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Cordycepin suppresses cell proliferation and migration by targeting CLEC2 in human gastric cancer cells via Akt signaling pathway

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ABSTRACT

Purpose: Gastric cancer is a common malignancy worldwide, and is associated with high morbidity and mortality rates. Cordycepin is a 3′-deoxyadenosine drug with significant anti-cancer effects. The aim of this study was to determine the molecular mechanisms underlying cordycepin action on gastric cancer cell proliferation and migration.

Methods: The human gastric cancer cell lines MGC-803 and HGC-27 were treated with different concentrations of cordycepin (25 μM, 50 μM, 100 μM and 5 μM, 25 μM and 50 μM) for 48 h. Cell proliferation was assessed by MTT and colony formation assays, and in vitro migration by the wound healing and transwell assays. In addition, Flow Cytometry was used to detect the cell cycle and apoptosis. RT-PCR and Western blotting were used to evaluate the expression levels of key factors.

Results: Cordycepin significantly inhibited gastric cancer cell proliferation and migration in a dose-dependent manner, in addition to inducing apoptosis and arresting the cell cycle at the G2 phase. Mechanistically, cordycepin targeted the PI3K/Akt signaling pathway by significantly altering the expression levels/activation of several key mediators, and upregulated the anti-metastatic factor CLEC2.

Conclusion: Cordycepin inhibited the proliferation and migration of gastric cancer cells by upregulating CLEC2 via the Akt signaling pathway.

1. Introduction

Gastric cancer is a malignant tumor of the digestive system with high morbidity and mortality rates [1]. According to the National Cancer Center, the rate of gastric cancer-related mortality in China is more than twice the global levels, and was the second most common malignancy and cause of deaths in 2016 [2]. Surgical resection is currently the primary treatment modality, but cannot significantly improve the prognosis or long-term survival of patients [3]. Chemotherapy also has limited effects on disease progression and overall survival of patients, and is accompanied by side effects such as high toxicity and bone marrow suppression [4]. Therefore, an effective and low-toxic treatment strategy is needed for treating gastric cancer.

Cordycepin (Fig. 1A), a 3′-deoxyadenosine ($C_{10}H_{13}N_5O_3$), was first isolated from *Cordyseps militaris* by Cunningham et al. [5]. It has documented antibacterial, anti-viral, immuno-regulatory, anti-inflammatory and anti-tumor effects [6–8]. In addition, cordycepin has shown potent anti-neoplastic effects against brain, liver, prostate and colon cancers [9–12]. Wong et al. [13] showed that cordycepin inhibited the adhesion of tumor cells by inhibiting the polyadenylation of critical mRNAs. Although studies show significant therapeutic effects of cordycepin on gastric cancer cells, the specific molecular mechanisms have not been fully elucidated.

The c-type lectin-like receptor 2 (CLEC-2) is a type II membrane protein with a c-type lectin like domain. CLEC-2 was first identified in a bioinformatic screen in search of c-type lectin receptors and CLEC-2 mRNA was found in the liver and myeloid cells including monocytes, dendritic cells, NK cells, and granulocytes [14]. Afterwards, CLEC-2 was recognized as a platelet activating receptor for the snake venom toxin rhodocytin inducing platelet aggregation. Recent studies have showed that the interaction between platelet-activating C-type lectin-like receptor-2 (CLEC-2) and its ligand podoplanin (PDPN) can activate platelets and affect the proliferation, migration, and metastasis of tumor cells. Therefore, using anti-CLEC-2

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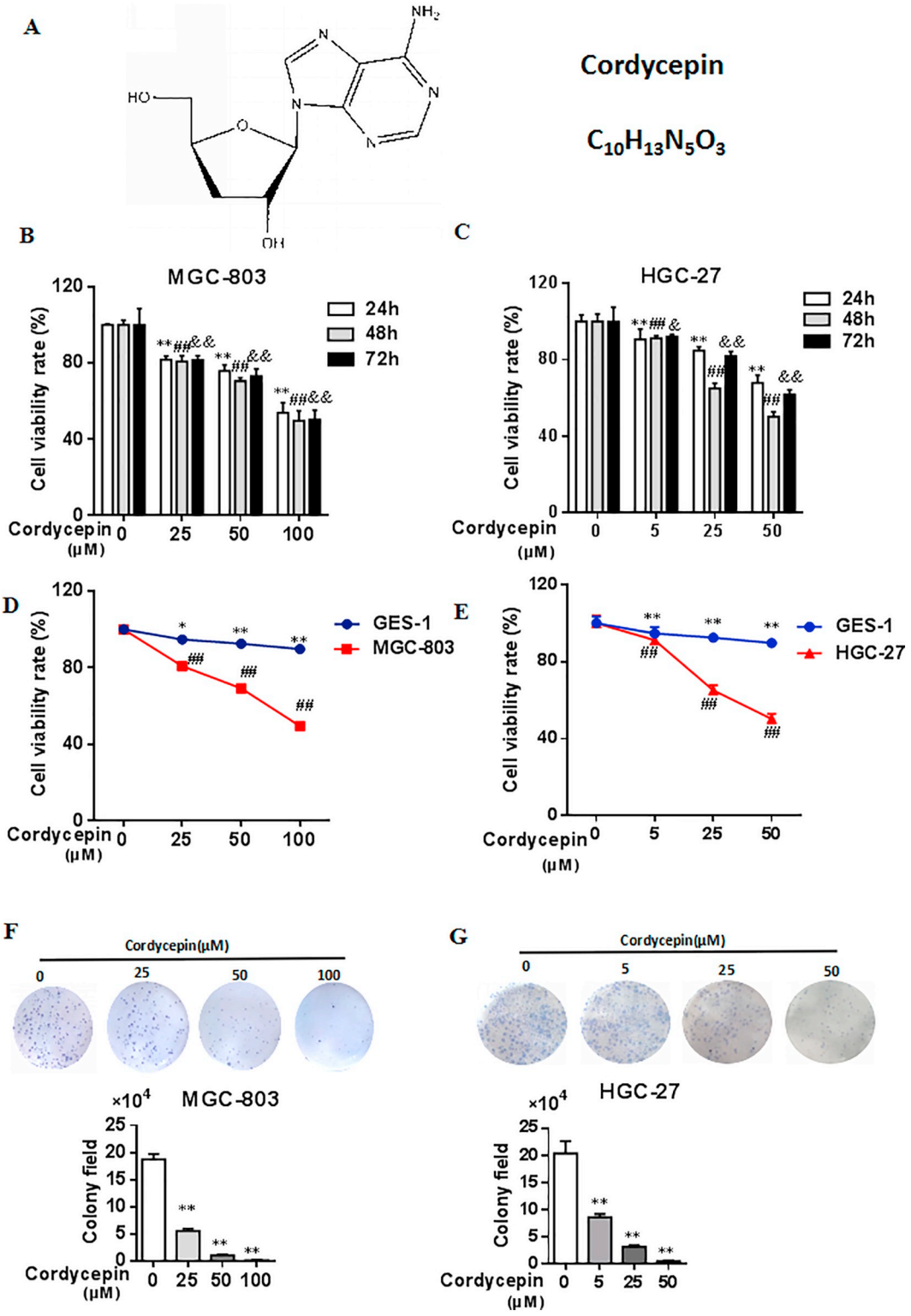

Fig. 1. Cordycepin inhibits proliferation and colony formation of human gastric cancer MGC-803 and HGC-27 cells
The chemical structural formula of cordycepin (A). The effect of cordycepin on the proliferation of MGC-803 and HGC-27 cells were detected by MTT assay (B and C). MTT assay was used to detect the viability of GES-1 cells and MGC-803 and HGC-27 cells (D and E). The effect of cordycepin on colony formation ability of the MGC-803 and HGC-27 cells (F and G). *: $P < 0.05$, **: $P < 0.01$; ##: $P < 0.01$, (vs 0 μM group, n = 3) (B and C). **:$P < 0.01$ vs 24 h 0 μM group; ##:$P < 0.01$ vs 48 h 0 μM group; &&$P < 0.01$ vs 72 h 0 μM group, n = 3 (D and E). **: $P < 0.01$, (vs 0 μM group, n = 3)(F and G).

antibody and blocking the CLEC-2/PDPN interaction maybe the novel anticancer treatments [15]. However, the role of CLEC2 in the progression of gastric cancer has not been fully elucidated.

The aim of this study therefore was to determine the possible mechanisms underlying cordycepin action on human gastric cancer cells.

2. Materials and methods

2.1. Cell lines and cell culture

The human gastric epithelial cell line GES-1, and human gastric cancer

cell lines MGC-803, SGC-7901 and HGC-27 were purchased from Shanghai Genechem Company. All cell lines were cultured in RPMI-1640 medium (Corning, America) containing 10% fetal bovine serum (FBS, Gibco) supplemented with 1% penicillin/streptomycin at 37 °C and 5% CO_2. Cordycepin was purchased from Sigma Company (Sigma, America).

2.2. MTT assay

MGC-803, HGC-27 and GES-1 cells were seeded in 96-well plates at the density of 10^4cells/well, and cultured with varying concentrations of cordycepin for 24, 48 and 72 h. The absorbance (OD) of the wells was measured at 490 nm using a microplate reader (TECAN-infinite M200 pro, Mannedorf, Switzerland).

2.3. Wound healing assay

Cells were seeded in 24-well plates and allowed to adhere overnight. A “wound” was created by scratching the monolayer with a sterile pipette tip, and then cultured with different concentrations of cordycepin. Cell migration to the wound area was monitored at 0 h and 48 h using a digital camera (Olympus, Japan). The distance of wound closure was measured in three independent wound sites per group.

2.4. Colony formation assay

Cells were seeded in six-well plates, and cultured with different concentrations of cordycepin for 14 days. The ensuing colonies were fixed with 4% paraformaldehyde for 30 min at RT, and stained with Giemsa for 30 min. After rinsing with tap water for 30 min, the colonies (> 50 cells) were counted, and the average of three independent experiments was calculated.

2.5. Cell migration assay

Cells were seeded in the upper chambers of transwell inserts at the density of 5×10^4 cells/well in medium supplemented with 1% FBS, and allowed to adhere for 4 h. The medium was replaced with fresh medium containing varying concentrations of cordycepin, and the lower chambers were filled with complete medium (10% FBS). After 48 h, the cells remaining on the upper surface of the filters were scraped with a cotton swab, and the migrated cells on the lower surface were stained with 0.1% hematoxylin. Images were taken under an OLYMPUS BX53 microscope.

2.6. Hoechst33342 staining

Cells were seeded in six-well plates and allowed to adhere overnight. After culturing with different concentrations of cordycepin for 48 h, the cells were fixed for 10 min at room temperature (RT) with 4% paraformaldehyde, stained with 1 μg/ml Hoechst33342 at RT for 5 min in the dark, and washed twice with PBS. The stained cells were imaged under a fluorescence microscope.

2.7. Transfection

Three siRNAs targeting CLEC2 si-h-CLEC2_001, si-h-CLEC2_002 and si-h-CLEC2_003 and a control siRNA (si-control) were purchased from RIBOBIO (China). The sequences are shown in Table 1. The cells were transfected with the respective siRNAs using Lipofectamine 3000, and cultured for 48 h at 37 °C under 5% CO_2 (Invitrogen, Carlsbad, CA).

2.8. Cell cycle and apoptosis assay

After 48 h treatment with varying concentrations of cordycepin, the cells were fixed overnight with 75% ethanol at −20 °C, washed twice with PBS and stained with propidium iodide (PI; 1:100, Propidium iodide cycle Detection kit II, BD Bioscience, USA) for 15 min. The stained cells were acquired using FACS Caliber (BD Company, USA) and analyzed with the standard software. To detect apoptosis, the cells were washed with PBS and re-suspended in the binding buffer provided with the FITC Annexin V Apoptosis Detection kit II (BD Bioscience, USA), and 100 μl cell suspension was incubated with 5 μl Annexin V-FITC (1:100) and 5 μl PI for 15 min in the dark. The reaction was stopped with 400 μl binding buffer, and the apoptotic cells were analyzed by flow cytometry as described.

Table 1
SiRNA sequences.

Product number	Product name	Target sequence
stB0011252A	si-h-CLEC2_001	GCAAAGCGCTTCTGTCAAT
stB0011252B	si-h-CLEC2_002	TGACCAAGGTGGACCAACT
stB0011252C	si-h-CLEC2_003	GCTCGGTTATCTCAGAAAA

Table 2
The information of antibodies.

Antibodies	Source company	Dilution ration
Anti-Cleaved-Caspase3	Cell signaling Technology, USA	1:1000
Anti-Cleaved-Caspase9	Cell signaling Technology, USA	1:1000
Anti-Cleaved-PARP	Cell signaling Technology, USA	1:1000
Anti-CyclinB1	Cell signaling Technology, USA	1:1000
Anti-Vimentin	Cell signaling Technology, USA	1:1000
Anti-snail	Cell signaling Technology, USA	1:1000
Anti-slug	Cell signaling Technology, USA	1:1000
Anti-E-cadherin	Cell signaling Technology, USA	1:1000
Anti-p-Akt	Cell signaling Technology, USA	1:1000
Anti-Akt	Cell signaling Technology, USA	1:1000
Anti-P-4EBP1	Cell signaling Technology, USA	1:1000
Anti-4EBP1	Cell signaling Technology, USA	1:1000
Anti-Survivin	Cell signaling Technology, USA	1:1000
Anti-CLEC2	Santa Cruz, USA	1:1000
Anti-β-actin	Abcam, USA	1:3000
Anti-CDK1	Proteintech, USA	1:1000
Anti-CDC25C	Proteintech, USA	1:1000
Anti-MMP2	Proteintech, USA	1:1000
Anti-MMP9	Proteintech, USA	1:1000

Table 3
The sequences of the primers and cycling condition.

Gene	Primer	Sequence(5′ to 3′)	Cycles	Annealing
CLEC2	Forward	5′-ATTCTGCTGATCCTGTGCGT-3′	40	58
	Reverse	5′-TCCAGTTTGTGTCACAGGGG-3′		
GAPDH	Forward	5′-GAGTCAACGGATTTGGTCGT-3′		
	Reverse	5′-TTGATTTTGGAGGGATCTC-3′		

2.9. Western blotting

Cordycepin-treated cells were lysed, and 20 μl lysates were resolved by SDS-PAGE. The protein bands were transferred to a PVDF membrane (Millipore, Eschbom, Germany), blocked with 5% non-fat milk and incubated with the primary antibodies (Table 2) at 4 °C. After incubation with the secondary antibody (Beyotime, China) for 1 h at RT, the positive bands were detected by enzyme-linked chemiluminescence (ECL), and quantified with a Chemiluminescent and Fluorescent Imaging System (Bio-Rad, USA).

2.10. RT-PCR

Total RNA was isolated using the TRIzol extraction kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions, and 20 μl per sample was reverse transcribed to cDNA using the PrimeScript RT-PCR kit (Takara, Japan). The sequences of the primers (5′-3′) are shown in Table 3. RT-PCR was performed in Real-

Time PCR Thermal Cycler (Applied Biosystems, USA) and cycling conditions are summarized in Table 3. The amplified products were run on a 2% agarose gel, and imaged using the gel doc XR system (BioSpectrum Imaging System, USA). All reactions were performed in triplicates.

2.11. Statistical analysis

All data analyses were performed using SPSS 17.0 software (SPSS, Chicago, IL) and GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA). LSD-*t*-test was used to compare two groups and one-way ANOVA was used for multiple comparisons. $P < 0.05$ was considered statistically significant. Average values of at least 3 independent experiments were used for analysis.

3. Results

3.1. Cordycepin inhibits proliferation and colony formation of human gastric cancer cells

The MGC-803, HGC-27 and GES-1 cells were treated with varying doses of cordycepin, and their viability and proliferative ability were determined by the MTT and colony formation assays respectively. Cordycepin significantly reduced the viability of both gastric cancer cell lines ($P < 0.05$ or $P < 0.01$; Fig. 1B and C), relative to that of GES cells ($P < 0.01$; Fig. 1D and E). In addition, the colony formation ability of the MGC-803 and HGC-27 cells were significantly reduced by cordycepin in a dose-dependent manner ($P < 0.01$; Fig. 1F and G). Taken together, cordycepin inhibits proliferation of gastric cancer cells, with minimal effects on the normal epithelial cells.

3.2. Cordycepin induces apoptosis in gastric cancer cells

Since apoptosis induction is the mode of action of most anti-cancer drugs, we also analyzed the potential apoptotic effects of cordycepin on the gastric cancer cells. Hoechst 33342 staining showed a significant increase in apoptosis in the MGC-803 and HGC-27 cells following cordycepin treatment (Fig. 2A and B). Consistent with this, Annexin-V and PI staining showed a significant increase in the percentage of apoptotic cells after cordycepin treatment ($P < 0.05$ or $P < 0.01$; Fig. 2C and D). Furthermore, cordycepin also resulted in a significant increase in the levels of the pro-apoptotic proteins like cleaved caspase-9, cleaved caspase-3 and cleaved PARP ($P < 0.05$ or $P < 0.01$; Fig. 2E and F).

3.3. Cordycepin blocks the cell cycle at the G2/M phase

To further determine the basis of the anti-proliferative action of cordycepin on the gastric cancer cells, we next analyzed the cell cycle profile of the treated cells. Cordycepin significantly increased the proportion of cells in the G2/M phase ($P < 0.05$ or $P < 0.01$; Fig. 3A and B), along with downregulating the G2/M-phase checkpoint proteins such as CDK1, CyclinB1 and CDC25C ($P < 0.01$; Fig. 3C and D). Taken together, cordycepin inhibits gastric cancer cell proliferation by inducing cell cycle arrest at the G2/M checkpoint.

3.4. Cordycepin inhibits migration and epithelial-mesenchymal transition (EMT) of gastric cancer cells

Cordycepin significantly reduced in vitro lateral and longitudinal migration of the gastric cancer cells ($P < 0.01$; Fig. 4A, B–D), as determined by the wound healing and transwell assays respectively. In addition, cordycepin increased the levels of the epithelial marker E-cadherin, and decrease that of the mesenchymal markers like Vimentin, Snail and Slug, as well as the matrix metalloproteases MMP2 and MMP9 ($P < 0.01$; Fig. 4E and F). These results clearly indicate that cordycepin blocks EMT in gastric cancer cells, thereby inhibiting their ability to migrate.

3.5. Cordycepin regulates PI3K/Akt signaling pathway

Several studies have shown the involvement of the PI3K/Akt pathway in tumor initiation and progression [16], making them suitable targets of several novel anti-cancer drugs. Cordycepin treatment significantly decreased the levels of p-AKT and p-4EBP1 levels, without affecting total Akt, 4EBP1 ($P < 0.01$; Fig. 5A and B). Therefore, cordycepin attenuates gastric cancer cell proliferation by targeting the PI3K/Akt pathway.

3.6. Cordycepin inhibits proliferation and migration of gastric cancer cells by regulating CLEC2

Study showed that CLEC-2 and PDPN can affect the proliferation, migration, and metastasis of tumor cells [15]. To further dissect the molecular mechanism of cordycepin action therefore, we analyzed CLEC2 expression levels in the GES and gastric cancer cells. As shown in Fig. 6A, CLEC2 was significantly downregulated in the cancer cell lines compared to GES-1 cells, whereas cordycepin treatment substantially increased its levels (Fig. 6B). To further confirm a potential anti-oncogenic role of CLEC2 in gastric cancer, we silenced CLEC2 in the MGC-803 cells (Fig. 6C), and observed a significant increase in their viability (Fig. 6D) and lateral migration ability (Fig. 6E). Furthermore, CLEC2 silencing upregulated p-Akt and p-4EBP1 ($P < 0.05$ or $P < 0.01$; Fig. 6F). Taking all the findings together, we can hypothesize that cordycepin inhibits the proliferation and migration of gastric cancer cells by upregulating CLEC2 via the PI3K/Akt signaling pathway.

4. Discussion

In recent years, natural plant extracts have gained considerable attention for their antibacterial, anti-inflammatory, immune-modulating, and even anti-tumor effects, in addition to their low toxicity, high efficacy and pleiotropy [17]. Cordycepin was discovered in the 1950s, and Chai et al. [18] first demonstrated its anti-cancer effects in brain tumors. We treated the human gastric cancer cell lines MGC-803 and HGC-27 with cordycepin and found that it significantly inhibited their proliferation and metastasis.

Various anti-cancer drugs target the factors regulating G2/M transition in the cancer cells, such as CDK1, cyclinB1 and CDC25C. Yu et al. [19] found that mTOR inhibitors suppressed the formation of the CDK1/CyclinB1 complex and arrested the cell cycle at the G2/M phase, thereby inhibiting the proliferation of oral cancer cells. Similarly, Ling et al. [20] showed that diallayl disulfide inhibited the proliferation of the gastric cancer BGC-823 cells by downregulating the expression of CDC25C and cyclinB1. Cordycepin also significantly decreased the proportion of cells at the G2/M phase, and downregulated CDK1, cyclinB1 and CDC25C, indicating that it inhibits gastric cancer cell proliferation by inducing cell cycle arrest. Another mechanism employed by anti-cancer drugs is apoptosis induction. For example, Wang et al. [21] showed that 6-gingenol induced apoptosis in the colon cancer HCT116 cells by upregulating caspase3 and PARP1 proteins. Similarly, Chai et al. [18] showed that cordycepin increased caspase protein levels and induced apoptosis in brain tumor cells. Consistent with these

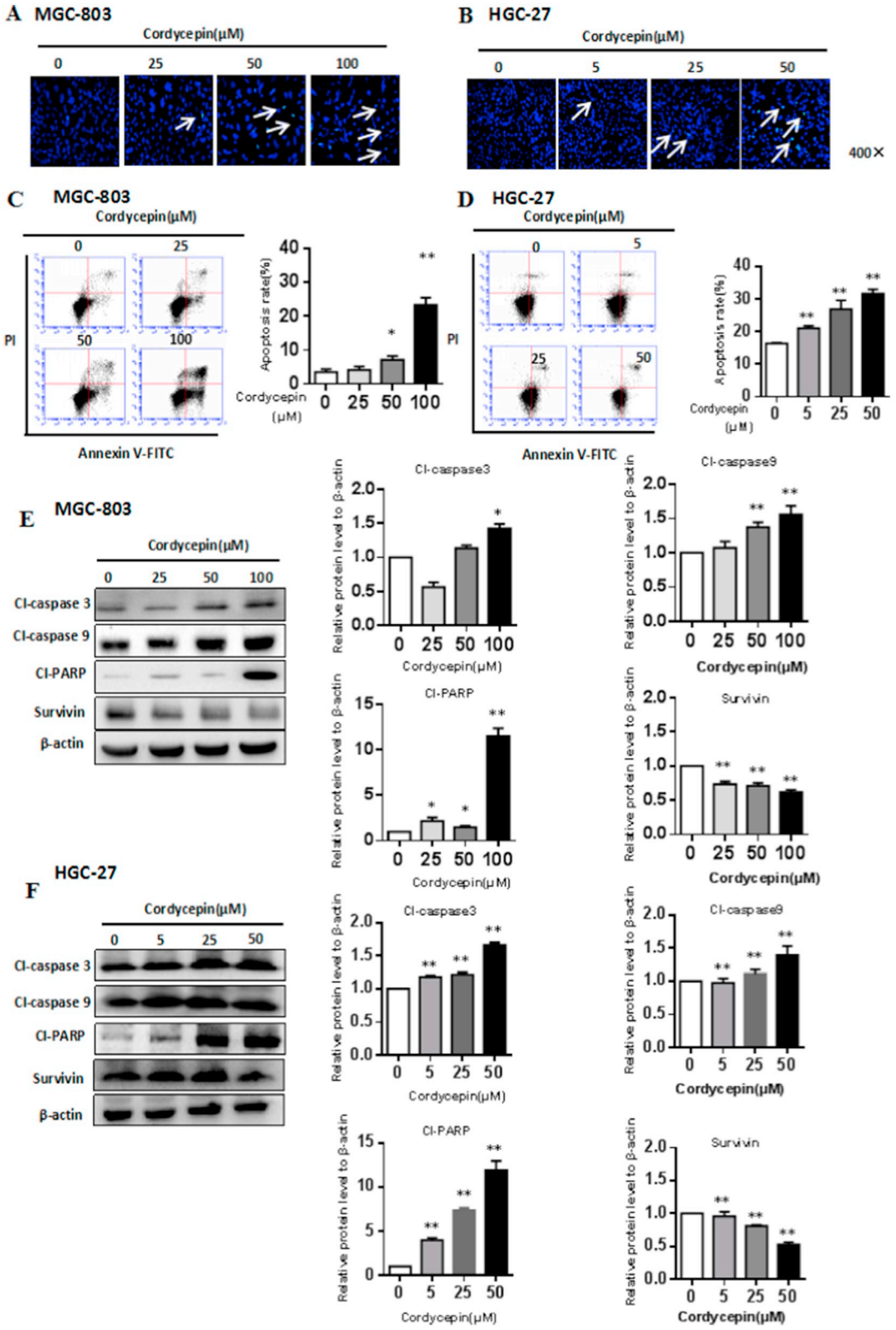

Fig. 2. Cordycepin induces apoptosis in MGC-803 and HGC-27 cells

The effect of cordycepin on the apoptosis of MGC-803 and HGC-27 cells were detected by Hoechst33342 staining (white arrow) (A and B) and flow cytometry (C and D). The effect of cordycepin on the expression of apoptosis-related proteins in MGC-803 and HGC-27 cells were detected by Western blot (E and F). *: $P < 0.05$; **: $P < 0.01$, (vs 0 μM group, n = 3) (C, D, E and F). Original magnifications, x200 (A and B).

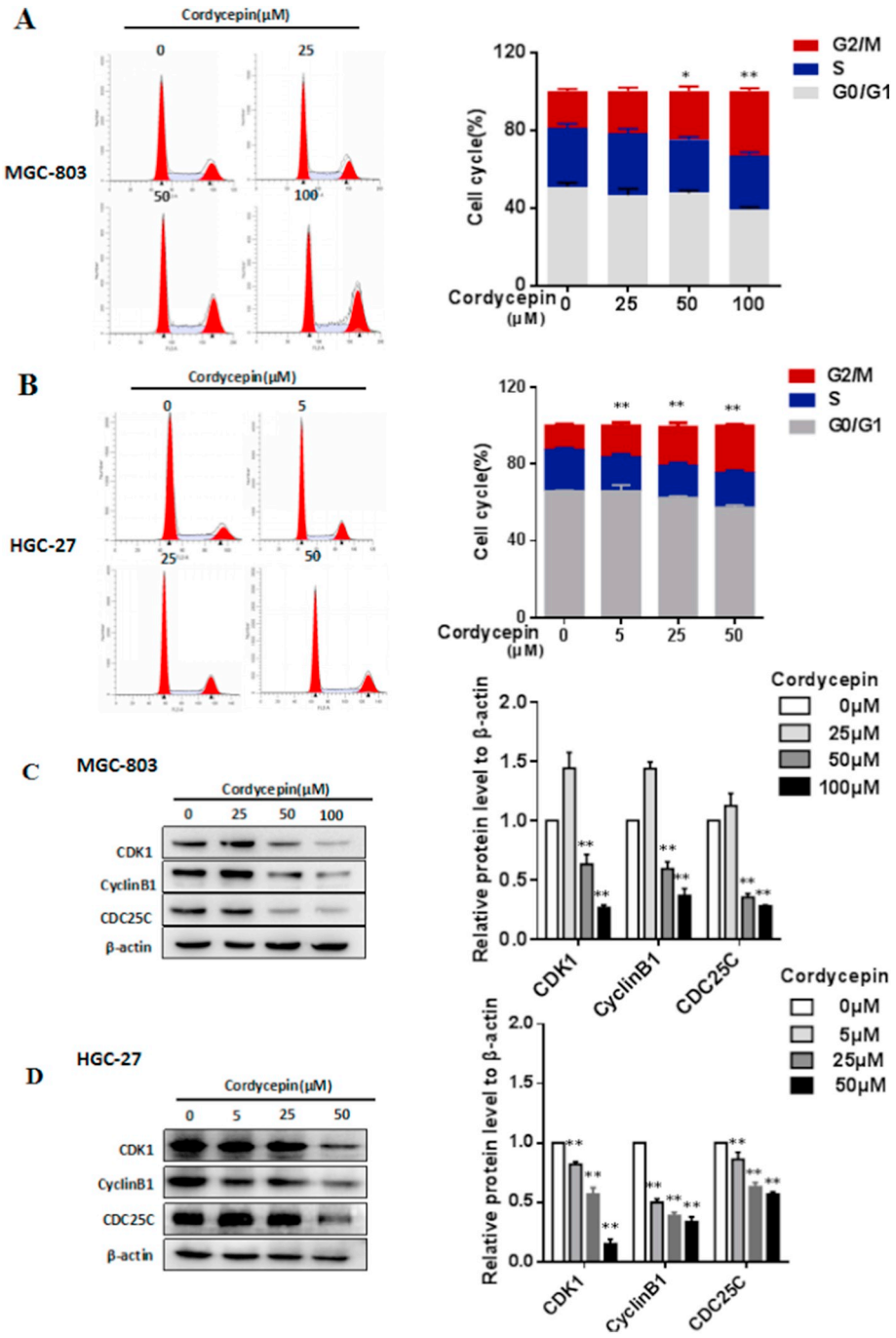

Fig. 3. Cordycepin blocks MGC-803 and HGC-27 cells in G2/M phase
Cell cycle was detected by flow cytometry after treatment with cordycepin for 48 h (A and B). The effect of cordycepin on the expression of cycle-associated proteins in MGC-803 and HGC-27 cells was detected by Western blot (C and D). *: $P < 0.05$; **: $P < 0.01$, (vs 0 μM group, n = 3) (A and B); **: $P < 0.01$, (vs 0 μM group, n = 3) (C and D).

reports, cordycepin significantly increased apoptosis rates in gastric cancer cells in a concentration-dependent manner, in addition to up-regulating cleaved-caspase 3, cleaved-caspase 9 and cleaved-PARP, and downregulating survivin. Tumor metastasis is a result of activation of the EMT pathway and high expression levels of the matrix metalloproteases (MMP)-2 and 9, which are often targeted by chemotherapeutic agents. Gao et al. [22] reported that berberine inhibited the invasion of the bladder cancer T24 cells by down-regulating MMP2 and MMP9. We found that both lateral and vertical migration of the gastric cancer cells decreased significantly after treatment with

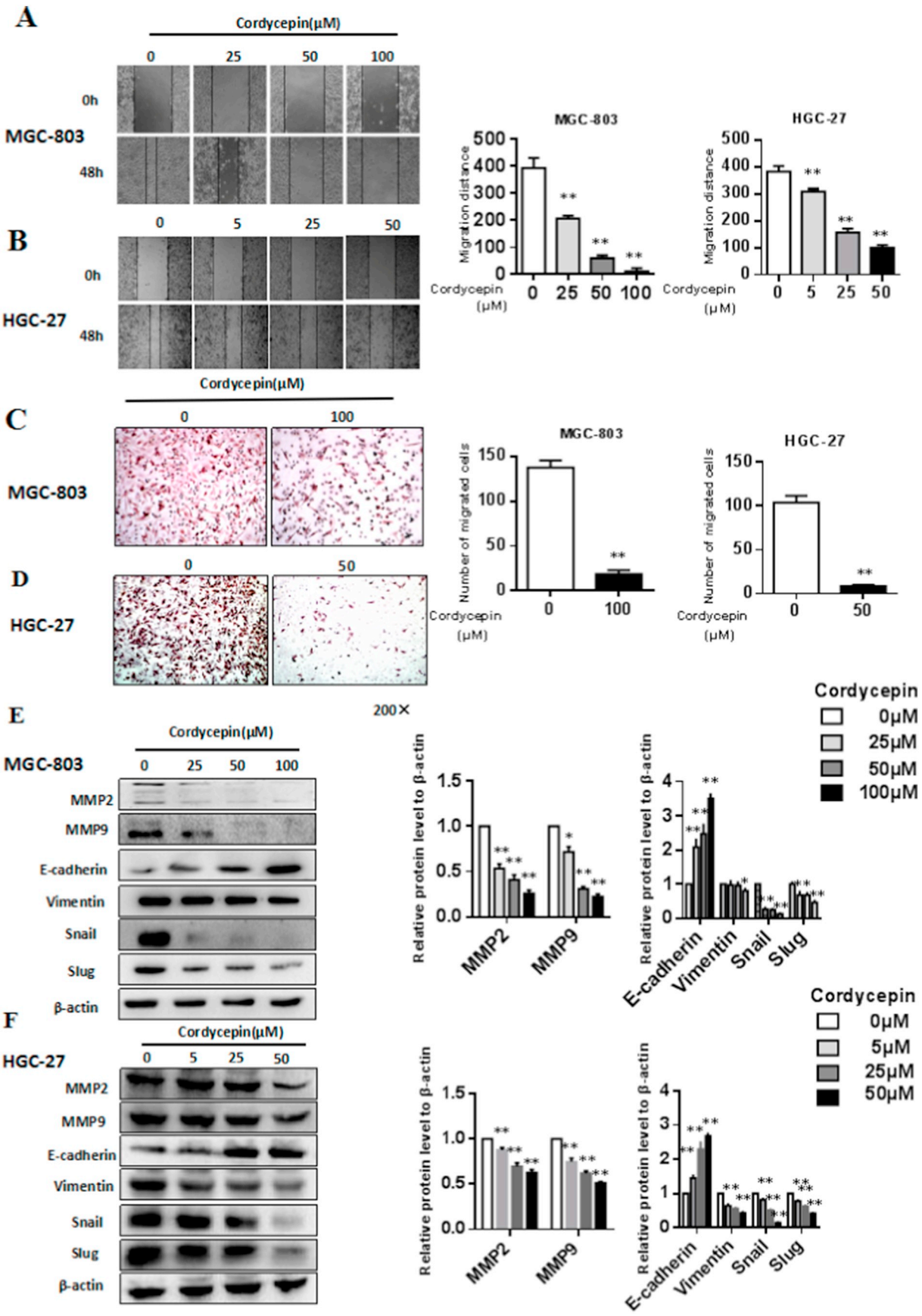

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Fig. 4. Cordycepin inhibits MGC-803 and HGC-27 cells migration and EMT
Effect of cordycepin on the lateral migration ability of MGC-803 and HGC-27 cells (A and B). Effect of cordycepin on the longitudinal migration ability of cells (C and D). The effect of cordycepin on the expression of EMT-related proteins, MMP2 and MMP9 proteins were detected by Western blot (E and F). **: $P < 0.01$, (vs 0 μM group, n = 3) (A and B); **: $P < 0.01$, (vs 0 μM group, n = 3) (C and D); **: $P < 0.01$, (vs 0 μM group, n = 3) (E and F). Original magnifications, x40(A and B), Original magnifications, ×200 (C and D).

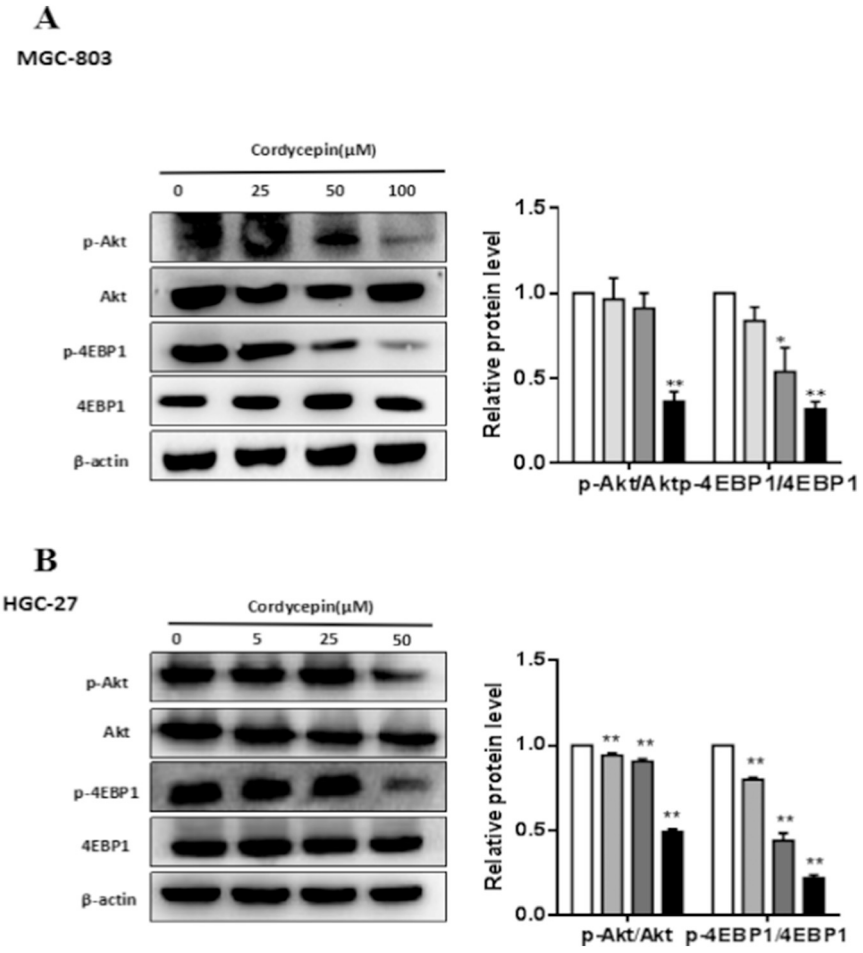

Fig. 5. Cordycepin regulates PI3K/Akt signaling pathway
The effect of cordycepin on the expression of p-Akt, Akt, p-4EBP1, 4EBP1 protein in Akt signaling pathway was detected by Western blot (A and B). *: $P < 0.05$, **: $P < 0.01$, (vs 0 μM group, n = 3).

cordycepin, which also upregulated the epithelial marker E-cadherin and downregulated the mesenchymal markers vimentin, snail and slug, and MMP2 and MMP9. Taken together, cordycepin inhibits the gastric cancer cells metastasis by inhibiting EMT and MMPs.

The aberrant activation of the PI3K/Akt signaling pathway is related to neoplastic transformation. Most gastric cancer patients also show abnormal upregulation of this pathway, and activation of the PI3K/Akt pathway is associated with distant metastasis and poor prognosis [23,24]. Cordycepin treatment significantly downregulated p-Akt and its downstream effector p-4EBP in the gastric cancer cells. Taken together, cordycepin inhibits gastric cancer cell proliferation and migration by targeting the PI3K/Akt signaling pathway. CLEC2, a C-type lectin expressed on the myeloid cells [25]. Xiong et al. found that High CLEC-2 expression associates with unfavorable postoperative prognosis of patients with clear cell renal cell carcinoma [26]. Interestingly, scholars have recently confirmed that the expression level of CLEC2 in various gastric cancer cell lines is lower than that of normal gastric mucosa cells, and its low expression is closely related to the metastasis of gastric cancer. Wang et al. proved that increases in the content of CLEC2 in gastric cancer cells inhibit the proliferation of gastric cancer cells and reversing the occurrence of migration [27]. In agreement with the above, CLEC2 levels were significantly lower in the MGC-803 compared to the GES-1 cells, and increased after cordycepin treatment. CLEC2 knockdown significantly enhanced the proliferation and lateral migration of MGC-803 cells, increased the levels of p-Akt and p-4EBP1. Taking all these findings together, we hypothesize that cordycepin inhibits gastric cancer cells by up-regulating CLEC2 via the PI3K/Akt signaling pathway.

In conclusion, we demonstrated potent anti-gastric cancer effects of cordycepin and identified its mechanistic basis, thus providing novel insights into gastric cancer therapy.

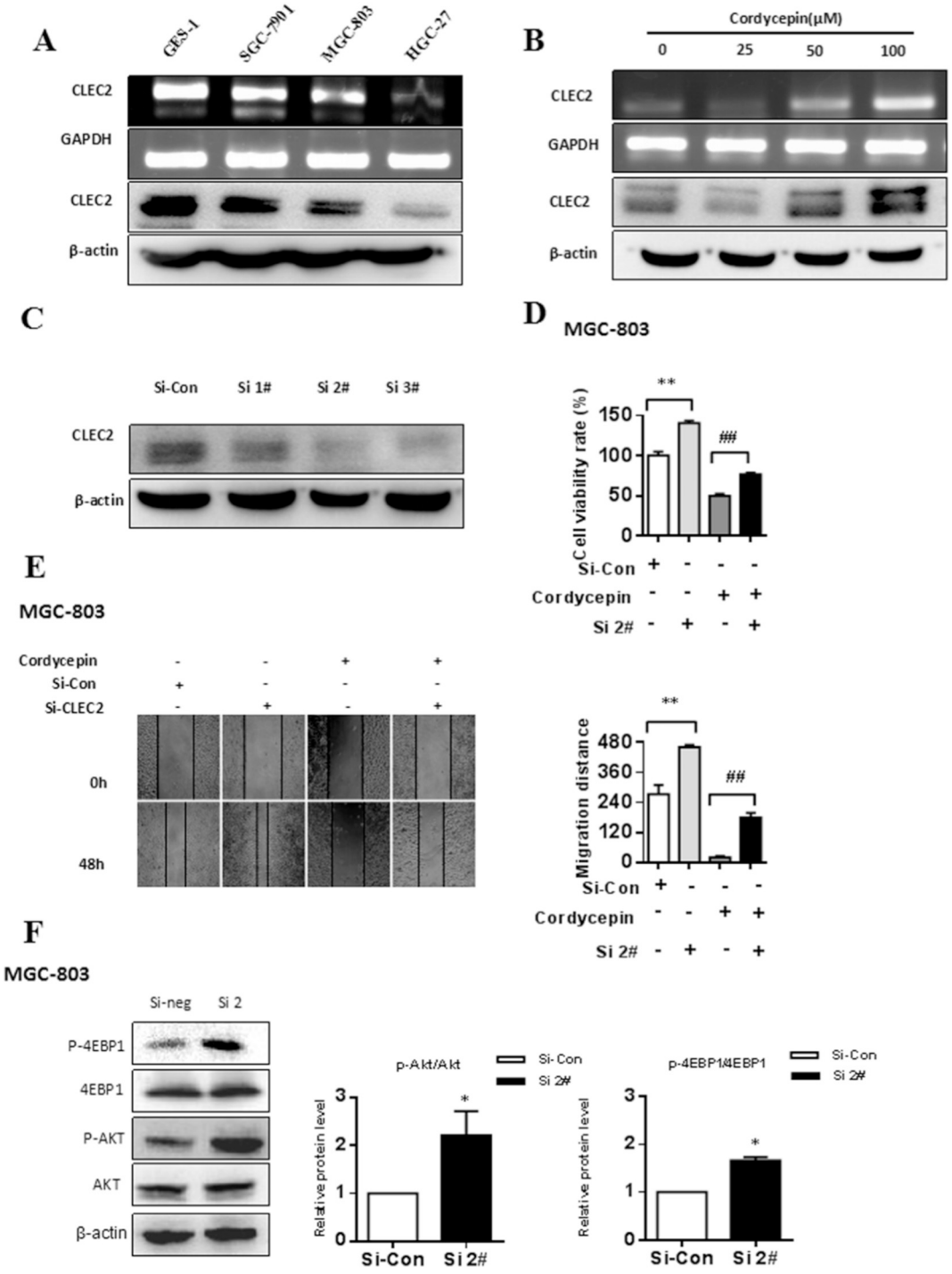

Fig. 6. Cordycepin inhibits proliferation and migration of gastric cancer cells by regulating CLEC2
RT-PCR and Western blot were used to detect the expression of mRNA and protein of CLEC2 in GES-1, SGC-7901, MGC-803, HGC-27 after treatment with cordycepin for 48 h (A and B). Western blot was used to verify the effect of gene silencing (C). Effects on cell proliferation, migration and expression of pathway-related proteins after the silencing of CLEC2 gene (D, E, and F). *: $P < 0.05$, **: $P < 0.01$, ##: $P < 0.01$, (vs si Control group, n = 3) (D, E, and F). Original magnifications, ×40 (E).

Disclosures

None of the authors has any conflicts of interest to report.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.03.025.

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