Downregulation of ENDOCAN in myeloid leukemia cells inhibits proliferation and promotes apoptosis by suppressing nuclear factor-κB activity

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Abstract. Previous studies have demonstrated that ENDOCAN is elevated in leukemia, and it has been reported to be associated with poor prognosis. However, the functional role of ENDOCAN in the development of leukemia remains to be fully elucidated. In the present study, the expression levels of ENDOCAN were detected in THP-1, U937, HL-60 and K562 cells, and it was found that ENDOCAN was increased in U937 and K562 cells, compared with the other two cell lines. Subsequently, ENDOCAN was knocked down in U937 and K562 cells via lentiviral infection. It was found that cell proliferation and the expression of proliferating cell nuclear antigen were inhibited in myeloid leukemia cells following the silencing of ENDOCAN. ENDOCAN knockdown induced G0/G1-phase cell cycle arrest in myeloid leukemia cells with a decreased expression of cyclin D1. Furthermore, cell apoptosis was increased in response to ENDOCAN silencing, which was accompanied by the downregulation of B-cell lymphoma (BCL2) and the upregulation of BCL2-associated X protein, cleaved caspases 3 and 9, and cleaved poly (ADP-ribose) polymerase. Furthermore, it was demonstrated that the knockdown of ENDOCAN inhibited nuclear factor-κB (NF-κB) activity, as evidenced by the increased expression of NF-κB inhibitor α (IκBα), decreased expression of phosphorylated (p-)IκBα, p-P65 and nuclear P65, and reduced NF-κB DNA-binding activity. In combination, the present findings suggested that ENDOCAN may serve as a potential therapeutic target in the treatment of leukemia.

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Introduction

Leukemia is the most common malignancy among children between birth and 14 years of age worldwide (1). There are four major types of leukemia: Acute myeloid leukemia (AML), chronic myeloid leukemia, acute lymphocytic leukemia (ALL) and chronic lymphocytic leukemia (2). Multiple factors have been implicated in the etiology of the disease, such as ionizing radiation, electromagnetic fields, infection, chemotherapy agents and certain inherited genetic disorders (3). The treatment of leukemia has been improved significantly through chemotherapy, interferon therapy, radiation therapy, stem cell transplantation and surgery, however, survival rates remain poor (4,5). It is therefore necessary to investigate novel therapeutic targets or strategies for the treatment of leukemia.

ENDOCAN, also known as endothelial cell-specific molecule-1 (ESM-1), was initially cloned from a cDNA library of human endothelial cells (6). Serum ENDOCAN levels are increased in essential hypertension, and are positively correlated with carotid intima-media thickness and high-sensitivity C-reactive protein (7). High levels of ENDOCAN have been shown to predict poor survival rate in liver cirrhosis (8). Circulating ENDOCAN may serve as an outcome measure in the development of Behcet's disease (9) and sepsis (10). Previous studies have reported that the expression levels of ENDOCAN were elevated in a wide range of tumor types, including non-small cell lung cancer (11), glioblastoma (12) and hepatocellular carcinoma (13), and was positively correlated with the severity of the disease. The overexpression of ENDOCAN can induce tumor formation (14). By contrast, ENDOCAN knockdown can inhibit tumor cell proliferation and promote tumor cell apoptosis (15). Xu *et al* (16) and Hatfield *et al* (17) identified elevated levels of ENDOCAN in patients with untreated AML and ALL. However, the functional role of ENDOCAN in myeloid leukemia cells remains to be fully elucidated.

In the present study, the expression of ENDOCAN in four myeloid leukemia cell lines (THP-1, U937, HL-60 and K562) was determined, following which ENDOCAN was silenced in two ENDOCAN-elevated cell lines (U937 and K562) via lentiviral infection. Subsequently, the effects of ENDOCAN

knockdown on cell proliferation, cell cycle distribution, cell apoptosis and NF-κB activity were investigated.

Materials and methods

Cells and cell culture. The THP-1 cells (derived from a patient with monocytic leukemia) and HL-60 cells (derived from a patient with promyelocytic leukemia) were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The K562 cells (derived from a patient with chronic myelogenous leukemia) were obtained from CHI Scientific (Jiangyin, China). The U937 cells (derived from a patient with histiocytic lymphoma) were purchased from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). The HL-60 cells were cultured in Iscove's modified Dulbecco's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Beit Haemek, Israel), 100 U/ml penicillin and 0.1 mg/ml streptomycin. The THP-1, K562, and U937 cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) containing 10% FBS (Biological Industries), 100 U/ml penicillin and 0.1 mg/ml streptomycin. All cell lines were maintained in an incubator (Shanghai Lishen, Shanghai, China) with 5% CO_2 at 37˚C.

Lentivirus generation. Lentiviruses containing two short hairpin RNA (shRNA) sequences specific to ENDOCAN and a negative control (NC) were generated by a lentiviral packaging system from Wanleibio Co., Ltd. (Shenyang, China). The sequences were as follows: ENDOCAN shRNA1, 5'-TGG TGAAGAGTTTGGTATCTTTCAAGAGAAGATACCAAA CTCTTCACCTTTTTTC-3'; ENDOCAN shRNA2, 5'-TCA TCTGGAGATGGCAATATTTCAAGAGAATATTGCCAT CTCCAGATGTTTTTTC-3'; NC shRNA, 5'-TTTCTCCGA ACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGA GAATTTTTTC-3'. The shRNA sequences were cloned into the *Age*I/*Eco*RI sites of the lentiviral vector (LV-shRNA). The recombinant lentiviral vector and packaging plasmids were co-transfected into 293T cells (Procell Life Science and Technology Co., Ltd., Wuhan, China) with Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) and the supernatant containing the virus was collected to infect target cells.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from cells using an RNApure High-purity Total RNA Rapid Extraction kit (BioTeke Corporation, Beijing, China) and reverse transcribed into cDNA using Super M-MLV reverse transcriptase (BioTeke Corporation). The cDNA was amplified by RT-qPCR using Exicycler™ 96 (Bioneer Corporation, Daejeon, Korea) with the following primers: ENDOCAN forward 5'-CTG GGAAACATGAAGAGCG-3' and 5'-GCCTGAGACTGT GCGGTAG-3' reverse; β-actin forward 5'-CTTAGTTGC GTTACACCCTTTCTTG-3' and 5'-CTGTCACCTTCACCG TTCCAGTTT-3' reverse. The reactions were performed in a 20-μl reaction mixture containing 1 μl template cDNA, 10 μl SYBR GREEN mastermix (2X), 0.5 μl forward and reverse primers and 8 μl ddH_2O. For the negative control, no primers were added. The thermocycling steps were as follows: Initial denaturation at 94˚C for 5 min, followed by 40 cycles of denaturation at 94˚C for 10 sec, annealing at 60˚C for 20 sec and elongation at 72˚C for 30 sec, and a final extension at 72˚C for 2 min 30 sec. Gene expression was analyzed using the $2^{-\Delta\Delta Cq}$ method (18).

Western blot analysis. Total protein was separated from cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) supplemented with 1% phenylmethylsulfonyl fluoride. The nuclear protein was extracted using a nuclear protein extraction kit (Beyotime Institute of Biotechnology). The protein concentration was determined using a BCA protein quantitative kit (Beyotime Institute of Biotechnology). Protein samples (20-40 μg/lane) were resolved by 8-15% SDS-PAGE, blotted onto polyvinylidene fluoride membranes, blocked with 5% skim milk and incubated with primary antibodies against ENDOCAN (1:300; cat. no. PAC463Hu01; Cloud-Clone Corp., Wuhan, China), proliferating cell nuclear antigen (PCNA; 1:500; cat. no. 10205-2-AP; Wuhan Sanying Biotechnology, Wuhan, China), cyclin D1 (1:500; cat. no. AP2612d; Abgent, Inc., San Diego, CA, USA), B-cell lymphoma 2 (BCL2; 1:500; cat. no. D160117; Sangon Biotech Co., Ltd., Shanghai, China), BCL-2-associated X protein (BAX; 1:1,000; D120073; Sangon Biotech Co., Ltd.), cleaved caspase 3 (1:1,000; cat. no. ab2302; Abcam, Cambridge, MA, USA), cleaved caspase 9 (1:1,000; cat. no. 9501; CST Biological Reagents Co., Ltd., Shanghai, China), cleaved poly (ADP-ribose) polymerase (PARP; 1:500; cat. no. ab32561; Abcam), NF-κB, P65 (1:1,000; cat. no. 10745-1-AP; Wuhan Sanying Biotechnology), phosphorylated (p-)P65 (1:400; cat. no. BM3994; Boster Biological Technology, Pleasanton, CA, USA), NF-κB inhibitor α (IκBα) (1:500; cat. no. bs-1287R; BIOSS, Beijing, China), p-IκBα (1:500; cat. no. bs-2513R; BIOSS), β-actin (1:500; bsm-33139M; BIOSS), and histone H3 (1:5,000; cat. no. GTX122148; GeneTex, Inc., Irvine, CA, USA) overnight at 4˚C. Subsequently, the membranes were treated with horseradish peroxidase (HRP)-labeled goat anti-rabbit antibodies (1:5,000; cat. no. A0208; Beyotime Institute of Biotechnology) at 37˚C for 45 min. The protein was visualized using enhanced chemiluminescence (ECL; Beyotime Institute of Biotechnology) with a gel imaging system (Beijing Liuyi Instrument Factory, Beijing, China). The protein expression levels were evaluated by densitometric analysis with Gel-Pro Analyzer 3.1 (Meyer Instruments, Inc., Houston, TX, USA) and each target protein was normalized to the corresponding β-actin or histone H3 band.

Cell counting kit-8 (CCK-8) assay. Cell proliferation ability was assessed using a CCK-8 assay (Wanleibio, Shenyang, China), based on the activity of mitochondrial dehydrogenases (19). Briefly, the cells were seeded into 96-well plates ($3x10^3$ cells/well). The cells were cultured for 0, 24, 48, 72 and 96 h at 37˚C with 5% CO_2. The supernatant was removed at each time point and replaced with 100 μl of complete medium, and 10 μl CCK-8 solution was added into each well for 1 h. The absorbance was measured at 450 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

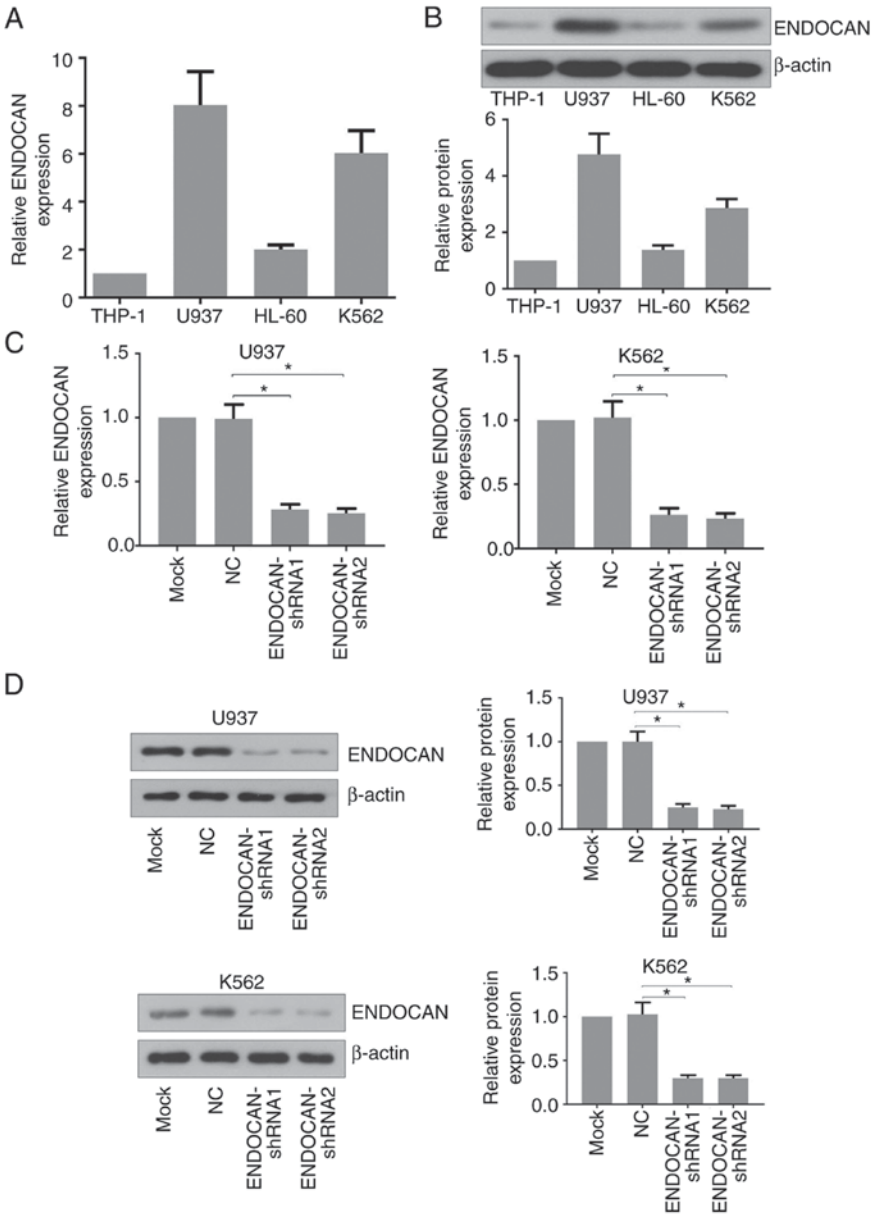

Figure 1. Silencing ENDOCAN in myeloid leukemia cells. The expression of ENDOCAN in THP-1, U937, HL-60 and K562 cells was determined by (A) RT-qPCR and (B) western blot analyses. The efficiency of ENDOCAN silencing in U937 and K562 cells was measured by (C) RT-qPCR and (D) western blot analyses. The results are expressed as the mean ± standard deviation. *P<0.05. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, negative control; shRNA, short hairpin RNA.

Cell cycle analysis. The cell cycle distribution was detected using a propidium iodide (PI)-based cell cycle analysis kit (Beyotime Institute of Biotechnology), as previously described (20). The principle of this method is that the nuclei of dead cells can be stained by PI. Briefly, the cells were collected and fixed in pre-cooled 70% ethanol for 2 h. The cells were then suspended in 500 μl of binding buffer containing 25 μl PI and 10 μl ribonuclease A (which can degrade RNA and prevent the PI staining of RNA) for 30 min at 37˚C in the dark. The cell cycle was analyzed using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), based on forward light scatter, side light scatter and PI (FL-2 channel, wavelength 575 nm) fluorescence.

Cell apoptosis. Cell apoptosis was evaluated using an Annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis detection kit (Wanleibio Co., Ltd.), as previously described (21). Annexin V binds to the externalized phosphatidylserine of cells undergoing programmed cell death, and the nuclei of dead cells can be stained by PI. Briefly, the cells were collected and resuspended in 500 μl binding buffer. Subsequently, 5 μl Annexin V-FITC and 10 μl PI were added and mixed well for 15 min at room temperature away from light. The cells were analyzed using a flow cytometer (BD Biosciences), based on forward light scatter, side light scatter, and FITC (FL-1 channel, wavelength 530 nm) and PI (FL-2 channel, wavelength 575 nm) fluorescence.

Electrophoretic mobility shift assay (EMSA). The principle of EMSA is that DNA-protein complexes migrate at a slower rate than unbound DNA in a gel. The DNA binding activity of NF-κB was determined using the EMSA kit (Viagene

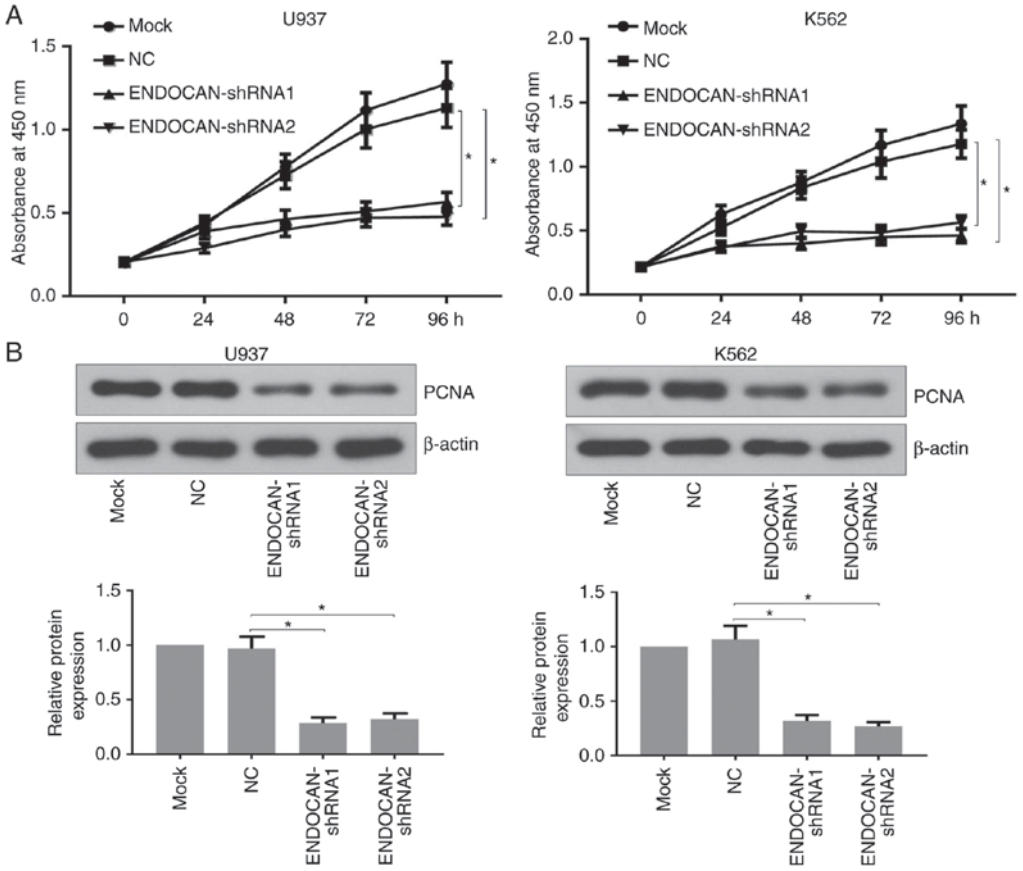

Figure 2. ENDOCAN knockdown inhibits the proliferation of myeloid leukemia cells. (A) Proliferation abilities of U937 and K562 cells in response to ENDOCAN silencing were evaluated using a CCK-8 assay. (B) Expression of PCNA in U937 and K562 cells with or without ENDOCAN knockdown was assessed by western blot analysis. The results are expressed as the mean ± standard deviation. *P<0.05. CCK-8, cell counting kit-8; PCNA, proliferating cell nuclear antigen; NC, negative control; shRNA, short hairpin RNA.

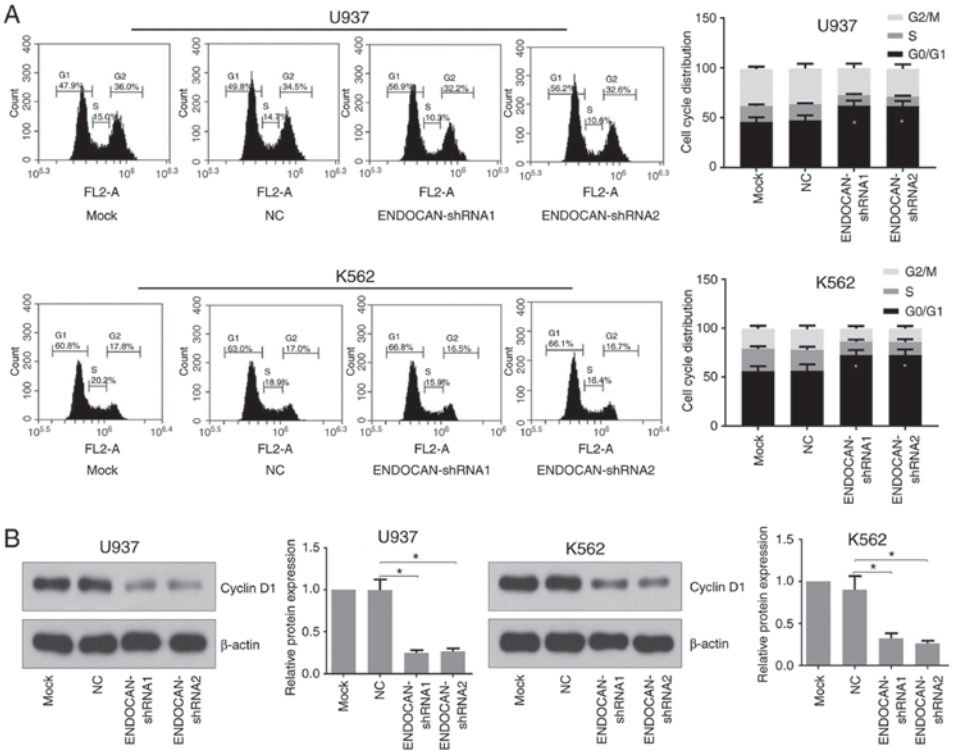

Figure 3. ENDOCAN knockdown leads to G0/G1 arrest of myeloid leukemia cells. (A) Cell cycle distribution in ENDOCAN-silenced U937 and K562 cells was assessed using flow cytometry following propidium iodide staining. (B) Western blot analysis was used to assess the expression of cyclin D1 in ENDOCAN-silenced U937 and K562 cells. The results are expressed as the mean ± standard deviation. *P<0.05. NC, negative control; shRNA, short hairpin RNA.

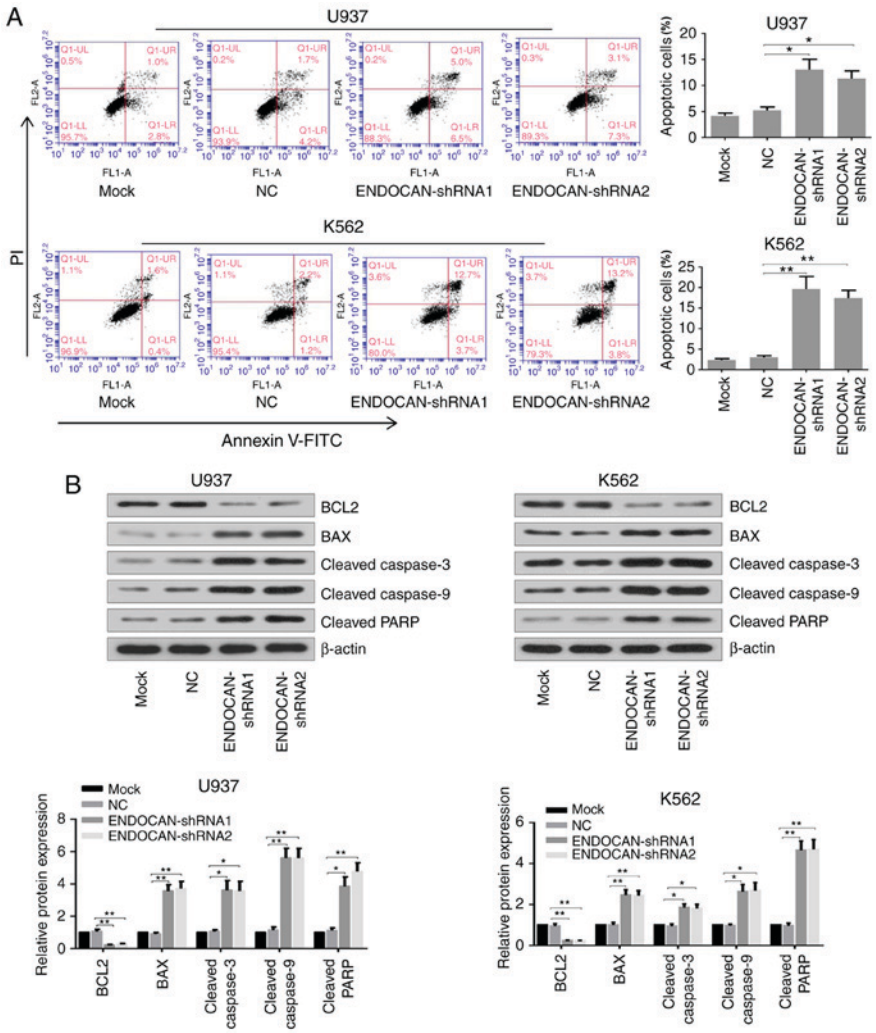

Figure 4. ENDOCAN knockdown promotes apoptosis in myeloid leukemia cells. (A) Apoptosis of U937 and K562 cells with or without ENDOCAN silencing was determined by flow cytometry following Annexin V-FITC/PI staining. (B) Expression levels of BCL2, BAX, cleaved caspases 3 and 9, and PARP in ENDOCAN-knocked down U937 and K562 cells were detected by western blot analysis. The results are expressed as the mean ± standard deviation. *P<0.05, **P<0.01. FITC, fluorescein isothiocyanate; PI, propidium iodide; BCL2, B-cell lymphoma 2; BAX, BCL2-associated X protein; PARP, cleaved poly (ADP-ribose) polymerase; NC, negative control; shRNA, short hairpin RNA.

Biotech, Ningbo, China), according to the manufacturer's protocol (22). Briefly, nuclear protein was extracted using a nuclear extraction kit (Beyotime Institute of Biotechnology). Protein concentration was determined using the BCA method. The nuclear protein (15 μg) was mixed with a biotin-labeled NF-κB probe for 20 min at room temperature. Subsequently, the DNA-protein complex was separated using a 6.5% polyacrylamide gel at 180 V for 80 min, transferred onto a nylon membrane at 360 mA for 1 h and crosslinked to the nylon membrane under UV light for 30 min. The bands were visualized using streptavidin-HRP and an ECL kit. The sequence of the probe used in EMSA was 5'-AGTTGAGGGGACTTT CCCAGGC-3'.

Statistical analysis. The results are presented as the mean ± standard deviation. Each experiment was repeated three times. Statistical differences were analyzed using Student's t-test, one-way or two-way analysis of variance or a Kruskal-Wallis test using GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Downregulation of ENDOCAN in U937 and K562 cells. To investigate the function of ENDOCAN in leukemia cells, its expression was determined in four myeloid leukemia cell lines. The results of the RT-qPCR and western blot analyses showed that the expression of ENDOCAN was higher in the U937 and K562 cells than in the THP-1 and HL-60 cells (Fig. 1A and B). The U937 and K562 cells were then infected with lentivirus carrying ENDOCAN shRNA-1, -2 or scramble shRNA for 48 h. The mRNA and protein expression levels of ENDOCAN were evaluated by RT-qPCR and western blot analyses. As shown in Fig. 1C and D, its expression was significantly reduced in these cells in response to the ENDOCAN shRNAs.

Effect of ENDOCAN silencing on the proliferation of myeloid leukemia cells. Subsequently, experiments were performed to determine whether ENDOCAN knockdown affected cell proliferation. The U937 and K562 cells were treated with lentivirus harboring ENDOCAN shRNA-1, -2 or scramble shRNA for 0, 24, 48, 72 and 96 h. A CCK-8 assay was then

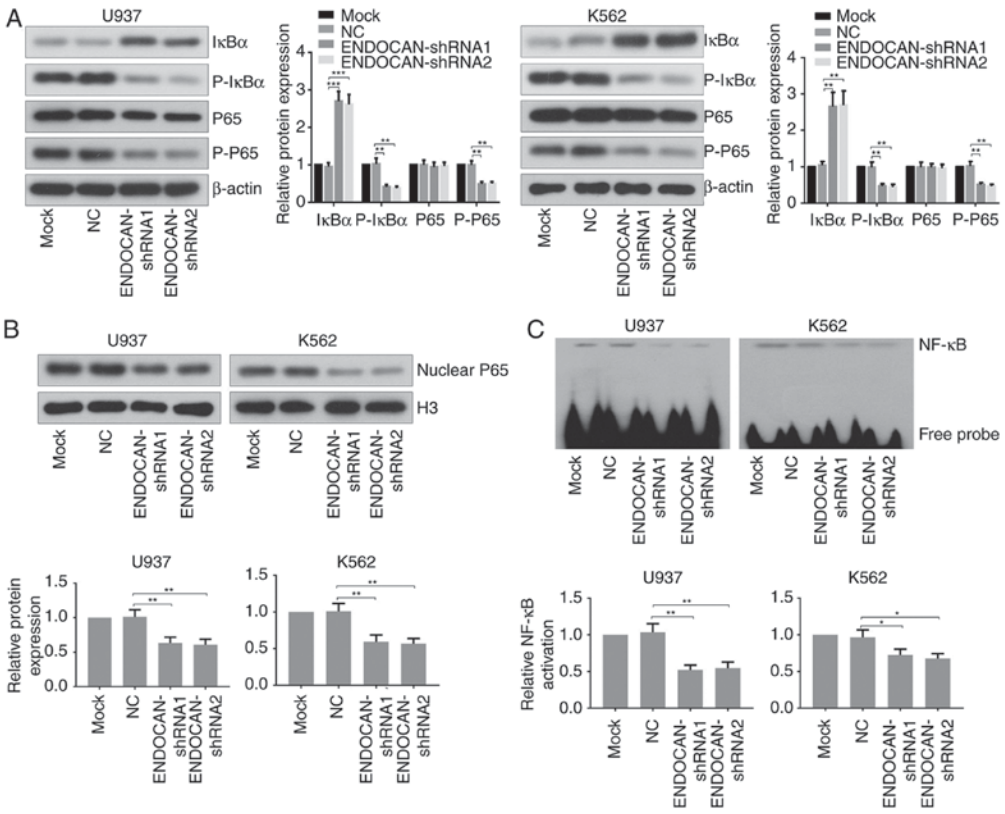

Figure 5. ENDOCAN knockdown inhibits NF-κB pathway activation. (A) Expression levels of NF-κB p65, p-p65, IκBα and p-IκBα in ENDOCAN-silenced U937 and K562 cells were determined by western blot analysis. (B) Nuclear expression of NF-κB p65 in U937 and K562 cells following ENDOCAN knockdown was detected by western blot analysis. (C) DNA binding activity of NF-κB in U937 and K562 cells following ENDOCAN knockdown was evaluated by electrophoretic mobility shift assay. The results are expressed as the mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001. NF-κB, nuclear factor-κB; IκBα, NF-κB inhibitor α; p-, phosphorylated; NC, negative control; shRNA, short hairpin RNA.

used to evaluate the proliferation ability of the U937 and K562 cells. As shown in Fig. 2A, the proliferation potential of the ENDOCAN-silenced cells was distinctly inhibited. Furthermore, reduced expression of PCNA was identified in these cells following ENDOCAN silencing for 48 h, as determined by western blot analysis (Fig. 2B). In addition, following infection for 48 h, the flow cytometry results showed that ENDOCAN knockdown led to G0/G1 cell cycle arrest in the U937 and K562 cells (Fig. 3A). The western blot analysis results showed that the expression level of cyclin D1 was decreased in the ENDOCAN-silenced cells (Fig. 3B).

Effect of ENDOCAN silencing on the apoptosis of myeloid leukemia cells. The apoptotic rates of ENDOCAN-silenced myeloid leukemia cells were then evaluated by flow cytometry using Annexin V/PI staining. Following infection of the U937 and K562 cells with lentivirus harboring ENDOCAN shRNA-1, -2 or scramble shRNA for 72 h, the apoptotic rate was significantly increased in the cells with ENDOCAN knockdown (Fig. 4A). In addition, the expression of apoptosis-related genes was detected by western blot analysis following infection for 72 h. As shown in Fig. 4B, the expression of BCL2 was decreased, whereas the levels of BAX, cleaved caspases 3 and 9, and cleaved PARP were increased in the ENDOCAN-silenced myeloid leukemia cells.

Effect of ENDOCAN silencing on NF-κB activity in myeloid leukemia cells. Finally, the effect of ENDOCAN knockdown on the activity of NF-κB was assessed in U937 and K562 cells infected with lentivirus carrying ENDOCAN shRNA-1, -2 or scramble shRNA for 48 h. As shown in Fig. 5A, the results of the western blot analysis showed that the expression of IκBα was increased, whereas the expression levels of p-IκBα and p-p65 were decreased following ENDOCAN knockdown in U937 and K562 cells. In addition, the expression of nuclear p65 was found to be reduced in the ENDOCAN-silenced myeloid leukemia cells, as determined by western blot analysis (Fig. 5B). The EMSA assay revealed that the DNA binding activity of NF-κB was attenuated in the myeloid leukemia cells following ENDOCAN silencing (Fig. 5C).

Discussion

The anticancer effect of ENDOCAN knockdown has been investigated in several types of cancer, including hepatocarcinoma (15), and prostate (23), head and neck (24), and gastric cancer (25). ENDOCAN has been found to be elevated in the serum and bone marrow blasts of patients with acute leukemia (16,17). In the present study, it was demonstrated that ENDOCAN silencing effectively suppressed cell proliferation, induced G0/G1 cell cycle arrest, stimulated cell apoptosis and attenuated NF-κB activity in myeloid leukemia cells *in vitro*.

Uncontrolled proliferation and suppression of apoptosis is a hallmark of tumor cells. PCNA has been widely used as a marker in the assessment of tumor cell proliferation (26). The

inhibition of cyclin D1, a key cell cycle regulatory protein, can induce cell-cycle arrest at the G0/G1 phase (27). Members of the BCL2 family are pivotal in the regulation of apoptosis (28). For example, reducing the ratio of BCL2 to BCX can trigger the release of cytochrome *c* from mitochondria, leading to an increase in cleaved caspases-3 and -9 and PARP, which contributes to cell apoptosis (29). By detecting the above factors, the antiproliferation and pro-apoptotic effects of ENDOCAN silencing on myeloid leukemia cells were confirmed in the present study.

The NF-κB family consists of five members, NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB and c-Rel, which form homo- or heterodimers. IκB sequesters NF-κB in the cytoplasm and prevents its translocation to the nucleus. Upon the appropriate stimulus, IκB is phosphorylated and degraded, resulting in the translocation of NF-κB into the nucleus, where it can bind to specific DNA sequences in the promoters of target genes and stimulate their transcription (30,31). Previous studies have shown that NF-κB is constitutively activated in several types of cancer, including thyroid (32) and breast cancer (33), melanoma (34) and leukemia (35). It has been reported that the inhibition of NF-κB can restrain the proliferation and migration, and promote the apoptosis of leukemia cells (36). The function of ENDOCAN in regulating the activity of NF-κB has been demonstrated in colorectal cancer and hepatocarcinoma (15,37). Kang *et al* (37) revealed that ENDOCAN can interact with NF-κB and activate NF-κB promoter. These reports, in combination with the present findings, suggest that the antitumor effect of ENDOCAN silencing may, at least in part, be attributed to a reduction in the activity of NF-κB in myeloid leukemia cells. Based on the present data, ENDOCAN inhibitors offer potential to be developed as therapeutic agents for leukemia.

Furthermore, NF-κB has been demonstrated to be an important mediator of autophagy in cancer (38). Autophagy is the process through which cellular contents are engulfed by autophagosomes and delivered to lysosomes for degradation, and has emerged as a key pathway in cancer development and therapy (39). A previous study has shown that the suppression of autophagy can assist in overcoming chemoresistance in leukemia (40). Wang *et al* (41) found that bardoxolone methyl, a potent NF-κB inhibitor, induced cell cycle arrest, apoptosis and autophagy in leukemia cells. Therefore, ENDOCAN may be involved in the autophagy of leukemia cells. Other tumor growth-related pathways, including p53, epidermal growth factor receptor (EGFR), vascular EGFR type 2, phosphoinositide 3-kinase, Akt and mammalian target of rapamycin are also regulated by ENDOCAN (42,43). These pathways may also be involved in ENDOCAN-mediated proliferation and apoptosis.

In conclusion, the results of the present study showed that the knockdown of ENDOCAN inhibited cell growth and induced cell apoptosis, which was accompanied by the inhibition of NF-κB activity in myeloid leukemia cells, indicating that ENDOCAN is a promising therapeutic target in leukemia.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

WY and LS conceived, designed and prepared the manuscript. LS performed the experiments, analyzed and interpreted the data. CS and JS performed part of the experiments and data collection. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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