasis. Therefore, we inferred that disrupting the interaction between CAFs and tumor cells may be beneficial for tumor treatment. Biologically active drugs, such as curcumin, resveratrol, and omega-3 polyunsaturated fatty acids, can affect the crosstalk between CAFs and tumor cells and, thus, influence tumor progression [39–41]. As a safer, less toxic, and more effective antitumor drug, melatonin functioning in the TME mainly focuses on the tumor immune microenvironment and rarely on CAFs [42]. Melatonin regulates angiogenic and inflammatory proteins in MDA-MB-231 breast cancer cells and in co-culture with CAFs [43]. However, the direct effects of melatonin on CAFs have not yet been reported. The results of the present study suggested that after direct melatonin treatment of CAFs, cell viability decreased, the morphology of CAFs gradually atrophied, and there was a tendency to transform to NFs morphology. The CAFs cell ability to synthesize and secrete MMP2 and MMP9 was suppressed. These results provide a new perspective for further studying the role of melatonin on CAFs.

Inflammation plays a vital role in gastric cancer. ROS and the TME can aggravate inflammation, and the NF-κB signaling pathway is an inflammation-related pathway [44]. Therefore, we further investigated the effect of melatonin on the NF-κB signaling pathway in gastric cancer cells. The NF-κB signaling pathway can directly and indirectly control inflammation, cancer cell proliferation and survival, invasive behavior, angiogenesis, and metastasis, as well as genetic and epigenetic alterations, cancer stem cell formation, cellular metabolism, and therapy resistance, furthermore, activation induces immunosuppression via several mechanisms [45]. In gastric cancer, many of the genes transcribed by NF-κB promote gastric carcinogenesis. In this study, we tested the expression of MMP2, MMP9, and cyclin D1, all of which are

regulated by the activation of NF-κB. The results showed that melatonin directly affected the expression of P—P65, MMP2, and cyclin D1 in gastric cancer cells; furthermore, MLT-CAFs CM could counteract the activation of P—P65, MMP2, MMP9, and cyclin D1 in CAFs CM group.

Metastasis is one of the essential characteristics of tumors, previous studies have shown that melatonin can affect tumor cell metastasis by influencing ECM rearrangement, cell-cell and cell-matrix interactions, etc. [46]. ECM remodeling occurs in tumors, mainly regulated by matrix metalloproteinases. On the one hand, ECM can maintain stiffness through proteins, such as fibronectin, thus, forming a natural barrier to prevent drugs and generating therapeutic resistance; On the other hand, ECM can facilitate tumor metastasis through the solubilization of MMPs, particularly MMP2 and MMP9, thus, exposing the target site to bind tumor cells [47]. Our results confirmed the reduced metastatic capacity combined with decreased MMP2 and MMP9 expression in gastric cancer cells. CAFs are one of the major cell types that produce MMPs, and MMP2 and MMP9 are not only structural proteins but also soluble proteins that can function through autocrine and paracrine forms [48]. This was also observed in the present study, melatonin directly reduced the production and secretion of MMP2 and MMP9 in CAFs, and MLT-CAFs CM could attenuate the high expression of MMP2 and MMP9 induced by CAFs CM in gastric cancer cells. This suggested that melatonin may attenuate crosstalk between tumor and stromal cells or regulate ECM rearrangement by reducing MMP2 and MMP9 expression in CAFs. Cyclin D1 is a key molecule in the regulation of the cell cycle, and its expression is directly or indirectly regulated by NF-κB. Our results showed that melatonin decreases the viability and proliferation ability of gastric cancer cells; the cell cycle was arrested at the G0/G1 stage and decreased the expression of cyclin D1. Therefore, our results suggest that melatonin can affect the proliferation and metastasis of gastric cancer cells directly or indirectly through the expression of the NF-κB signaling pathway-related proteins MMP2, MMP9, and cyclin D1.

Although this study initially explored the effect of melatonin on gastric cancer cells and CAFs, we have only conducted in vitro studies, and further in vivo experiments are needed. Moreover, we can also apply proteomics, microarray technology, single-cell sequencing, and other high-throughput technologies to detect the expression profile changes after melatonin treatment of CAFs, thus, providing a more comprehensive understanding of the role of melatonin in CAFs. CAFs are internally heterogeneous with different subtypes, and some subtypes of CAFs have tumor-suppressive effects; thus, further consideration of the effects of melatonin on different subtypes and changes in the TME are needed [49]. In addition, in future clinical research, issues of dose and time of melatonin administration require exploration [13]. Despite the limitations of the current study, it is likely that melatonin has great potential in gastric cancer therapy, especially since this study suggests a dual effect of melatonin on both gastric cancer cells and CAFs.

5. Conclusion

Melatonin not only inhibits cell proliferation and metastasis by reducing the production of ROS in gastric cancer cells but also inhibits CAF-induced gastric cancer cell progression by reducing the production of MMP2 and MMP9 in CAFs. The direct and indirect inhibitory effects of melatonin on gastric cancer cells are involved in NF-kB signaling pathway-related proteins, and melatonin has great potential for gastric cancer treatment.

Declaration of competing interest

The authors declare that they have no conflict of interest to disclose.

Acknowledgments

This study was supported by the Technology Development Foundation of Pudong District (grant no. PKJ2016-Y54), the National Cultivation Project of Shanghai University of Medicine & Health Sciences (grant no. SFP-18-20-14-005).

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