Effects of celastrol on enhancing apoptosis of ovarian cancer cells via the downregulation of microRNA-21 and the suppression of the PI3K/Akt-NF-κB signaling pathway in an *in vitro* model of ovarian carcinoma

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Abstract. Celastrol has previously been used to treat rheumatoid arthritis, bruises, back pain and additional diseases. At present, efficacy studies predominantly focus on the anti-inflammatory, antioxidative and antitumor effects of celastrol. However, the effect of celastrol on ovarian cancer cells is not fully elucidated. In the present study, the effects of celastrol were investigated in ovarian cancer cells and the mechanisms involved were explored. In OVCAR3 cells, celastrol was observed to suppress cellular proliferation, induce apoptosis and increase caspase-9 and -3 activity in a dose- and time-dependent manner. The expression levels of microRNA-21 (miRNA-21) were reduced, in addition to a reduction in the levels of phosphoinositide 3-kinase (PI3K)/p-Akt-NF (NF)-κB following treatment with celastrol. Notably, reduced expression of miRNA-21 replicated the effect of celastrol on OVCAR3 cells and inhibited the PI3K/p-Akt-NF-κB signaling pathway in an *in vitro* model of ovarian carcinoma. To the best of our knowledge this is the first study to indicate that celastrol may represent a potential agent for the treatment of human ovarian carcinoma, via the induction of apoptosis through the downregulation of miRNA-21 and the PI3K/Akt-NF-κB signaling pathway in an *in vitro* model of ovarian carcinoma.

Introduction

Ovarian carcinoma is a common female gynecological cancer, and its incidence ranks third to cervical cancer and endometrial cancer in China (1). However, due to the lack of obvious signs and symptoms in the early stages of ovarian carcinoma, the majority of patients are diagnosed with ovarian cancer during the treatment of cancer in other organs (2). Therefore, ovarian carcinoma is commonly identified only at advanced stages, resulting in the greatest mortality rate of all types of gynecological cancer and representing a serious threat to female health (3).

MicroRNAs (miRNAs) are non-coding single-stranded small RNA molecules, which are highly conserved and exist in animals and plants. miRNAs consist of a single-chain 21-25 nt in length (4). miRNAs are produced by gene transcription, following which miRNAs bind to their target gene and regulate its expression. miRNA-21 has been observed to be associated with tumor occurrence and development in numerous types of cancer, including liver, non-small cell lung, stomach, breast, and esophageal cancer, and tumors of the nervous system (5,6).

Numerous previous studies have indicated that the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway serves an important role in the proliferation, angiogenesis and metastasis of ovarian carcinoma, and the tumor resistance to radiotherapy and chemotherapy (7,8). The PI3K/Akt-nuclear factor-κB (NF-κB) signaling pathway stimulates an increase in the expression levels of vascular endothelial growth factor (VEGF) to promote angiogenesis in ovarian carcinoma (9).

Celastrol is a herb found in numerous regions of China, with extensive pharmacological effects (10). Celastrol is a pentacyclic triterpene monomer extracted from the root of *Tripterygium wilfordii*. In an *in vitro* study, celastrol was indicated to exert inhibitory effects on angiogenesis in vascular endothelial cells and on the proliferation of endothelial cells (11). In the present study, the effects of celastrol were investigated in OVCAR3 cells and the anticancer mechanisms of celastrol were explored.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco, Thermo Fisher Scientific (Waltham, MA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and celastrol (Fig. 1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The

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Annexin V/propidium iodide (PI) staining kit was purchased from BD Biosciences (San Jose, CA, USA). The bicinchoninic acid (BCA) protein assay kit was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell culture. OVCAR3 human ovarian carcinoma cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultivated in DMEM with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Sangon Biotech Co., Ltd., Shanghai, China) at 37°C in a 5% CO_2 incubator.

MTT assay. OVCAR3 cells (4,000 cells/well) were seeded and cultured in 96-well microplates overnight. Subsequently, OVCAR3 cells were treated with varying concentrations of celastrol (0, 0.25, 0.5, 1, 2, 4 and 6 μM) for 0, 1, 2 and 3 days. A total of 10 μl MTT (5 mg/ml) solution was added to each well and incubated at a temperature of 37°C in a humidified atmosphere of 5% CO_2. Following this, 100 μl of the resolving solution [10% sodium dodecyl sulfate (SDS) and 0.1 mM HCl] was added to each well and incubated for 10 min at room temperature whilst the plate was agitated. The absorbance of the plate was measured at 570 nm using the Multiskan EX Primary EIA V.2.1-1 spectrophotometer.

Apoptosis levels using Annexin V/PI staining. Following treatment with celastrol (1, 2 and 4 μM) for 2 days, OVCAR3 cells were harvested and washed with phosphate-buffered saline. OVCAR3 cells were re-suspended with 1X binding buffer prior to staining with Annexin V for 30 min at room temperature in the dark. OVCAR3 cells were then double-stained with PI for 30 min at room temperature in the dark. The apoptotic OVCAR3 cells were quantitatively counted by a flow cytometer.

Caspase-3 and -9 activity assay. OVCAR3 cells (1x10^6 cells/well) were seeded and cultured in 6-well microplates overnight at 37°C in a 5% CO_2 incubator. Subsequently, the OVCAR3 cells were treated with varying concentrations of celastrol (1, 2 and 4 μM) for 2 days. Following treatment with celastrol, the OVCAR3 cells were prepared in cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) for 30-60 min and centrifuged at 12,000 x g for 10 min at 4°C. The protein concentration was measured using a BCA protein assay kit. An equal quantity of total protein extract was mixed with the reaction buffer (acetyl-Leu-Glu-His-Asp-p-nitroanilide for caspase-9 and acetyl-Asp-Glu-Val-Asp-p-nitroanilide for caspase-3) (Beyotime Institute of Biotechnology) for 4-6 h. Caspase-9 and -3 activity was measured at an absorbance of 405 nm.

Quantitative polymerase chain reaction (qPCR) analysis of miRNA-21 expression. OVCAR3 cells (1x10^6 cells/well) were seeded and cultured in 6-well microplates overnight at 37°C. Subsequently, OVCAR3 cells were treated with varying concentrations of celastrol (1, 2 and 4 μM) for 2 days. Following treatment with celastrol, OVCAR3 cells were prepared in cell lysis buffer for 30-60 min and centrifuged at 12,000 x g for 10 min at 4°C. Total RNA was extracted from the cell lysate using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (1-2 μg) was reversed transcribed into to cDNA using PrimeScript RT Master Mix (Takara Bio, Inc.,

Otsu, Japan). qPCR was conducted using the ABI 7500 system (Takara Bio, Inc.). The cycling conditions were as follows: One cycle at 94°C for 5 min, followed by 30 cycles at 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. The relative expression of miRNA-21 was measured using a Bulge-Loop miRNA qRT-PCR kit (Invitrogen; Thermo Fisher Scientific, Inc.). The primers used were as follow: miRNA-21, forward 5'-GCC CGCTAGCTTATCAGACTGATG-3' and reverse 5'-GCC CGCTAGCTTATCAGACTGATG-3'; and U6 forward 5'-GTT GACATCCGTAAAGACC-3' and reverse 5'-GGAGCCAGG GCAGTAA-3'.

Western blot analysis. OVCAR3 cells (1x10^6 cells/well) were seeded and cultured in 6-well microplates overnight at 37°C in a 5% CO_2 incubator. Subsequently, OVCAR3 cells were treated with varying concentrations of celastrol (1, 2 and 4 μM) for 2 days. Following treatment with celastrol, the OVCAR3 cells were prepared in radioimmunoprecipitation assay lysis buffer with protease and phosphatase inhibitors (Beyotime Institute of Biotechnology) for 30-60 min and centrifuged at 12,000 x g for 10 min at 4°C. The protein concentration was measured using a BCA protein assay kit. A total of 30 μg of total protein lysate was loaded and electrophoresed onto 10% SDS-polyacrylamide gels (Sangon Biotech Co., Ltd.) and the separated proteins were transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% fat-free milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 2 h at room temperature. The membranes were then incubated with anti-PI3K (sc-67306; 1:500; Santa Cruz Biotechnology, Inc.), anti-phosphorylated-Akt (p-Akt; sc-7985-R and sc-1618; 1:1,000; Santa Cruz Biotechnology, Inc.), anti-NF-κB (sc-109; 1:1,000; Santa Cruz Biotechnology, Inc.) and anti-β-actin (sc-130657; 1:500; Santa Cruz Biotechnology, Inc.) overnight at 4°C. Following washing with TBST three times for 2 h at room temperature, secondary fluorescent antibodies were incubated with the membranes at room temperature for 2 h. Proteins were visualized using an LI-COR Odyssey scanner (LI-COR, Inc., Lincoln, NE, USA).

Transfection plasmid. Anti-miRNA-21 plasmids were chemically synthesized by BeastBio Co., Ltd. (Shanghai, China). OVCAR3 cells (1x10^6 cells/well) were seeded and cultured in 6-well microplates overnight. A total of 100 pmol/l anti-miRNA-21 plasmid was transfected into the OVCAR3 cells with Lipofectamine 2000 serum medium (Invitrogen; Thermo Fisher Scientific) for 24 h. Subsequently, the transfected OVCAR3 cells were treated with celastrol for 48 h.

Statistical analysis. Statistical analysis was conducted with SPSS software, version 18.0 (SPSS, Inc., Chicago, IL, USA). Values are presented as the mean ± standard error. Experiments were conducted a minimum of three times. Differences between the groups were assessed by two-way analysis of variance and no post hoc tests were used. $P < 0.01$ was considered to indicate a statistically significant difference.

Results

Effects of celastrol on the cellular proliferation of ovarian carcinoma cells. The effects of celastrol on the cellular prolif-
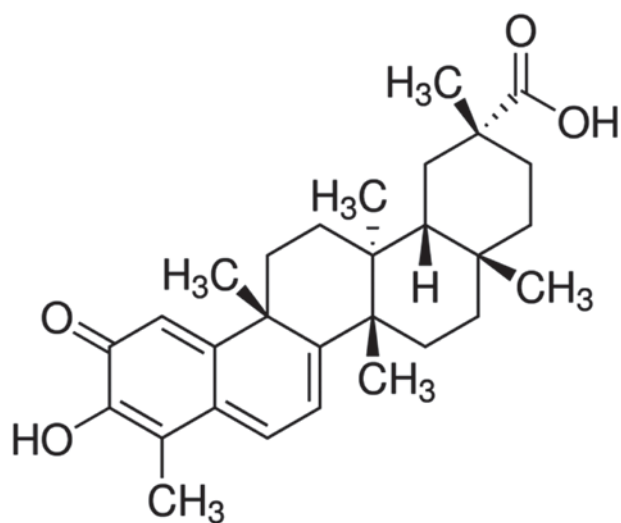
Figure 1. Chemical structure of celastrol.

eration of ovarian carcinoma cells was investigated using an MTT assay. OVCAR3 cells were treated with a range of celastrol concentrations (0, 0.25, 0.5, 1, 2, 4 and 6 μM) for 0, 1, 2 and 3 days. As presented in Fig. 2, the proliferation of OVCAR3 cells was inhibited following treatment with celastrol in a dose- and time-dependent manner. Treatment with celastrol (1, 2, 4 and 6 μM) for 3 days or celastrol (2, 4 and 6 μM) for 2 days resulted in significant reductions in cellular proliferation (P<0.01; Fig. 2). These results suggest that celastrol exerted clear anticancer effects on human ovarian carcinoma cells.

Effects of celastrol on apoptosis in ovarian carcinoma cells. The effects of celastrol on apoptosis in ovarian carcinoma cells was investigated using Annexin V/PI staining. As presented in Fig. 3, celastrol induced apoptosis in ovarian carcinoma cells in a dose-dependent manner, with a significant increase in the levels of apoptotic cells following celastrol treatment for 2 days at 2 and 4 μM (P<0.01; Fig. 3).

Effects of celastrol on caspase-9 and -3 activity in ovarian carcinoma cells. To further investigate the effects of celastrol treatment on apoptosis in ovarian carcinoma cells, the activity levels of caspase-9 and -3 was investigated. As presented in Fig. 4, celastrol increased the activity levels of caspase-9 and -3 in ovarian carcinoma cells in a dose-dependent manner. Treatment with celastrol (2 and 4 μM) for 2 days resulted in a significant increase in the activity levels of caspase-9 and -3 (P<0.01; Fig. 4).

Effects of celastrol on miRNA-21 expression in ovarian carcinoma cells. To explore the mechanisms involved in the effect of celastrol on ovarian carcinoma cells, miRNA-21 expression was measured using qPCR. The relative expression levels of miRNA-21 in OVCAR3 cells was reduced following treatment with celastrol (2 and 4 μM) for 2 day (P<0.01; Fig. 5). These results indicated that the anticancer effect of celastrol may be involved with reducing miRNA-21 levels.

Effects of celastrol on the expression levels of PI3K/Akt in ovarian carcinoma cells. To investigate the mechanisms

involved in the effect of celastrol treatment on ovarian carcinoma cells, the expression levels of PI3K/Akt were measured in ovarian carcinoma cells using western blot analysis. Following treatment with celastrol (2 and 4 μM) for 2 days, the protein expression levels of PI3K and p-Akt were reduced in OVCAR3 cells (P<0.01; Fig. 6). These results indicate that the anticancer effects of celastrol may be associated with the suppression of the PI3K/Akt signaling pathway.

Effects of celastrol on NF-κB protein expression in ovarian carcinoma cell. To further investigate the mechanisms involved in the effect of celastrol treatment on ovarian carcinoma cells, NF-κB protein expression was measured by western blot analysis. Following treatment with celastrol (2 and 4 μM) for 2 days, the expression levels of NF-κB in OVCAR3 cells were significantly reduced (P<0.01; Fig. 7). These results indicated that the anticancer effect of celastrol may be associated with the suppression of the NF-κB signaling pathway.

Effect of downregulation of miRNA-21 on PI3K/Akt/NF-κB expression. To further analyze the mechanisms involved in the effect of celastrol treatment on ovarian carcinoma cells, anti-miRNA-21 plasmids were transfected into OVCAR3 cells. As presented in Fig. 8A, the anti-miRNA-21 plasmids significantly reduced the relative expression levels of miRNA-21 in OVCAR3 cells. In addition, the anti-miRNA-21 plasmids were able to reduce the cellular proliferation of OVCAR3 cells (Fig. 8B). Notably, the anti-miRNA-21 plasmids suppressed the PI3K/Akt-NF-κB signaling pathway in ovarian carcinoma cells (Fig. 8C-F).

Discussion

Ovarian carcinoma is the most common ovarian cancer, and has the greatest associated mortality of all types of female gynecological cancer (12). Despite the progress made in the treatments available for ovarian carcinoma, the prognosis remains poor, with a mortality rate of 22/100,000 and a 5-year survival rate of 25-30% (13). Therefore, ovarian carcinoma is an important disease in the field of gynecology, of which the etiology, pathogenesis, biological characteristics and exploration of novel effective treatments are the focus of research (14). To the best of our knowledge, the current study is the first to demonstrate that celastrol is an effective and potent agent in treating ovarian carcinoma cells *in vitro*. Celastrol exerted anticancer effects on ovarian carcinoma cells via a reduction in the cellular proliferation and the activation of caspase-dependent apoptosis in OVCAR3 cells. Previous reports have indicted that celastrol significantly inhibits the cellular proliferation and induces apoptosis in gastric cancer cells (15,16) and prostate cancer cells (17).

miRNA are a type of non-coding single-stranded small RNA molecules, which are highly conserved and exist widely in animals and plants. Previous studies have indicated that there are significant differences in the expression profile of miRNA between cancer cells and normal tissues (18,19). The alterations in the expression of miRNA are associated with tumorigenesis, and the treatment and prognosis of cancer (20). A previous study indicated that the expression levels of
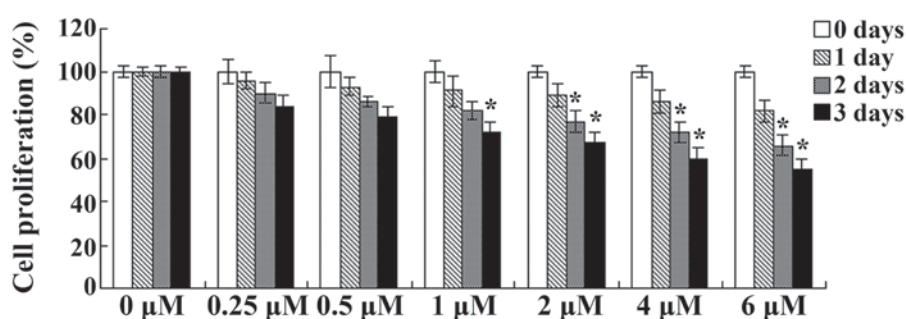
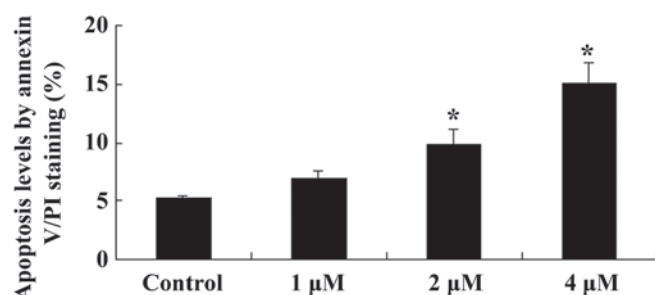Figure 2. Effect of celastrol on the proliferation of ovarian carcinoma cells. *P<0.01 vs. the 0 μM group.

Figure 3. Effect of celastrol on apoptosis in ovarian carcinoma cells. *P<0.01 vs. the control group. PI, propidium iodide.

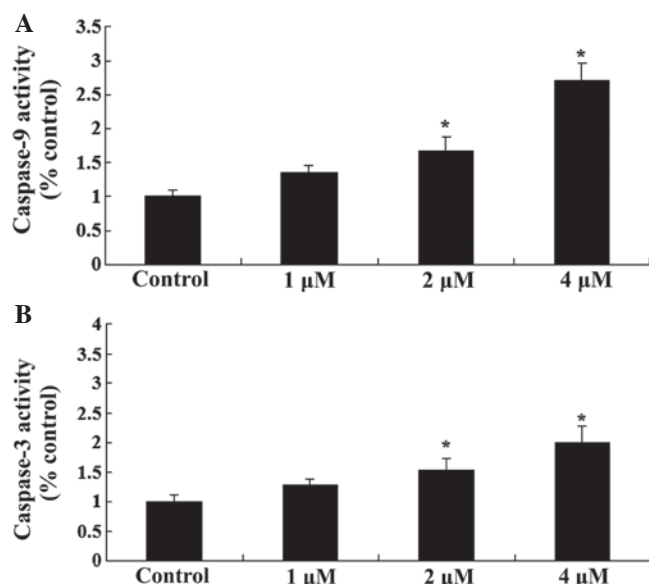Figure 4. Effect of celastrol on (A) caspase-9 and (B) caspase-3 activity in ovarian carcinoma cells. *P<0.01 vs. the control group.

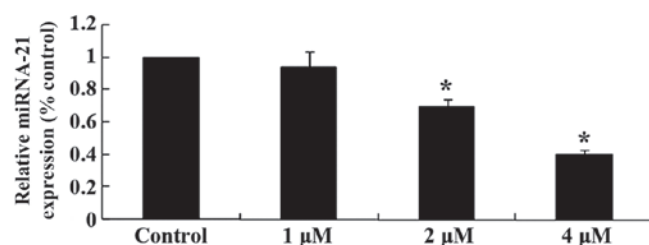Figure 5. Effect of celastrol on miRNA-21 expression levels in ovarian carcinoma cells. *P<0.01 vs. the control group. miRNA-21, microRNA-21.
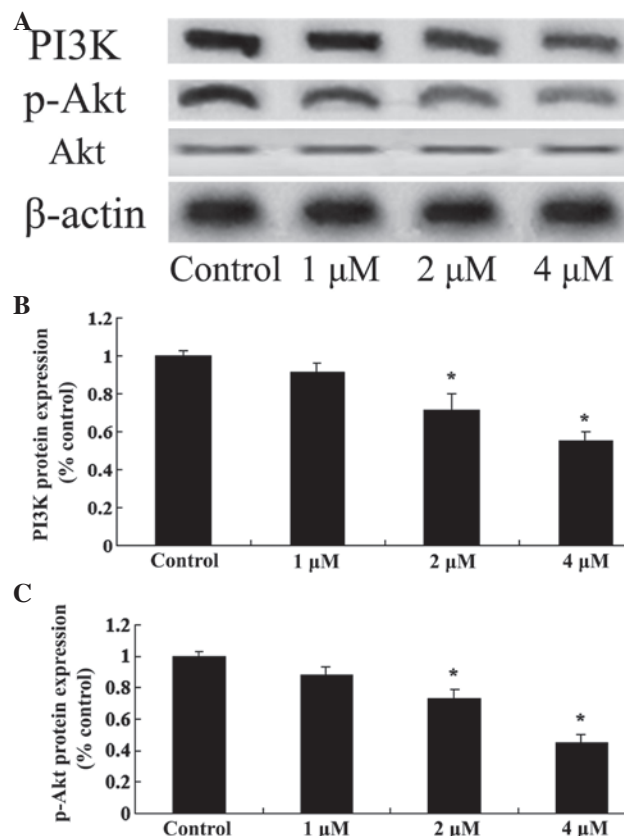

Figure 6. Effect of celastrol on PI3K/Akt protein expression levels in ovarian carcinoma cells. (A) Western blot images and the quantification of the expression levels of (B) PI3K and (C) p-Akt. *P<0.01 vs. the control group. PI3K, phosphoinositide 3-kinase; p-, phosphorylated.

miRNA-21 were increased in the tissues of breast, stomach, liver and cervical cancer, indicating that miRNA-21 may serve a role as an oncogene in tumorigenesis (21). However, reports of miRNA-21 expression in ovarian carcinoma tissue are inconsistent, though the majority indicate upregulated expression (22). In a previous study, microarrays and additional methods were used to screen miRNA expression in ovarian carcinoma tissue, and demonstrated that 12 miRNAs were upregulated, including miRNA-21 (23). The current study demonstrated that treatment with celastrol inhibited the relative expression levels of miRNA-21 in OVCAR3 cells. Sha *et al* (16) reported that celastrol induced apoptosis in gastric cancer cells via the miRNA-21-mediated inhibition of the PI3K/Akt-NF-κB signaling pathway.
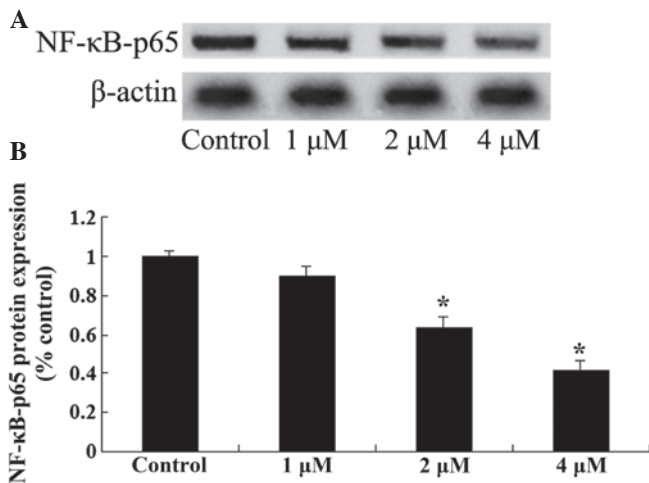
Figure 7. Effect of celastrol on the expression levels of NF-κB in ovarian carcinoma cells. (A) Western blot images and (B) the quantification of the expression levels of NF-κB. *P<0.01 vs. the control group. NF-κB, nuclear factor-κB.

PI3K is a member of the lipid kinase family and is involved in the regulation of cellular metabolism, survival and proliferation. Akt is an important protein kinase downstream of PI3K, and its continuous activation is closely associated with tumor development (24,25). In breast and ovarian cancer, as well as additional malignancies, the PI3K/Akt pathway has been observed to be resistant to the induction of apoptosis by chemotherapy and radiotherapy (26,27). Selective inhibition of PI3K or Akt activity reduces the phosphorylation levels of Akt, which is able to increase the sensitivity of the cells to the induction of apoptosis by chemotherapy and radiotherapy (28). The current study indicates that celastrol treatment reduced the protein expression levels of PI3K and p-Akt in OVCAR3 cells. In a previous study, Lee et al (29) indicated that treatment with celastrol was able to suppress cell growth and increase apoptosis in melanoma cells through the suppression of PI3K/AKT signaling. Sha et al (16) demonstrated that celastrol induces apoptosis in gastric cancer cells via the inhibition of the PI3K/Akt-NF-κB signaling pathway by miRNA-21.

The PI3K/Akt pathway directly or indirectly affects downstream processes that are associated with cellular proliferation, protein synthesis and certain apoptosis-associated factors. NF-κB is a key factor downstream of Akt, and under physiological conditions is bound to its inhibitor, IκB, and is localized in the cytoplasm in an inactive form (30). The activation of NF-κB results in the regulation of gene transcription associated with the promotion of cellular proliferation and the inhibition of apoptosis (31). The present study indicated that treatment with celastrol reduced the expression levels of NF-κB in OVCAR3 cells. Youn et al (32) reported that celastrol treatment ameliorated human immunodeficiency virus-1 Tat-induced inflammatory responses via the inhibition of NF-κB (32).

The current study demonstrated that the downregulation of miRNA-21 expression was able to replicate the anticancer effect of celastrol on OVCAR3 cells, resulting in a reduction in the expression levels of PI3K/Akt-NF-κB in OVCAR3 cells. In conclusion, the current study indicates that celastrol is able to significantly suppress cellular proliferation and induce
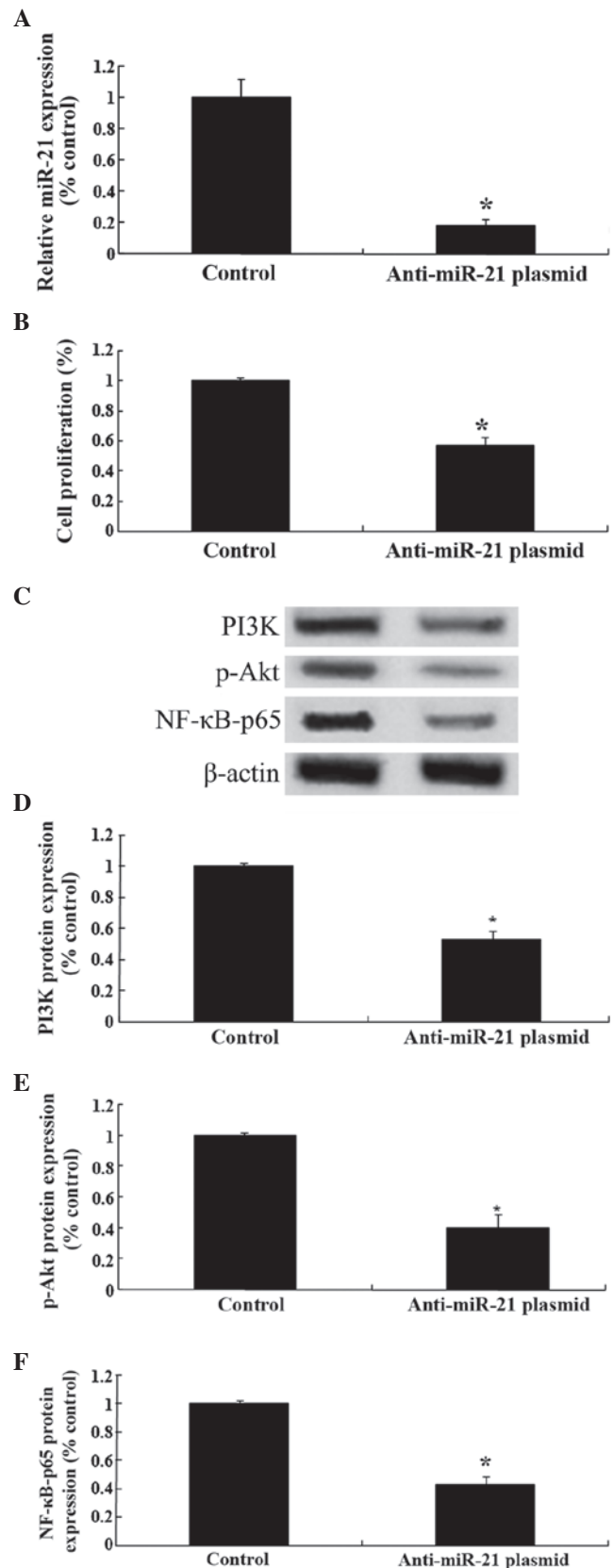

Figure 8. Effect of downregulation of miRNA-21 on the expression levels of PI3K/Akt/NF-κB in ovarian carcinoma cells. (A) The expression levels of miRNA-21 are reduced by the miRNA-21 plasmid. (B) The effect of the miRNA-21 plasmid on cellular proliferation in ovarian carcinoma cells. (C) Western blot images of the protein expression of PI3K, p-Akt and NF-κB, and the quantification of (D) PI3K, (E) p-Akt and (F) NF-κB protein expression levels. *P<0.01 vs. the control group. miRNA-21, microRNA-21; PI3K, phosphoinositide 3-kinase; p-Akt, phosphorylated protein kinase B; NF-κB, nuclear factor-κB.

apoptosis of OVCAR3 cells. These results demonstrated that celastrol may represent a potential novel anticancer treatment, with its mechanisms associated with the downregulation of microRNA-21 and the suppression the PI3K/Akt-NF-κB signaling pathway in an *in vitro* model of ovarian carcinoma.

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