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Original Articles

Melatonin inhibits MLL-rearranged leukemia via RBFOX3/hTERT and NF-κB/COX-2 signaling pathways

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ABSTRACT

MLL-rearranged leukemia is an aggressive malignancy associated with poor outcome, which is refractory to conventional treatment. Melatonin has been proven to exert anti-tumor activity, but the effect of melatonin on MLL-r leukemia and the underlying mechanism remain poorly understood. In this study, melatonin inhibited cell proliferation and induced apoptosis by activating the caspase-dependent apoptotic pathway in MLL-r leukemia cells. Mechanistic investigations revealed that melatonin suppressed the expression of hTERT by abrogating the binding activity of RBFOX3 to the hTERT promoter. Melatonin also blocked NF-κB nuclear translocation and suppressed NF-κB binding to the COX-2 promoter, thereby suppressing the expression of COX-2. In addition, clinical samples revealed that melatonin exerts anti-leukemic activity in primary MLL-r leukemia blasts ex vivo. In vivo, the mice treated with melatonin experienced a larger reduction in leukemic burden than the control group in a MLL-r leukemia xenograft mouse model. Collectively, these results suggest that melatonin inhibits MLL-rearranged leukemia through suppressing the RBFOX3/hTERT and NF-κB/COX-2 signaling pathways. Our findings provide new insights into the role of melatonin for MLL-r leukemia treatment.

1. Introduction

Translocations of the mixed lineage leukemia (MLL) gene occur in 5%–10% of pediatric acute leukemia patients and are often associated with poor prognosis [1,2]. Though improvements in five-year event-free survival among children with acute leukemia have been achieved, drug resistance remains the major cause of relapse and therapeutic failure in MLL-rearranged (MLL-r) leukemia [3,4]. Therefore, investigation of novel therapeutic agents is urgently needed for more effective treatment of MLL-r leukemia.

Melatonin is synthesized in the pineal gland and has extensive biological activities including anti-oxidation and anti-inflammation. Recently, more and more studies have suggested that melatonin exhibited a strong anti-tumor activity in human cancers through several mechanisms, including inhibiting cell proliferation, and inducing cell apoptosis [5–10]. It has been shown that melatonin could overcome drug resistance in several types of cancers [11–13], however, the functions and the underlying molecular mechanisms of melatonin on the cell growth of MLL-r leukemia remain poorly understood.

A number of transcriptional factors and oncogenes have been identified to play important roles in drug resistance [14,15]. Previous studies have indicated that human telomerase reverse transcriptase (hTERT) and COX-2 were highly expressed in more than 80% of cancers, and their expression levels were associated with high tumorigenesis and poor prognosis of patients with acute leukemias [16,17]. Therefore, inhibition of hTERT and COX-2 expression might be an

Abbreviations: MLL-r MLL, rearranged; MLL, mixed lineage leukemia; hTERT, human telomerase reverse transcriptase; RBFOX3, RNA binding protein fox-1 homolog 3

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Table 1
The characteristics of the cell lines and their IC50 values of melatonin.

	Cell Lines		
Characteristics	RS4-11	MOLM-13	Nalm-6
Cell type	B-ALL	AML-M5	B-ALL
Primary site	BM	PB	PB
Fusion gene	MLL-AF4	MLL-AF9	-
24h IC50 (mM)	1.523	0.957	1.869
48h IC50 (mM)	0.728	0.748	1.877

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BM, bone marrow; PB, peripheral blood; IC50, half-inhibitory concentration, calculated using Graphpad Prism 6 software with three independent values from CCK-8 assay.

effective strategy to inhibit the growth of MLL-r leukemia. Whether melatonin could suppress hTERT and COX-2 expression to inhibit cell growth remains unclear.

In the present study, we attempted to investigate the effect of melatonin in MLL-r leukemia cells *in vitro* and *in vivo*. We analyzed the functions of melatonin on cell proliferation and cell apoptosis in MLL-r leukemia cell lines and primary cells from patients. To identify the underlying mechanisms, we detected the action of melatonin in the regulation of the hTERT and COX-2 signaling pathways in MLL-r leukemia cells. Our results show that melatonin could be used as a potential agent to treat MLL-r leukemia through hTERT and COX-2 signaling pathways.

2. Methods

2.1. Cell lines and primary cells

Human leukemia cell lines RS4-11 (MLL-AF4$^+$ B-ALL), MOLM-13 (MLL-AF9$^+$ AML), and Nalm-6 (non MLL-r B-ALL) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The information is detailed in Table 1. These cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin. The primary samples were obtained from patients at the First Affiliated Hospital of Sun Yat-sen University. Clinical information is detailed in Table 2. Informed consent was obtained from all patients, and the study was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University. Mononuclear cells from bone marrow were separated using Ficoll–Paque PREMIUM (GE Healthcare, Uppsala, Sweden). All samples were collected at the time of diagnosis before treatment. All cells were cultured in an incubator with 5% CO_2 at 37 °C under standard culture condition.

2.2. Reagents and antibodies

Melatonin and celecoxib were purchased from Sigma (St Louis, MO, USA) and dissolved in a small amount of dimethyl sulfoxide (DMSO).

Table 2
Clinical and biological features for patients in the study.

Patient	Sex	Age	WBC (10^9/L)	FAB category	Fusion gene	Blast cells (%)
1	M	6 m	52.10	L2, BIII	MLL/AF4	87.40
2	F	2y	30.50	L2, BIII	MLL/ENL	86.70
3	M	9 m	34.65	L1, BII	MLL/AF4	92.50
4	M	6 m	20.30	L1, BIII	MLL/AF4	90.60
5	F	5y	8.54	L1, BII	None	85.90
6	F	3y	22.28	L2, BIII	None	85.30
7	M	8y	10.10	L2, BII	None	89.34
8	F	10y	20.50	L2, BIII	TEL/AML1	87.73

F, female; M, male; m, month; y, year; WBC, white blood cells; FAB, French – American – British.

The final concentration of DMSO in the culture media was 0.01%. Antibodies for RBFOX3 and COX-2 were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibodies of β-actin, H3, and TERT were from Abcam (Cambridge, UK). The antibodies of NF-κB p50 and p65 were from Santa Cruz Biotechnology (CA, USA).

2.3. Lentivirus infection

The hTERT short-hairpin RNA (shRNA)-expressing constructs, and the RBFOX3 shRNA-expressing constructs were purchased from Clontech (Mountain View, CA, USA). The pLP1, pLP2 and pLP/VSVG packaging plasmids were from Invitrogen (Carlsbad, CA, USA). The constructs were transfected into HEK293T cells along with the packaging plasmids. The leukemia cells were infected with either the lentiviral vectors encoding shRNA sequences or the negative control vector. Puromycin selection to establish stable cells began 24 h after virus infection.

2.4. Cell viability assay

Cell viability was determined using the Cell Counting Kit-8 assay (CCK-8, Dojindo, Kumamoto, Japan). The cells were seeded at 5×10^3 cells/well in 96-well plates and treated with the indicated concentrations of melatonin. After 24 h or 48 h incubation, cell viability was tested using the CCK-8 assay according to the manufacturer's instructions. All experimental process was performed in triplicate.

2.5. Apoptosis assay

The cells plated in 6-well plates were treated with different concentrations of melatonin for 24, 48 or 72 h. The cells were stained with Annexin V Apoptosis Detection Kit (BD ebioscience, CA, USA) according to the manufacturer's protocol. The rate of apoptosis was analyzed by the Accuri™ C6 Flow Cytometer (BD Biosciences, CA, USA).

2.6. Caspase activity

The caspase-3/9 activity were determined with the Caspase Colorimetric Assay Kit (Keygen Biotech, Nanjing, China), according to the manufacturer's protocol. In brief, cells were washed, lysed, and then centrifuged 12,000 × *g* for 10 min at 4 °C. The supernatant was collected and the protein concentration was tested. Cell lysates were mixed with Caspase substrate and reaction buffer, and incubated at 37 °C for 4 h. Absorbance values at 400 nm were recorded on a Microplate Reader (Thermo Fisher Scientific, MA, USA).

2.7. Quantitative real-time reverse transcription PCR (qRT-PCR)

According to the manufacturer's instructions, total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), then qRT-PCR was performed using the Mx3005 P Real-Time PCR System (Agilent, CA, USA). The relative mRNA expression was normalized to β-actin RNA levels and analyzed using the $2-\Delta\Delta CT$ method. Each sample was performed in triplicate. The primers were synthesized by Invitrogen (Carlsbad, CA, USA). Sequences of primers (forward and reverse, respectively) were as follows: RBFOX3: 5′-CCAGGCTCCGAGG CCAGCACAC-3′ and 5′-TGTAGGGTCGGAGGGGTGGAG-3'; β-actin: 5′-GGCACCCAGCACAATGAA-3′ and 5′-TAGAAGCATTTGCGGTGG-3'; hTERT: 5′-TCACGGAGACCACGTTTCAAA-3′ and 5′-TTCAAGTGCTGTC TGATTCCAAT-3'; COX-2: 5′-TCACAGGCTTCCATTGACCAG-3′ and 5′-CCGAGGCTTTTCTACCAGA-3'.

2.8. Western blot assay

Whole cell lysates were performed by using Complete Lysis-M Reagent Kit (Roche, Basel, Switzerland). Nuclear–cytoplasmic

fractionation was determined using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce, Illinois, USA) according to the manufacturer's protocol. Proteins were separated by electrophoresis in a 10% sodium dodecyl suplfate-polyacrylamide minigel (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride membranes. Then they were immunoblotted with specific antibodies. The protein bands were detected by enhanced chemiluminescence.

2.9. Streptavidin-agarose pull-down assay

The hTERT promoter binding proteins were analyzed by the streptavidin-agarose pull-down assay described previously [18]. In brief, the nuclear proteins (400 μg) from human leukemia cells were incubated with double-strand biotin-labeled hTERT promoter probes (10 μg) and 100 μl of streptavidin-agarose beads (Sigma, St Louis, MO, USA) at 4 °C overnight. The mixture was centrifuged to pull-down the DNA-protein complex. The DNA-bound protein was analyzed by Western blot.

2.10. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed using the ChIP-IT® Express Enzymatic Kit (Active Motif, Carlsbad, CA, USA) based on the previously published protocol [19]. Transcription factor binding was assessed by qRT-PCR using the following primers: hTERT promoter, forward: 5′- TGGCCCCTCCCTCGGGTTAC-3′ and reverse: 5′-TGAAGGGGCAGGACGGGTGC-3'; COX-2 promoter, forward: 5′-ACG TGACTTCCTCGACCCTC-3′ and reverse: 5′-AAGACTGAAAACCAAGC CCA-3'.

2.11. Animal experiments

All of the mouse studies were conducted in accordance with the guidelines established by the Animal Research Committee of Sun Yat-sen University. Female Balb/c mice (4–5 weeks old) were maintained under specific pathogen-free conditions. The mice were treated with busulfan and cyclophosphamide (Sigma, St Louis, MO, USA) to inhibit the immune system before experiment according to the previously published protocol [20]. 1×10^7 RS4-11 cells were inoculated intravenously via the tail vein, and the body weight was monitored every 3 days. Seven days after inoculation, mice were randomly separated into control and treatment groups. The treatment group received daily intravenous injections of melatonin (25 mg/kg) and the control group received the vehicle only. Mice were sacrificed when hunched posture, ruffled fur, or inactivity was observed. On day 7 and 22, blood was drawn and collected for complete blood counts (CBC). Peripheral blood smears were stained with Wright's Giemsa stain and spleens were fixed and stained with hematoxylin and eosin. The proteins extracted from spleens were analyzed by Western blot. The study was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University.

2.12. Statistical analysis

All data were presented as the mean ± standard deviation (s.d.) from at least three independent experiments. Statistical analysis was carried out using SPSS17.0 software (Chicago, IL, USA). Differences were considered statistically significant at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Results

3.1. Melatonin inhibited the proliferation of MLL-r leukemia cells in a dose and time-dependent manner

The molecular structure of melatonin was showed in Fig. 1A. To investigate whether melatonin affected the proliferation of MLL-r leukemia cells, we examined the viability of MLL-r (RS4-11 and MOLM-13) and non-MLL-r (Nalm-6) leukemia cell lines. As shown in Fig. 1B and C, melatonin remarkably inhibited cell viability in a dose-dependent manner in RS4-11 and MOLM-13 cells for 24 and 48 h. Compared to MLL-r cell lines, melatonin didn't remarkably inhibit the viability of Nalm-6 cell for 24 and 48 h (Fig. 1D). In addition, the IC50 of melatonin in RS4-11 and MOLM-13 cells were 0.728 mM and 0.748 mM respectively, much lower than that in Nalm-6 cell (Table 1). These results demonstrated that melatonin was more effective for MLL-r cell lines than non-MLL-r cell line.

3.2. Melatonin induced apoptosis of MLL-r leukemia cells

To determine whether the inhibition of cell growth induced by melatonin was associated with cell apoptosis, we analyzed the rate of apoptosis in RS4-11, MOLM-13 and Nalm-6 cells, exposed to 1.0, 2.0 and 3.0 mM melatonin for 24, 48, and 72 h. Treatment with melatonin resulted in a significant increase of apoptosis rate in RS4-11 and MOLM-13 cells. However, the rate of apoptosis was not significantly increased in Nalm-6 cells for 24 h (Fig. 2A). Similar results were obtained in 48 h (Figs. 2B) and 72 h (Fig. 2C). To further confirm the effect of melatonin on apoptosis induction, we detected the caspase-3 and caspase-9 activities in RS4-11, MOLM-13 and Nalm-6 cells 48 h after melatonin treatment. As shown in Fig. 2D and E, different concentrations of melatonin (0, 1.0, 2.0 or 3.0 mM) resulted in a significant increase of caspase-3 and caspase-9 activities in RS4-11 and MOLM-13 cells, but both caspase-3 and caspase-9 activities did not increase in Nalm-6 cells. These results suggested that melatonin induced cell apoptosis and activated the caspase-dependent apoptotic pathway in MLL-r cell lines but not in non-MLL-r cell line.

3.3. Melatonin inhibited hTERT expression by suppressing RBFOX3 binding to the hTERT promoter in MLL-r cell lines

Melatonin has been reported to inhibit proliferation of cancer cells by targeting the hTERT signaling pathway [21–25]. We examined the effect of melatonin on the expression of the hTERT in RS4-11, MOLM-13 and Nalm-6 cells by qRT-PCR and Western blot. Melatonin markedly suppressed the mRNA expression of hTERT in RS4-11 and MOLM-13 cells, while almost had no effect on the Nalm-6 cell (Fig. 3A). As shown in Fig. 3B, Western blot also showed that melatonin significantly inhibited the expression of hTERT protein in RS4-11 and MOLM-13 cells, but the expression of hTERT protein did not change with different concentrations of melatonin in Nalm-6 cells.

The hTERT expression was regulated by several transcription factors on its promoter region, such as RBFOX3 (RNA binding protein fox-1 homolog 3) [18]. To further evaluate the mechanisms of the dose-dependent decrease of hTERT expression in the melatonin treated MLL-r cell lines, we constructed lentiviral shRNA of hTERT in RS4-11, MOLM-13 and Nalm-6 cells. As shown in Fig. 3C, hTERT knockdown promoted the proliferation inhibition mediated by melatonin at different concentrations in RS4-11 and MOLM-13 cells (MLL-r cell lines), but not in Nalm-6 cell (non-MLL-r cell line). We carried out streptavdin-agarose pull-down assay to evaluate the enhancement of melatonin treatment on RBFOX3 binding activities to hTERT promoter in MLL-r cell lines. The results showed that treatment with melatonin significantly reduced RBFOX3 binding to hTERT promoter (Fig. 3C). We further evaluated the effect of melatonin on the binding activity of RBFOX3 on hTERT promoter by ChIP assay. As shown in Fig. 3D, treatment with melatonin significantly inhibited the binding of RBFOX3 on the hTERT promoter in RS4-11 and MOLM-13 cells. These results supported that melatonin suppressed hTERT expression by inhibiting RBFOX3 binding to hTERT promoter in MLL-r cell lines.

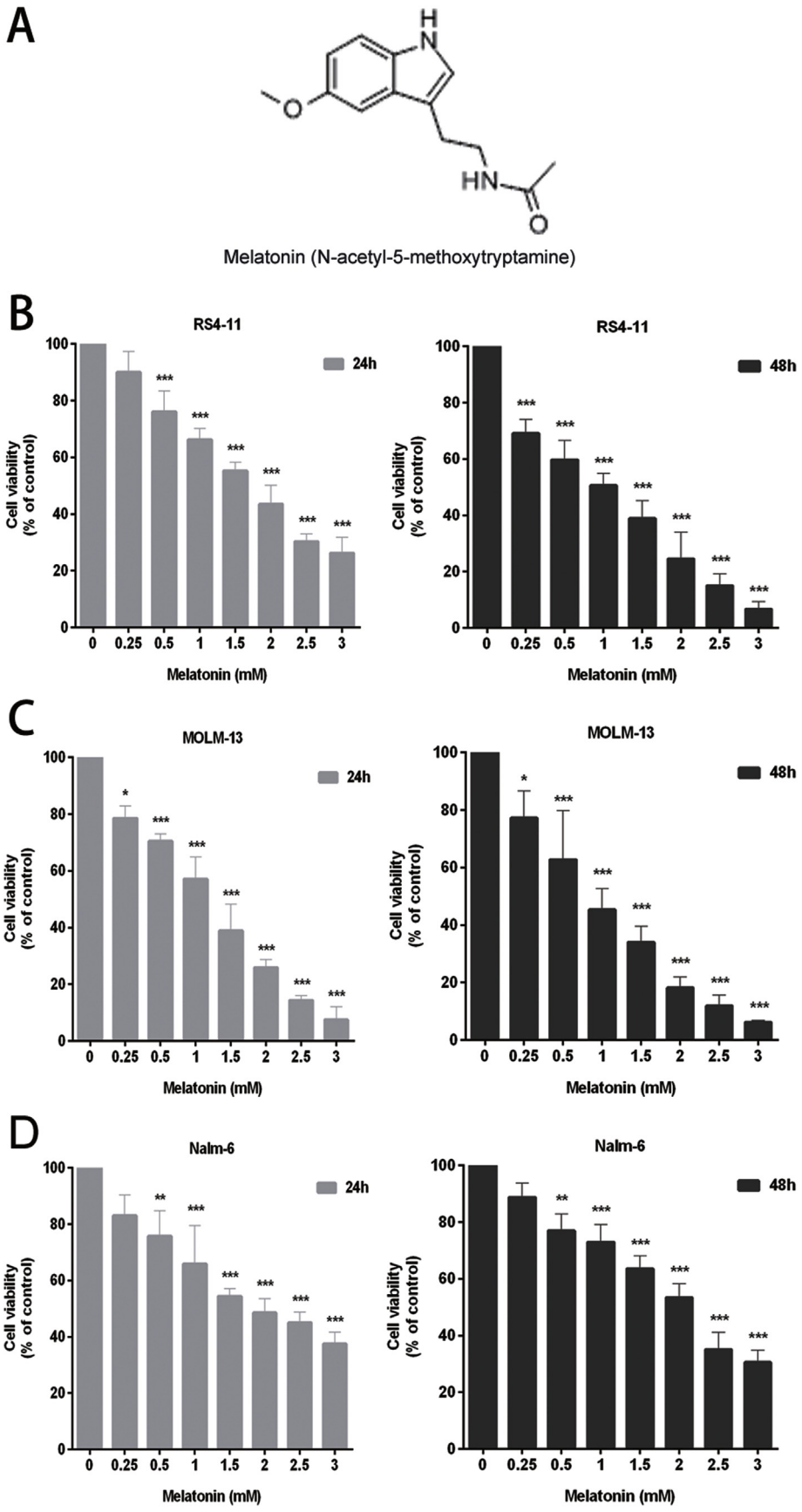

Fig. 1. Melatonin affected the cell viability of MLL-r and non-MLL-r leukemia cell lines. **(A).** The molecular structure of melatonin. **(B–D).** RS4-11 **(B)**, MOLM-13 **(C)**, and Nalm-6 **(D)** cell lines were treated with 0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 or 3.0 mM melatonin. At 24 and 48 h after treatment, the cell viability was determined by the CCK-8 assay. The control group was used as referent group with cell viability set at 100%. Results were represented as the mean ± s.d. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs control.

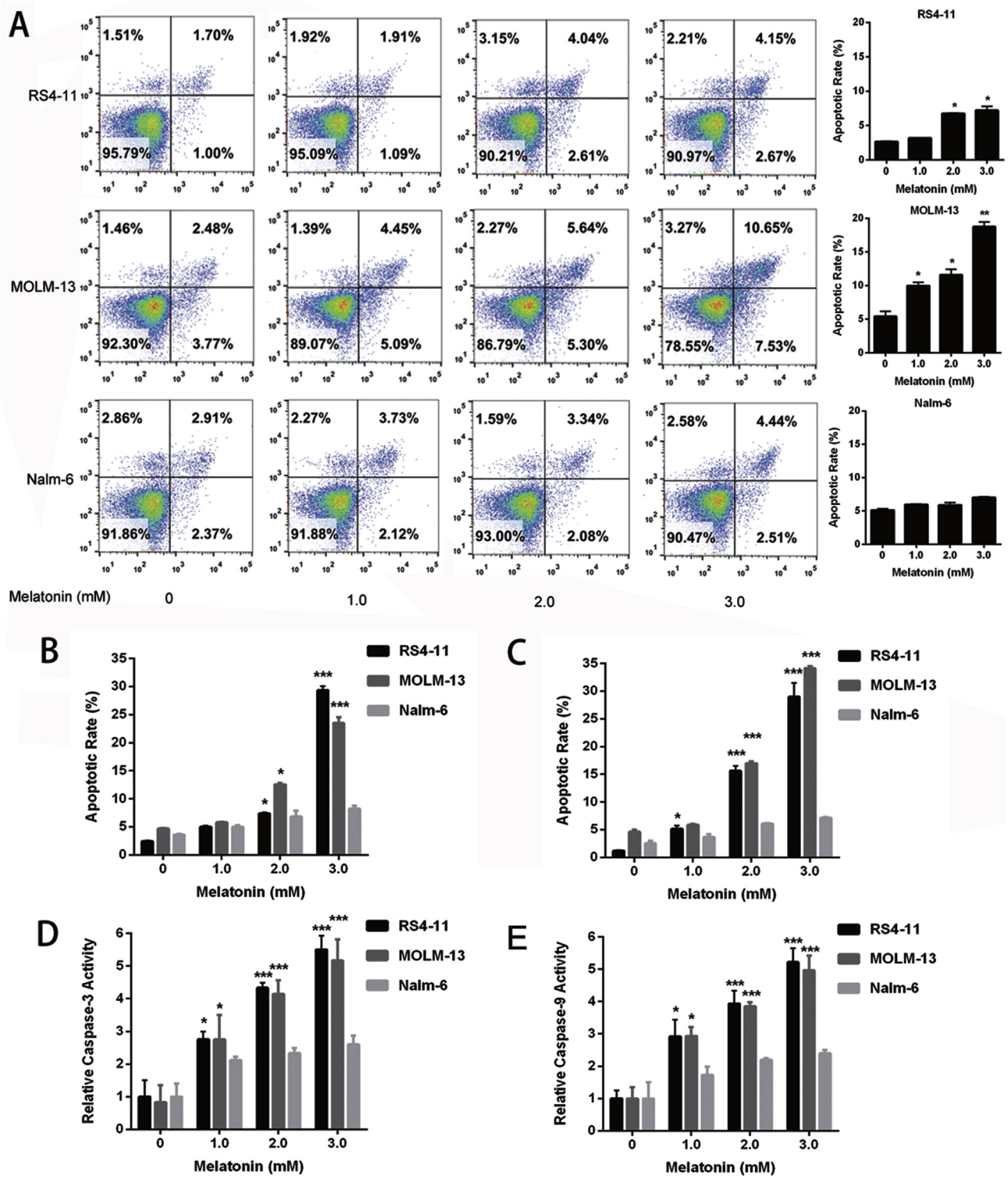

Fig. 2. Effect of melatonin on cell apoptosis in MLL-r leukemia cell lines via caspase pathways. **(A).** RS4-11, MOLM-13, and Nalm-6 cells were treated with melatonin (0, 1.0, 2.0 or 3.0 mM). The apoptosis was examined by a FACS analysis at 24 h after treatment, and the percentage of apoptotic cells was analyzed relative to that in control group. **(B–C).** The apoptosis was examined at 48 h **(B)** and 72 h **(C)** after 0, 1.0, 2.0 or 3.0 mM melatonin treatment. **(D–E).** The activities of the caspase-3 **(D)** and caspase-9 **(E)** in RS4-11, MOLM-13, and Nalm-6 cells were analyzed at 48 h. The experiments were examined three times and data were represented as the mean ± s.d. The level of significance was indicated by *P < 0.05, **P < 0.01, ***P < 0.001.

3.4. Melatonin suppressed RBFOX3/hTERT signaling pathway in MLL-r cell lines

To determine whether the inhibition of cell growth by melatonin in MLL-r cell lines was through the RBFOX3/hTERT signaling pathway, we examined the effect of melatonin on RBFOX3 expression in RS4-11, MOLM-13 and Nalm-6 cells by qRT-PCR and Western blot. As shown in Fig. 4A, melatonin significantly inhibited the mRNA expression of RBFOX3 in RS4-11 and MOLM-13 cells after treatment with melatonin (0, 1.0 or 2.0 mM) for 48 h, while the expression of RBFOX3 mRNA did

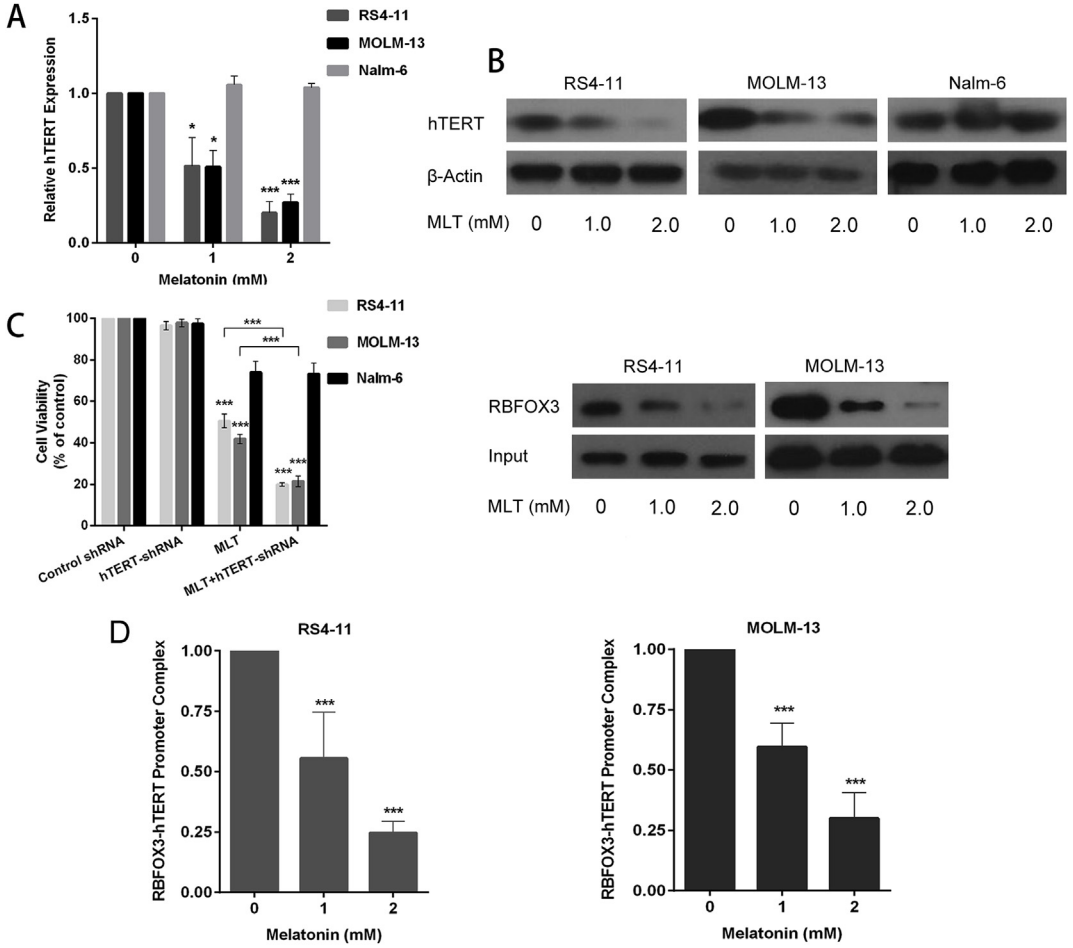

Fig. 3. Melatonin affected the hTERT expression by suppressing RBFOX3 binding activities to hTERT promoter in MLL-r cell lines. **(A–B).** RS4-11, MOLM-13, and Nalm-6 cells were treated with melatonin (0, 1.0 or 2.0 mM), the levels of hTERT mRNA and protein were respectively analyzed by qRT-PCR **(A)** and Western blotting **(B)** at 48 h after treatment. **(C).** RS4-11, MOLM-13, and Nalm-6 cells were constructed lentiviral shRNA of hTERT, the cell viability was determined by CCK-8 assay in control and hTERT knockdown cells treated with 0, 1 or 2 mM melatonin at 48 h after treatment. The binding of RBFOX3 to hTERT promoter probe was detected via streptavidin-agarose pulldown assays in RS4-11 and MOLM-13 cells treated with 0, 1 or 2 mM melatonin at 48 h after treatment. **(D).** RBFOX3 binding to hTERT promoter sequences was analyzed by quantitative ChIP assay in RS4-11 and MOLM-13 cells exposed to 0, 1 or 2 mM melatonin. The experiments were tested three times and data were represented as the mean ± s.d. The level of significance was indicated by *P < 0.05, ***P < 0.001.

not change in the Nalm-6 cell (non-MLL-r cell line). Further, western blot assay was performed to examine the expression of RBFOX3 protein in RS4-11 cells. We found that different concentrations of melatonin (0, 1.0 or 2.0 mM) reduced the expression of RBFOX3 protein. To further confirm the function of RBFOX3/hTERT signaling pathway in MLL-r cell lines treated with melatonin, we constructed lentiviral shRNA of RBFOX3 in RS4-11 and MOLM-13 cells. As shown in Fig. 4B and C, RBFOX3 knockdown decreased the expression of RBFOX3 and hTERT at mRNA level in RS4-11 and MOLM-13 cells. We also found that RBFOX3 and hTERT protein expression were both inhibited by RBFOX3 knockdown. Moreover, RBFOX3 knockdown promoted the proliferation inhibition mediated by melatonin (0, 1.0 or 2.0 mM) in RS4-11 and MOLM-13 cells (Fig. 4D). These results demonstrated that RBFOX3 signaling pathway was a potential target of melatonin treatment in MLL-r cell lines to inhibit hTERT expression.

3.5. Melatonin inhibited NF-κB/COX-2 signaling pathway in MLL-r cell lines

The COX-2 signaling is implicated in cell proliferation and apoptosis in cancer cells [13,26]. To determine the effect of melatonin on COX-2 signaling in MLL-r cell lines, we analyzed the effect of melatonin on the expression of COX-2 in RS4-11, MOLM-13 and Nalm-6 cells by qRT-PCR. As shown in Fig. 5A, melatonin significantly decreased the mRNA expression of COX-2 in RS4-11 and MOLM-13 cells after treatment with melatonin (0, 1.0 or 2.0 mM) for 48 h, while the expression of COX-2 mRNA did not change in the Nalm-6 cell (non-MLL-r cell line). To further confirm that melatonin inhibited COX-2 signaling, the cells were treated with melatonin (1.0 mM) after pretreatment with a COX-2 selective inhibitor celecoxib (20 μM). The cell viability was detected by CCK-8 assay after 48 h. As shown in Fig. 5B, the combined treatment of celecoxib with melatonin significantly enhanced the inhibition of cell viability in RS4-11 and MOLM-13 cells compared with melatonin treatment alone. However, the inhibition of cell viability did not change in the Nalm-6 cell (non-MLL-r cell line). The result indicated that melatonin might also partially inhibit the COX-2 signaling in MLL-r cell lines. COX-2 expression was regulated by several transcription factors on its promoter region, such as NF-κB [8,27,28]. We next examined the effect of melatonin on NF-κB p50 and p65 in the cytoplasm and nucleus in MLL-r cell lines by Western blot. The results showed that melatonin decreased the expression of NF-κB p50 and p65 in the nucleus, whereas the levels of NF-κB p50 and p65 were not changed in the cytoplasm (Fig. 5C).

The expression of COX-2 is regulated by the binding of NF-κB on the COX-2 promoter in many cancers [8,26], so we further evaluated the effect of melatonin on the binding of NF-κB on COX-2 promoter by ChIP

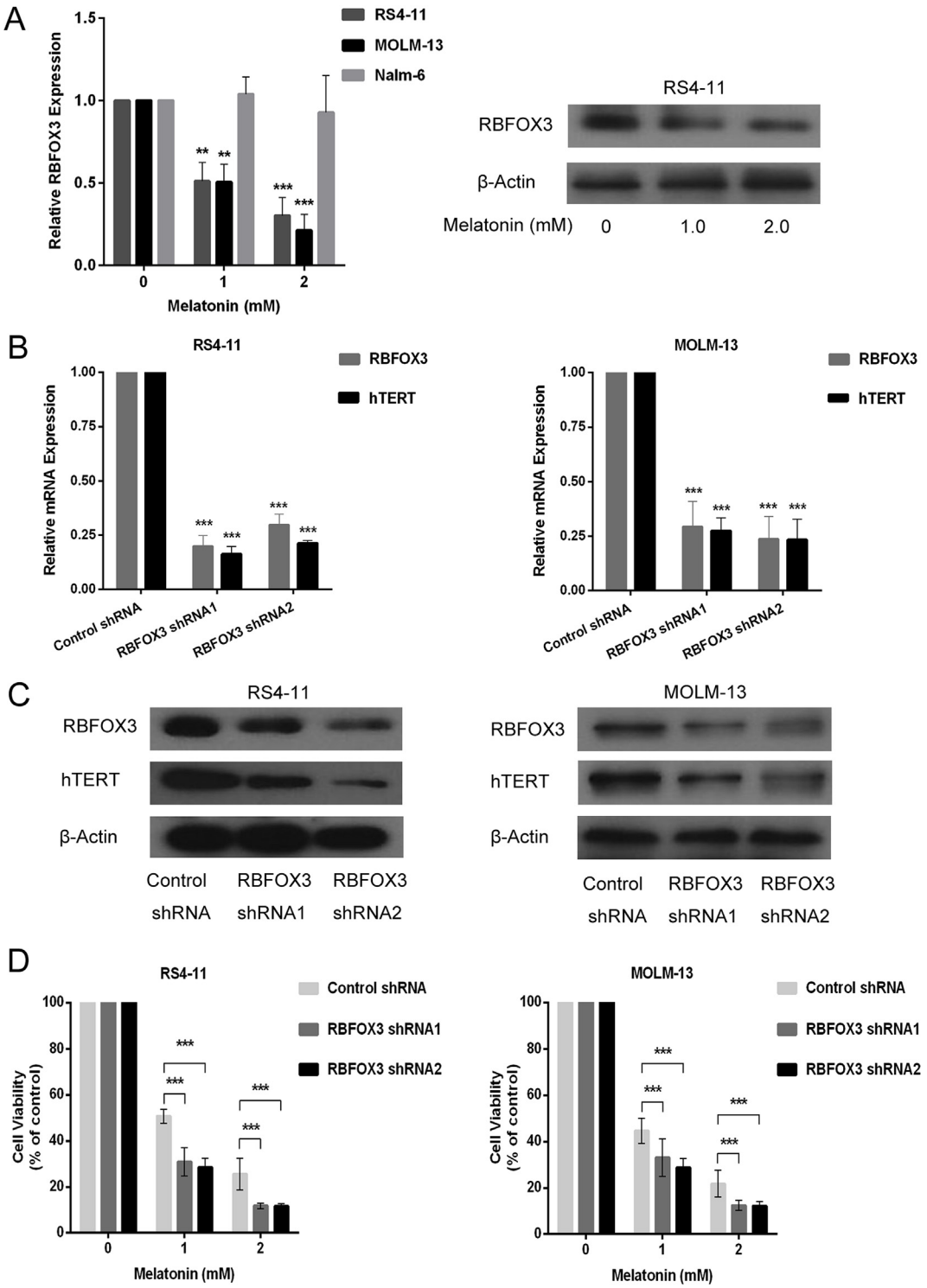

Fig. 4. Effect of melatonin on the RBFOX3/hTERT signaling pathway in MLL-r cell lines. **(A).** The expression of RBFOX3 mRNA in RS4-11, MOLM-13, and Nalm-6 cells treated with 0, 1 or 2 mM melatonin at 48 h were detected by qRT-PCR assay. Furthermore, the level of RBFOX3 protein was analyzed by Western blotting in RS4-11 cell treated with 0, 1 or 2 mM melatonin at 48 h. β-Actin was served as the loading control. **(B–C).** RS4-11 and MOLM-13 cells were constructed lentiviral shRNA of RBFOX3, the levels of RBFOX3 and hTERT mRNA and protein were respectively analyzed by qRT-PCR **(B)** and Western blotting **(C)**. **(D).** The cell viability was examined using CCK-8 assay in control and RBFOX3 knockdown cells treated with 0, 1 or 2 mM melatonin at 48 h after treatment in RS4-11 and MOLM-13 cells. The data are presented as the mean ± s.d. of three separate experiments. The level of significance was indicated by $^{**}P < 0.01$, $^{***}P < 0.001$.

assay. As shown in Fig. 5D, The results indicated that treatment with melatonin for 48 h markedly inhibited the binding of NF-κB p65 subunit to the COX-2 promoter. These results showed that melatonin might also inhibit NF-κB/COX-2 signaling pathway in MLL-r cell lines.

3.6. Melatonin inhibited cell proliferation and induced cell apoptosis via RBFOX3/hTERT and NF-κB/COX-2 signaling pathways in primary MLL-r ALL blasts ex vivo

To examine the effect of melatonin on primary ALL blasts, we

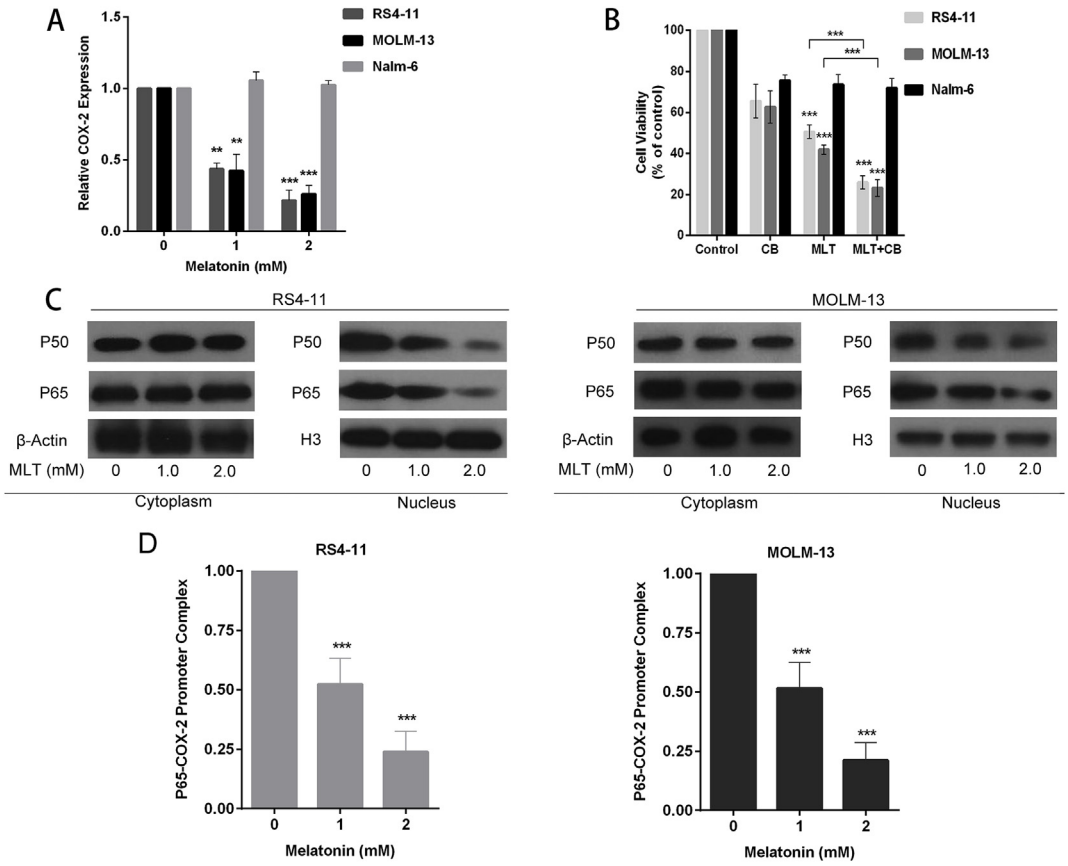

Fig. 5. Effect of melatonin on the NF-κB/COX-2 signaling pathway in MLL-r cell lines. **(A).** The expression of COX-2 mRNA were detected by qRT-PCR assay in RS4-11, MOLM-13, and Nalm-6 cells treated with 0, 1 or 2 mM melatonin at 48 h. **(B).** The cell viability was tested by the CCK-8 assay in melatonin (1.0 mM) alone, celecoxib (20 μM) alone, and melatonin (1.0 mM) after pretreatment with celecoxib (20 μM) for 48 h in RS4-11, MOLM-13, and Nalm-6 cells. **(C).** The expression of NF-κB p50 and p65 proteins in the cytoplasm and nucleus were detected by Western blot in RS4-11 and MOLM-13 cells treated with 0, 1 or 2 mM melatonin at 48 h. **(D).** Quantitative ChIP assay of NF-κB p65 subunit binding to COX-2 promoter sequences in RS4-11 and MOLM-13 cells exposed to 0, 1 or 2 mM melatonin at 48 h. The experiments were performed three times and experimental values were represented by mean ± s.d. The level of significance was indicated by $^{**}P < 0.01$, $^{***}P < 0.001$.

analyzed the cell proliferation treated with different concentrations of melatonin (0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 or 3.0 mM) in primary ALL blasts for 48 h. As shown in Fig. 6A, the proliferation of the MLL-r ALL blasts (Patient 1–4) was markedly inhibited by melatonin in a dose-dependent manner. However, the inhibition of cell viability was modest in the non-MLL-r ALL blasts (Patient 5–8) than the MLL-r ALL blasts. As shown in Fig. 6B, we analyzed the apoptotic rate and the relative caspase-3 activity in MLL-r ALL blasts (Patient 1–2) and non-MLL-r ALL blasts (Patient 5–6) that were exposed to 0, 1.0, 2.0 and 3.0 mM melatonin for 48 h. The apoptotic rates and the relative caspase-3 activities were less in the non-MLL-r ALL blasts than the MLL-r ALL blasts. To further confirm that melatonin inhibited RBFOX3/hTERT and NF-κB/COX-2 signaling pathways in primary MLL-r ALL blasts, we evaluated the binding of RBFOX3 on hTERT promoter and NF-κB on COX-2 promoter by ChIP assay in MLL-r ALL blasts exposed to 1.0, 2.0 and 3.0 mM melatonin for 48 h. As shown in Fig. 6C, the results suggested that melatonin markedly inhibited the binding of RBFOX3 to the hTERT promoter and NF-κB p65 subunit to the COX-2 promoter. We further determined the effect of melatonin on RBFOX3, hTERT, P65 and COX2 expression in primary MLL-r ALL blasts, the results indicated melatonin significantly suppressed the mRNA expression of RBFOX3, hTERT, P65 and COX2 after treatment with melatonin (Fig. 6D). These results indicated that melatonin inhibited cell proliferation and induced cell apoptosis via RBFOX3/hTERT and NF-κB/COX-2 signaling pathways in primary MLL-r ALL blasts ex vivo.

3.7. Melatonin inhibited tumor growth in a MLL-r ALL mouse model

We further investigated the effect of melatonin on the tumor growth in a MLL-r ALL xenograft mouse model. We injected RS4-11 cells systemically into Balb/c female mice and treated the mice with melatonin. The mice had an excess of circulating leukemia cells before treatment, they were divided into melatonin treatment group and control group. After treatment with melatonin for 15 days, we found that the weight loss of control mice was significantly greater than that of melatonin-treated mice (Fig. 7A). In addition, the weight of the spleens in the melatonin-treated mice was significantly less than in the control group (Fig. 7B). As shown in Fig. 7C and D, the melatonin-treated mice had fewer white blood cells (WBC) count, but the blood platelet counts were not significantly different compared with the control mice. Furthermore, we observed that spleen samples from melatonin-treated mice had less leukemic infiltration than the control mice (Fig. 7E and F). Moreover, the Western blot analysis of spleen lysates also showed that treatment with melatonin suppressed the expression of RBFOX3, hTERT, and COX-2 proteins *in vivo* (Fig. 7G). These results supported that melatonin could inhibit the growth of MLL-r ALL xenograft.

4. Discussion

Although many previous studies have reported that melatonin suppressed cell growth in different types of cancers, the role of melatonin in MLL-r leukemia and the underlying regulatory mechanisms remain unclear. In this study, we demonstrated melatonin effectively

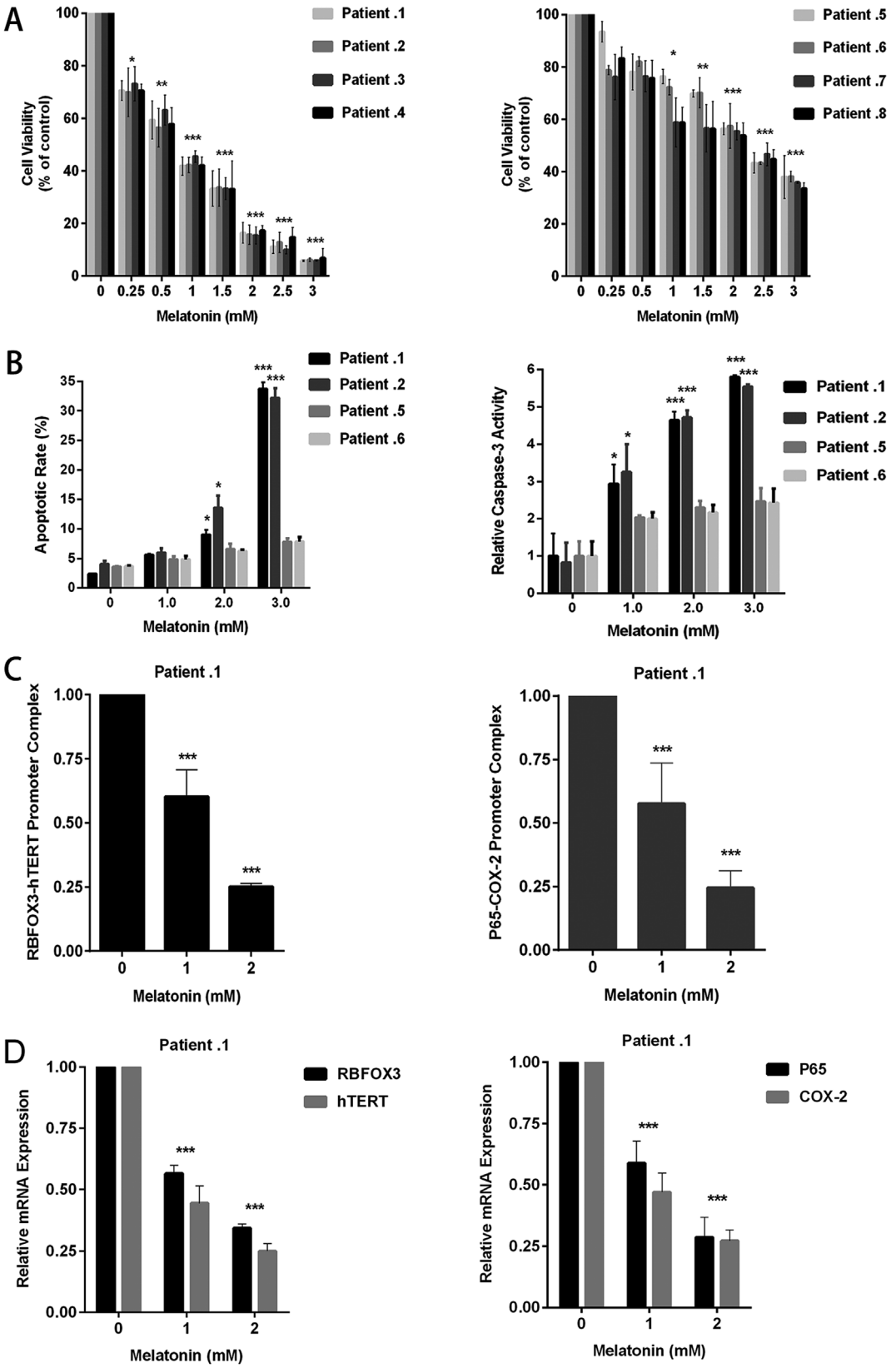

Fig. 6. Effect of melatonin on the cell proliferation and apoptosis in primary MLL-r ALL blasts ex vivo. **(A).** Cells isolated from the bone marrows of 4 MLL-r ALL patients (Patient 1–4) and 4 non MLL-r ALL patients (Patient 5–8) were exposed to 0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 or 3.0 mM melatonin for 48 h. Cell viability was measured using CCK-8 assay. **(B).** The apoptosis was tested by a FACS analysis and the caspase-3 activity was analyzed in Patient 1, 2, 5, 6 exposed to 0, 1, 2 or 3 mM melatonin for 48 h. **(C).** The RBFOX3 binding to hTERT promoter and NF-κB p65 subunit binding to COX-2 promoter were tested by quantitative ChIP assay in Patient 1 exposed to 0, 1 or 2 mM melatonin for 48 h. **(D).** The expression of RBFOX3, hTERT, P65 and COX2 mRNA in Patient 1 treated with 0, 1 or 2 mM melatonin at 48 h were detected by qRT-PCR assay. The results represent the means ± s.d. of triplicates. The level of significance was indicated by *P < 0.05, **P < 0.01, ***P < 0.001.

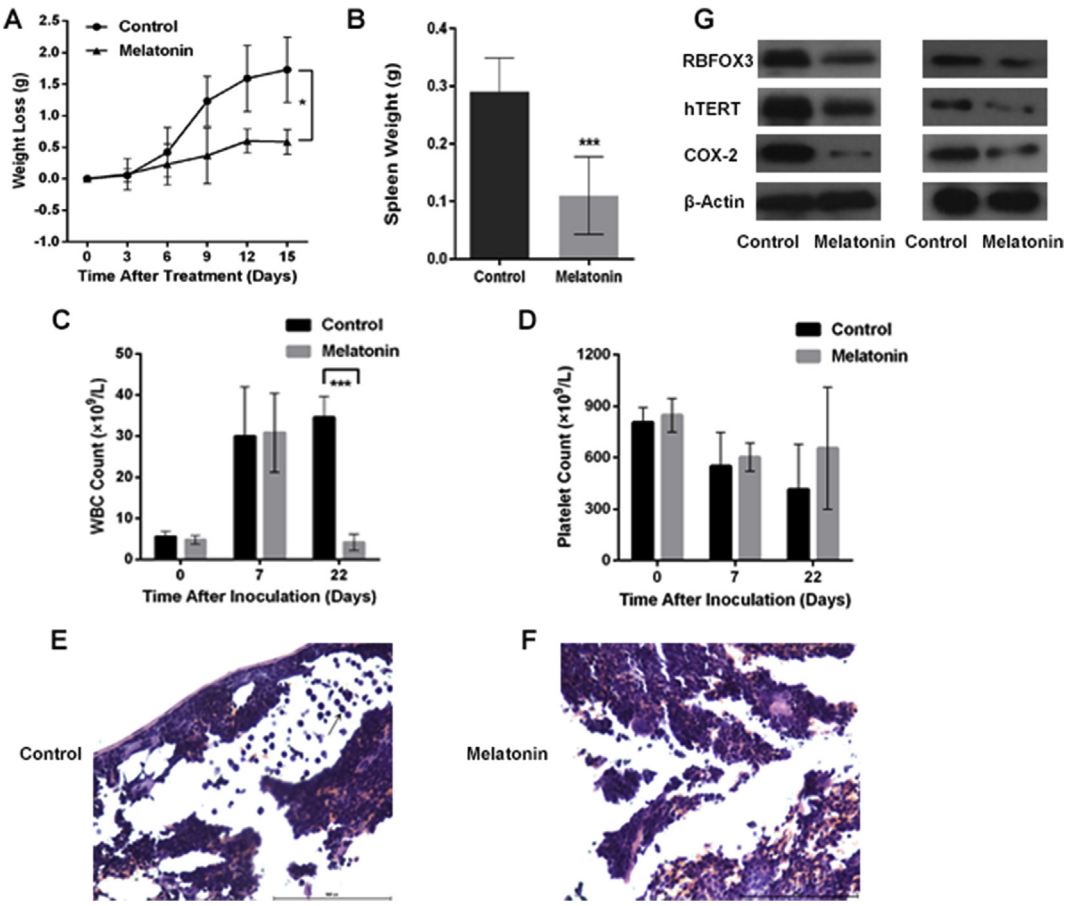

Fig. 7. Effect of melatonin on tumor growth in a MLL-r ALL mouse model. The female Female Balb/c mice aged with 3- to 4-weeks were used in the study. RS4-11 cells (1×10^7 in 100 μl PBS) were injected intravenously via the tail vein. **(A).** The body weight was measured once every 3 days. **(B).** Spleen weight was calculated after all the mice were sacrificed. **(C–D).** On the 7 and 22 days after inoculation, the WBC count **(C)** and blood platelet counts **(D)** were analyzed. **(E–F).** The spleens were fixed and stained with hematoxylin and eosin in the control group **(E)** and melatonin-treated group **(F)**. **(G).** The expression of RBFOX3, hTERT and COX-2 proteins extracted from spleens were analyzed by Western blot. The data are presented as the means ± s.d. The level of significance was indicated by $^*P < 0.05$, $^{***}P < 0.001$. N = 5 mice/group. Magnification, 500 × .

inhibited cell proliferation and induced apoptosis in MLL-r leukemia cells through RBFOX3/hTERT and NF-κB/COX-2 signaling pathways.

Based on previous reports indicating that melatonin could inhibit proliferation and induce apoptosis [16,29–31], we analyzed the anti-leukemic effect of melatonin on MLL-r leukemia cells. In our study, we demonstrated that melatonin was more effective in MLL-r leukemia cells than non MLL-r leukemia cells. Moreover, melatonin inhibited the proliferation and induced apoptosis in MLL-r leukemia cells in a dose and time-dependent manner. Melatonin increased the expression of cleaved caspase-9 and caspase-3 and induced the activation of caspase-dependent apoptotic signaling pathway. The fact that MLL-r leukemia cells are more sensitive to melatonin treatment than the non MLL-r leukemia cells suggests that melatonin could be a specific and effective therapeutic drug for the treatment of non MLL-r leukemia.

Our previous studies have revealed that two signaling pathways, hTERT and COX-2, might contribute to the anti-tumor action of melatonin. Acute myeloid leukemia patients with high telomerase expression and high levels of telomerase activity could not reach complete remission. In addition, a high level of full-length hTERT transcript negatively affected the survival in patients with acute myeloid leukemia. hTERT was a hallmark of cancer through controlling genes involved in proliferation, invasion, and apoptosis [31–34]. Therefore, the inhibition of hTERT expression was a new target for leukemia therapy. More and more evidence has demonstrated that several transcriptional factors may specifically bind to the hTERT promoter to regulate hTERT expression. Our report has indicated that melatonin displayed its anti-tumor effect by decreasing the expression of AP-2beta and the binding on hTERT promoter, resulting in reducing hTERT expression and telomerase activity [28]. Additionally, we have identified RBFOX3 as a regulatory factor that regulated hTERT signaling and tumor growth in hepatocellular carcinoma through binding to hTERT promoter. In this study, we demonstrated that melatonin inhibited RBFOX3 expression, and suppressed RBFOX3 binding to hTERT promoter in MLL-r leukemia cell lines, which suggests that the RBFOX3/hTERT signaling pathway could serve as a potentially therapeutic target for MLL-r leukemia therapy.

High COX-2 expression appears in many cancers, including acute leukemia. Activating COX-2 signaling pathway could induce cell proliferation, invasion, and metastasis of cancer cells. It has been reported that COX-2 expression is transcriptionally regulated by the binding of multiple transcription factors such as NF-κB p65/p50 to the core promoter regions [13,27]. Recent reports revealed that melatonin inhibited nuclear translocation of NF-κB and blocked the binding of p65 to COX-2 promoter, leading to inhibition of COX-2 expression [35–39]. In our study, we found that melatonin markedly suppressed the binding of NF-κB to COX-2 promoter and inhibited COX-2 expression by stimulating NF-κB translocation from cell nuclear to cytoplasm in MLL-r leukemia. These results suggested melatonin-mediated proliferation inhibition was at least partially mediated by the NF-κB/COX-2 signaling.

In our study, we also found that pediatric MLL-r leukemia cells were more sensitive to melatonin than primary non MLL-r leukemia cells. In addition, these results indicated that melatonin suppressed cell

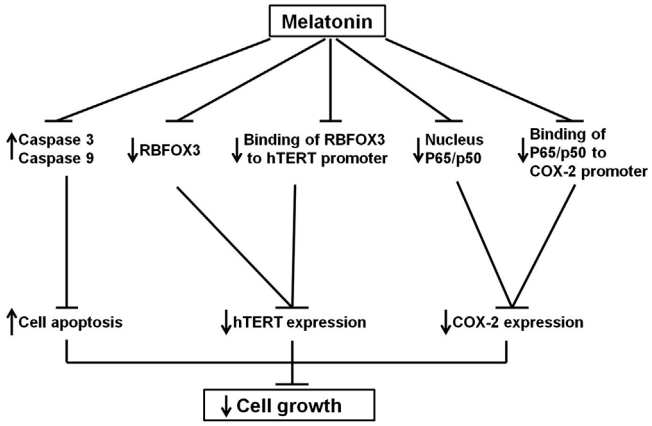

Fig. 8. Melatonin suppresses the cell growth of MLL-r ALL by targeting multiple signaling pathways. Melatonin induced cell apoptosis by activating the pro-apoptotic proteins caspase-3 and caspase-9, inhibited the expression of RBFOX3 and nucleus NF-κB p65/p50 proteins, abrogated the binding of RBFOX3 to the hTERT promoter and NF-κB to the COX-2 promoter, thereby suppressed the hTERT and COX-2 expression.

proliferation and promoted cell apoptosis in primary MLL-r ALL blasts ex vivo through RBFOX3/hTERT and NF-κB/COX-2 signaling pathways. In order to examine the effect of melatonin on MLL-r leukemia *in vivo*, we have validated the anti-tumor effect of melatonin in a human MLL-r leukemia mouse model. In this study, melatonin significantly suppresses tumor growth by inhibiting the RBFOX3/hTERT and NF-κB/COX-2 signaling pathways. Our data support recent reports melatonin effectively inhibits tumor growth in human cancers.

Our study suggests that melatonin inhibits the growth by enhancing the anti-proliferation and pro-apoptotic activities in MLL-r leukemia via RBFOX3/hTERT and NF-κB/COX-2 signaling pathways. The study shows that melatonin activates the pro-apoptotic proteins caspase-3 and caspase-9, suppresses the expression of RBFOX3 and nucleus NF-κB p65/p50 proteins, abrogates the binding of RBFOX3 to the hTERT promoter and NF-κB to the COX-2 promoter, resulting in inhibiting the hTERT and COX-2 expression (Fig. 8). Sum up, the study suggests that melatonin inhibits MLL-rearranged leukemia through suppressing the RBFOX3/hTERT and NF-κB/COX-2 signaling pathways.

5. Conclusions

Our findings indicate melatonin might serve as a more effective way to enhance therapeutic efficiency and overcome drug resistance in MLL-r leukemia, which provide new insights into the role of melatonin for MLL-r leukemia treatment.

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Authors' contributions

This work was carried out in collaboration between all authors. Y-L T, XS, X-Q L and WD defined the research theme, designed the experiments and revised the manuscript critically. Y-L T, XS, L-B H, X-J L, GQ, L-N W, X-L Z, Z-Y K, J-S L, CL, C-J P, W—Y T and Y-L T designed methods and experiments, carried out most of the experiments. Y-L T, WH and WD analyzed data. Y-L T, XS, L-B H, X-J L, X-Q L and WD wrote the manuscript. All authors approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the clinical research ethics committee of the First Affiliated Hospital, Sun Yat-sen University.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

Not applicable.

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