

pPeOp from *Omphalia lapidescens* Schroet induces cell cycle arrest and inhibits the migration of MC-4 gastric tumor cells

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Abstract. The aim of the present study was to investigate the effect of purified *Omphalia lapidescens* protein (pPeOp) extracted by polyvinylpyrrolidone from the fungus *Omphalia lapidescens* Schroet on the proliferation and cell cycle progression of MC-4 human gastric tumor cells. Using polyvinylpyrrolidone, pPeOp was extracted from *O. lapidescens* Schroet. MC-4 cells were cultured with 30, 60 or 90 $\mu\text{g/ml}$ pPeOp, with 5-fluorouracil used as a positive control. Survival rates of treated cells were significantly decreased compared with those of the untreated control group in a dose-dependent manner. Using flow cytometric analysis, cells treated with pPeOp were demonstrated to arrest in S phase and exhibit abnormal G_0/G_1 and G_2/M phase cell cycle distribution. In addition, a wound healing assay demonstrated that pPeOp significantly inhibited the migration of MC-4 cells. The mRNA and protein expression levels of cyclin D1/cyclin-dependent kinase (CDK) 4, cyclin B/CDK1, cyclin A/CDK2, matrix metalloproteinase (MMP)-2 and MMP-9 were determined using reverse transcription-quantitative polymerase chain reaction analysis and western blotting. The mRNA expression level of CDK4 and cyclin A was significantly increased compared with the untreated control; however, cyclin D1, CDK1, CDK2, cyclin B, MMP-2, and MMP-9 exhibited a significantly decreased mRNA expression level, indicating that there is a negative association between concentration and cyclin D1 expression levels. The expression of the cycle arrest-associated proteins and migration-associated proteins examined were similar to the observed mRNA expression levels. In conclusion, pPeOp was identified to inhibit migration of and cause S phase cell cycle arrest in MC-4 cells.

Introduction

Gastric cancer is one of the most common gastrointestinal cancers worldwide, ranking second in terms of cancer-associated

mortality rates (1,2), and causing marked morbidity and mortality in China (3). Early detection is important in the treatment of this type of cancer, as advanced-stage diagnosis results in a poor prognosis (4,5). Western medicine prioritizes surgical removal and other conventional treatment methods, which directly kill tumor cells or inhibit their proliferative and metastatic capabilities. However, these treatments cannot completely eliminate the tumor cells, and cause serious sideeffects in normal cells (5).

The treatment of tumors using traditional Chinese medicine has unique advantages (6,7). The use of traditional Chinese medicine in the treatment of cancer is able to significantly improve the quality of life and survival rate of patients (8). *Omphalia lapidescens* Schroet is widely used as a traditional anthelmintic in China. Previous studies have demonstrated that *O. lapidescens* Schroet effectively induces tumor necrosis; consequently, the China Food and Drug Administration has approved Lei Wan Pian and Lei Wan Jiao Nang as antitumor auxiliary drugs (9,10). Research has revealed that the antitumor effects of the active ingredients from *O. lapidescens* Schroet are associated with certain polysaccharides and proteins (11-14), with mechanisms that include the direct killing of tumor cells through induction of apoptosis, and the enhancement of immune and anti-inflammatory responses. In a previous study (14), purified *Omphalia lapidescens* protein (pPeOp) was extracted from dried sclerotoids with polyvinylpyrrolidone (PVP) extraction buffer, and it was verified that a single protein band was retained following isolation of the major fraction using molecular-sieve chromatography. The major constituent, pPeOp, was identified with relatively high chromatographic purity. pPeOp increased apoptosis in human gastric tumor cells compared with conventional Western treatments, which induce tumor cell (MC-4 and SGC-7901) death and apoptosis significantly, but also trigger minor apoptosis of normal gastric cells (MC-1) (14). To further understand the underlying molecular mechanisms of the antitumor activities of pPeOp, its effects on tumor cell migration and cell cycle progression were investigated.

Materials and methods

Drugs and reagents. *O. lapidescens* Schroet powder was purchased from Fang Hui Chun Tang (Hangzhou, Zhejiang, China); the protein pPeOp was extracted from dried sclerotoids of *O. lapidescens* Schroet using PVP extraction buffer [15% 1.0 M Tris-HCl (pH 8.0), 2% PVP and 25% glycerol],

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Table I. Primers used in reverse transcription-quantitative polymerase chain reaction analysis.

Target	Forward primer (5'-3')	Reverse primer (5'-3')
β -actin	ACCTGCAGCAATACCATTGAC	AAGGTGAGGGACTCAAAGTGC
CDK1	TGCTGGGGTTCAGCTCGTTACTCA	TGGGATGCTAGGCTTCCTGGTT
CDK2	GTGGGCCCGGCAAGATTTTAG	GCCGAAATCCGCTTGTTAGGG
CDK4	CTTGATCTGAGAATGGCTACCTCT	CATGAAGGAAATCTAGGCCTCTTA
Cyclin D1	CATCTCTGTACTTTGCTTGCTCAT	CGCTATTTTCTACACCTATTGGAC
Cyclin B	TCGAGCAACATACTTTGG	GCAAAAAGCTCCTGCTGC
Cyclin A	AGACCCTGCATTTGGCTGTG	ACAAACTCTGCTACTTCTGG
MMP-2	TGATGGTGTCTGCTGGAAAG	GACACGTGAAAAGTGCCTTG
MMP-9	GGAGACCTGAGAACCAATCTC	TCCAATAGGTGATGTTGTGG

CDK, cyclin-dependent kinase; MMP, matrix metalloproteinase.

with 100 μ g/ml 5-fluorouracil (5-FU) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) as a positive control. Antibodies against cyclin-dependent kinase (CDK) 2 (cat. no. 2546T), cyclin B (cat. no. 4138T), CDK4 (cat. no. 12790T) and cyclin D1 (cat. no. 2922S) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against CDK1 (cat. no. ab131450), cyclin A (cat. no. ab181591), and MMP-2 (cat. no. ab37150) and -9 (cat. no. ab73734) were purchased from Abcam (Cambridge, UK). Anti- β -actin was used as a control and horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) was used as a secondary antibody. Both antibodies were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Antibodies against CDK2, cyclin B, CDK4, cyclin D1, CDK1, MMP-2, MMP-9 and β -actin were diluted 1:1,000 in TBST containing 3% BSA, and antibody against cyclin A was diluted 1:2,000 for use.

Cell lines and cell culture. The human gastric cancer cell line MC-4 was obtained from the Zhejiang Provincial Center for Disease Control and Prevention (Hangzhou, China). MC-4 cells were cultured in RPMI-1640 medium (Genome Biotechnology, Hangzhou, China) supplemented with 10% (v/v) fetal bovine serum (Zhejiang Tianhang Biotechnology Co. Ltd., Hangzhou, China), 100 units/ml penicillin and 100 units/ml streptomycin (Genome Biotechnology) at 37°C in a humidified atmosphere containing 5% CO₂. Every 1-2 days, cells were used when >80% cells were in the exponential growth phase. The control group was untreated MC-4 cells, MC-4 cells treated with 90 μ g/ml PVP were used as a negative control and MC-4 cells treated with 100 μ g/ml 5-FU were used as a positive control.

Cell counting kit (CCK)-8 assay. Exponential growth phase MC-4 cells were seeded in a 96-well plate at a density of 2x10⁵ cells/ml/well, and were placed in an incubator at 37°C overnight to allow for attachment and recovery. Cells were pretreated with pPeOp (30, 60 or 90 μ g/ml), 90 μ g/ml PVP or 100 μ g/ml 5-FU (each drug was dissolved in RPMI-1640 medium), with 6 wells for each treatment and a total volume of 200 μ l/well. Following incubation at 37°C for 24 h in a humidified atmosphere containing 5% CO₂, 20 μ l CCK-8 reagent was

added to each well, prior to reincubating for 2 h. The optical density of each well was measured at 490 nm using a multimode plate reader, with the viability of cells from three independent biological replicates calculated for each experiment.

Cell cycle analysis. Cells were quantified using a Cell Cycle and Apoptosis Analysis kit (cat. no. C1052; Beyotime Institute of Biotechnology), according to the manufacturer's protocol. MC-4 cells were pretreated with pPeOp (30, 60 or 90 μ g/ml), 90 μ g/ml PVP or 100 μ g/ml 5-FU prior to culturing at 37°C for 24 h in a humidified atmosphere containing 5% CO₂. The cells were subsequently washed with phosphate-buffered saline (PBS) three times and fixed with 70% ice-cold ethanol at 4°C overnight. The cells were centrifuged, washed with PBS, treated with 20 μ l RNase A in a water bath at 37°C for 30 min, and placed in the dark at 4°C for 30 min with 300-500 μ l propidium iodide solution. The cell cycle distribution of the cells was determined using a Beckman FC500 flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) with red fluorescent light at a wavelength of 488 nm.

Wound healing assay. MC-4 cells at a density of 2x10⁵ cells/ml were seeded into 6-well plates. The cells were cultured at 37°C in an atmosphere containing 5% CO₂ for ~24 h, at which time they were ~80% confluent. Cells layers were scratched with a 200 μ l pipette tip. The cells were treated with 30, 60 or 90 μ g/ml pPeOp, 90 μ g/ml PVP, or 100 μ g/ml 5-FU and cultured at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h to allow for migration into the cell-free area. Images of the cells were captured with a light microscope (magnification, x40) 24 h following treatment.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA from the MC-4 cells treated with pPeOp (30, 60 or 90 μ g/ml), 90 μ g/ml PVP or 100 μ g/ml 5-FU was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. cDNA was synthesized using a Maxima First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The thermocycling conditions were as follows: 42°C for 60 min for the reverse transcription reaction;

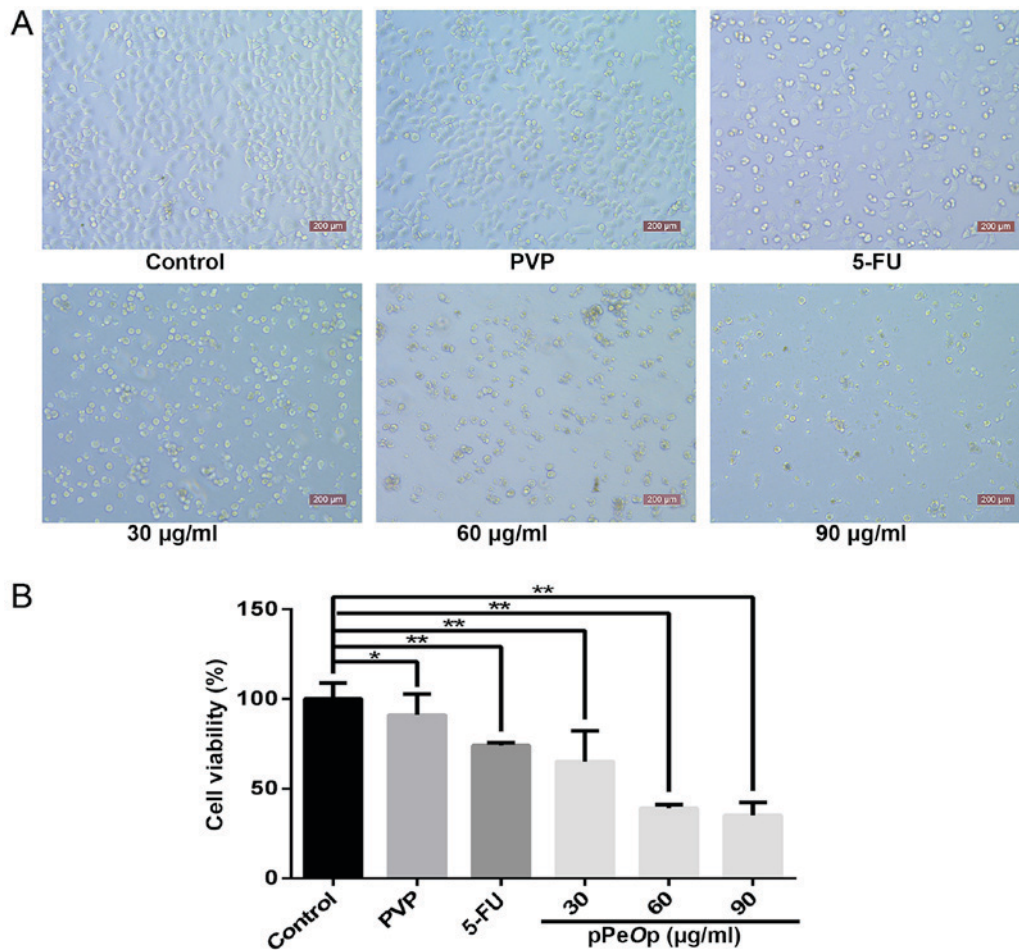


Figure 1. pPeOp inhibits MC-4 cell proliferation. (A) MC-4 cells were treated with pPeOp (30, 60 or 90 µg/ml), 90 µg/ml PVP (negative control) or 100 µg/ml 5-FU (positive control) for 24 h, then visualized using light microscopy. In addition, untreated cells were used as the control group. (B) Cell viability rates were analyzed using a cell counting kit-8 assay following treatment. *P<0.05, **P<0.01. pPeOp, purified *Omphalia lapidescens* protein; PVP, polyvinylpyrrolidone; 5-FU, 5-fluorouracil.

70°C for 5 min to terminate the reaction; and finally immediate cooling on ice. The cDNA reaction solution was used as a template for subsequent qPCR. Primers used for qPCR are presented in Table I and were purchased from Sangon Biotech (Shanghai, China). qPCR was performed using a MasterCycler RealPlex⁴ Real-Time PCR instrument (Eppendorf, Hamburg, Germany). β -actin was used as an internal normalization control. The PCR amplification conditions using a two-step method were as follows: 1 cycle at 95°C for 2 min (pre-denaturing); 40 cycles of 95°C for 15 sec (denaturing); and 62.9°C (β -actin), 57.8°C (MMP2, MMP9, cyclin A, cyclin B, cyclin D1) or 61°C (CDK1, 2 and 4) for 1 min (annealing). At the end of the PCR cycle, a dissociation curve was created to confirm amplification of a single product. The results are represented as the fold change in gene expression relative to that of β -actin ($2^{-\Delta\Delta Cq}$) (15).

Western blotting. MC-4 cells were collected following treatment with pPeOp (30, 60 or 90 µg/ml), 90 µg/ml PVP or 100 µg/ml 5-FU, subsequently lysed with radioimmunoprecipitation lysis buffer (Beyotime Institute of Biotechnology) for 30 min on ice. The lysates were separated by centrifugation at 12,000 x g for 15 min at 4°C. The total protein concentration in the supernatants was determined using a bicinchoninic

acid assay kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China), according to the manufacturer's protocol. SDS-PAGE was performed using 10% gels with ~20 µg protein per lane. Proteins were subsequently transferred onto polyvinylidene fluoride membranes, which were blocked with 5% dried skimmed milk in Tris-buffered saline containing 1% Tween-20 (TBST) and incubated with primary antibodies (described above) overnight at 4°C. Membranes were washed three times with TBST and incubated with HRP-conjugated goat anti-rabbit IgG at room temperature for 2 h, followed by washing three times with TBST. Images of the blots were captured on film in a darkroom using BeyoECL Plus Substrate kit (Beyotime Institute of Biotechnology) and were scanned and quantified using the Quantity One 1-D image analysis software (version 4.6.2; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. All experiments were conducted in triplicate. Results are presented as the mean \pm standard error of the mean. Statistical analyses were performed using SPSS software for Windows (version 16.0; SPSS, Inc., Chicago, IL, USA). Statistical differences were assessed using the Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

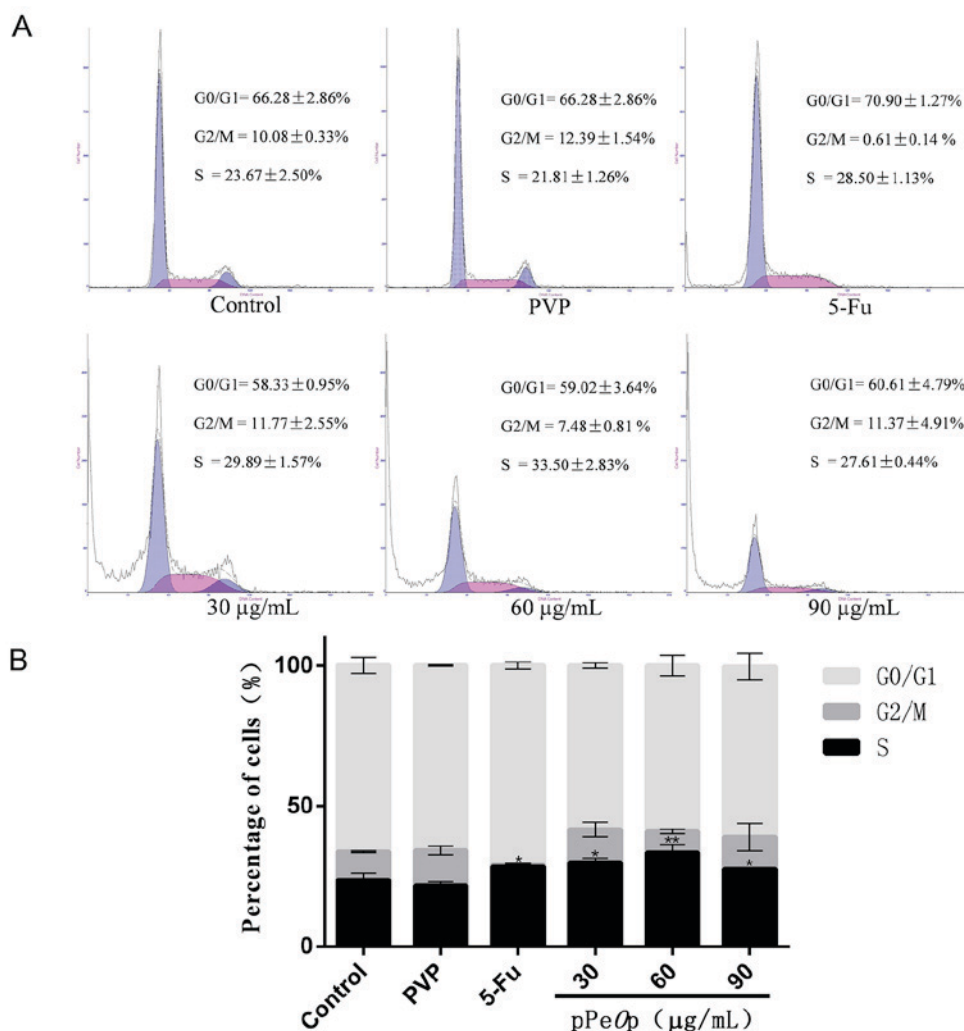


Figure 2. pPeOp arrests MC-4 cells in the S phase of the cell cycle. (A) MC-4 cells were treated with pPeOp (30, 60 or 90 $\mu\text{g/ml}$), 90 $\mu\text{g/ml}$ PVP (negative control) or 100 $\mu\text{g/ml}$ 5-FU (positive control) for 24 h, and analyzed using flow cytometry. In addition, untreated cells were used as the control group. (B) Summary graph of the cell cycle distribution of MC-4 cells following treatment. Results are presented as the mean \pm standard deviation of ≥ 3 independent experiments performed in triplicate. * $P < 0.05$, ** $P < 0.01$ vs. the untreated control group. pPeOp, purified *Omphalia lapidescens* protein; PVP, polyvinylpyrrolidone; 5-FU, 5-fluorouracil.

Results

pPeOp inhibits MC-4 cell proliferation. As presented in Fig. 1A, cell proliferation was decreased in MC-4 cells 24 h following treatment with 30, 60 or 90 $\mu\text{g/ml}$ pPeOp, and 100 $\mu\text{g/ml}$ 5-FU (positive control) compared with the untreated control group and 90 $\mu\text{g/ml}$ PVP-treated cells (negative control). This decrease was significant for all concentrations of pPeOp compared with the untreated control group ($P < 0.01$; Fig. 1B). As presented in Fig. 1B, the relative cell viability of each group was as follows: 30 $\mu\text{g/ml}$ pPeOp, $64.97 \pm 0.172\%$; 60 $\mu\text{g/ml}$, $39.42 \pm 0.021\%$; 90 $\mu\text{g/ml}$, $35.23 \pm 0.074\%$; PVP-treated negative control, $91.00 \pm 0.017\%$; and 5-FU-treated positive control, $73.81 \pm 0.118\%$. Compared with the 5-FU positive control group, an increase in pPeOp concentration led to increased inhibition of proliferation in a dose-dependent manner.

pPeOp arrests the MC-4 cell cycle in S phase. Treatment of MC-4 cells with various concentrations of pPeOp led

to alterations in cell cycle distribution following 24 h, as presented in Fig. 2. Compared with the untreated control, following treatment with pPeOp there was a significantly increased proportion of cells in S phase ($P < 0.05$), but a decreased proportion of cells in G_0/G_1 phase (Fig. 2B). pPeOp at a concentration of 30 and 90 $\mu\text{g/ml}$ also increased the proportion of cells in G_2/M phase compared with the untreated control, whereas 60 $\mu\text{g/ml}$ pPeOp decreased the proportion of cells in G_2/M phase (Fig. 2B). In addition, compared with the 5-FU-treated group, the proportion of cells in S phase in the 30 and 60 $\mu\text{g/ml}$ pPeOp-treated groups increased; however, cells treated with 90 $\mu\text{g/ml}$ pPeOp exhibited a decreased proportion of cells in S phase. These results indicate that in S phase cell cycle arrest is induced by pPeOp.

pPeOp decreases cell migration. A wound healing assay was used to assess the role of pPeOp in the migration of MC-4 cells. Microscopic analysis (Fig. 3) demonstrated that pPeOp decreased the migratory rate of MC-4 cells in a concentration-dependent manner, compared with the untreated control

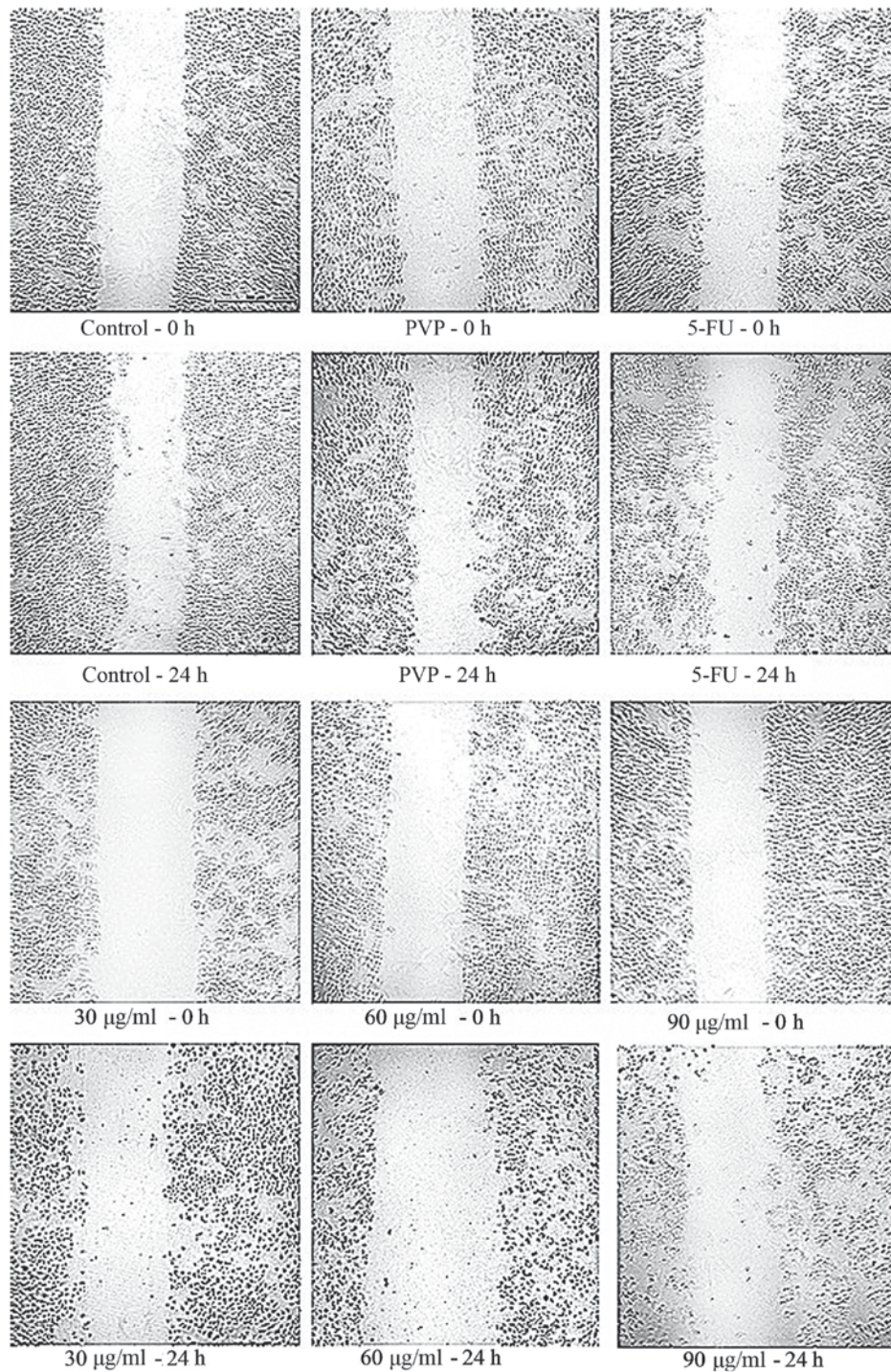


Figure 3. Effect of pPeOp on cell migration. Representative microscopic images of the wound healing assay demonstrating that in the untreated control and PVP-treated (negative control) groups there was no marked inhibition of migration. By contrast, treatment with 30, 60 or 90 $\mu\text{g/ml}$ pPeOp inhibited this migration in a concentration-dependent manner. Positive control consisted of treatment with 100 $\mu\text{g/ml}$ 5-FU. Magnification, x40. pPeOp, purified *Omphalia lapidescens* protein; PVP, polyvinylpyrrolidone; 5-FU, 5-fluorouracil.

cells. These results indicate that pPeOp has a negative effect on MC-4 cell migration.

pPeOp affects the mRNA and protein expression of cell cycle- and migration-associated proteins in MC-4 cells. As presented in Fig. 4A, the mRNA expression levels of the cyclin B, cyclin D1, CDK1 and CDK2 were decreased in MC-4 cells treated with pPeOp in a concentration-dependent manner compared with the untreated control group. However,

the mRNA expression levels of cyclin A and CDK4 mRNA were increased compared with the untreated control in a concentration-dependent manner (Fig. 4A). In addition, the mRNA expression levels of cell migration-associated MMP2 and MMP9 in MC-4 cells were decreased significantly at all concentrations of pPeOp used ($P < 0.001$; Fig. 4B). Furthermore, the expression of the cycle arrest-associated proteins cyclin D1, CDK4, cyclin B, CDK1, cyclin A and CDK2, and migration-associated proteins MMP-2 and MMP-9, were

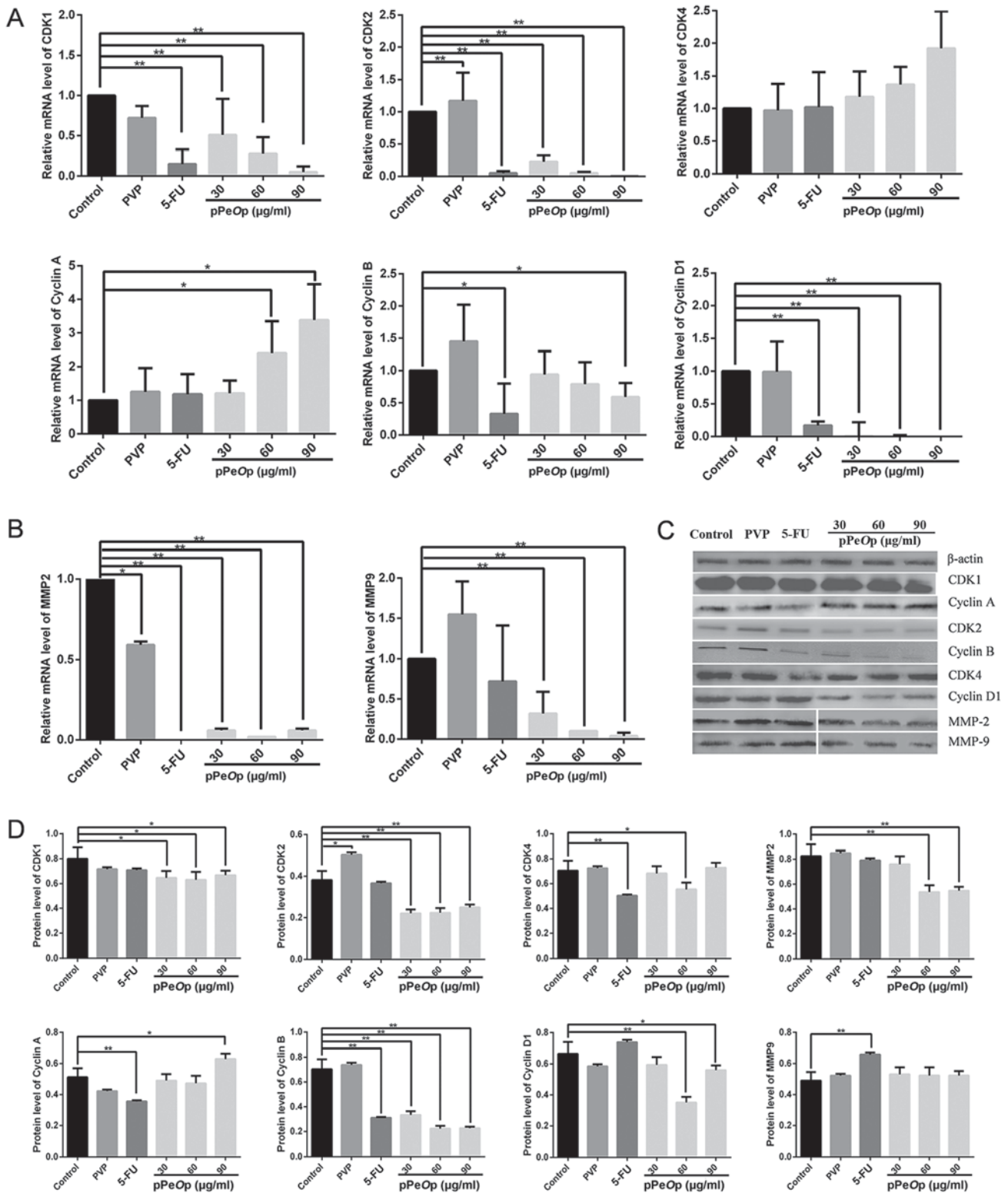


Figure 4. pPeOp affects the expression of cell cycle- and migration-associated genes/proteins in MC-4 cells. Cells were treated with pPeOp (30, 60 or 90 $\mu\text{g/ml}$), 90 $\mu\text{g/ml}$ PVP (negative control) or 100 $\mu\text{g/ml}$ 5-FU (positive control) for 24 h. In addition, untreated cells were used as the control group. Reverse transcription-quantitative polymerase chain reaction analysis was used to assess cell cycle-associated gene expression levels. All data were normalized to the untreated control. (A) Effects of pPeOp on the mRNA expression levels of the cell cycle-associated genes CDK1, CDK2, CDK4, cyclin A, cyclin B and cyclin D1. (B) Effects of pPeOp on the mRNA expression levels of the migration-associated genes MMP-2 and MMP-9. (C) Western blotting was conducted to examine the effects of pPeOp on the expression of cell cycle- and migration-associated proteins, with β -actin used as a loading control. (D) Quantification of protein expression levels of cell cycle- and migration-associated proteins normalized to β -actin. * $P < 0.05$, ** $P < 0.01$. pPeOp, purified *Omphalia lapidescens* protein; PVP, polyvinylpyrrolidone; 5-FU, 5-fluorouracil; CDK, cyclin-dependent kinase; MMP, matrix metalloproteinase.

analyzed by western blotting, with similar results to their corresponding mRNA expression (Fig. 4C). Quantification of

band density relative to that of β -actin demonstrated statistically significant differences in protein expression between

the untreated control cells and cells treated with pPeOp, as presented in Fig. 4D.

Discussion

Migration, an important biological characteristic of cancer cells, is observed in gastric cancer (16,17). The decreased survival rate of patients with gastric cancer may be attributed to the migration of gastric cancer cells into the lymph nodes and peritoneum (18,19). MMP-2 and MMP-9 are the matrix metalloproteinases primarily involved in degradation of type IV collagen in the basement membrane, and are associated with malignant tumor infiltration and migration (20-22). MMPs are important regulatory molecules in tumor migration, and exhibit extensive and increased expression in various types of human malignant tumor (23). In tumorigenesis MMPs regulate the degradation of the ECM, regulate tumor angiogenesis, alter the function of cell adhesion molecules and mediate the proliferation of tumor cells (24). MMP-2 and MMP-9 may be biomarkers of the migratory ability of cancer cells (25-27).

An uncontrolled cell cycle is closely associated with tumor occurrence, development and malignancy (28,29). Once cell proliferation or inappropriate cell death occurs, it typically results in a tumor, and it has been demonstrated that the regulation of the cell cycle is an important mechanism in tumorigenesis. Arrest of the various phases of the cell cycle may be used to inhibit cell proliferation and induce differentiation or apoptosis (30,31). Cancer is a disease that causes disorders in the cell cycle and uncontrolled proliferation due to the combined effects of hereditary and environmental factors (32). Therefore, the exploration of the fundamental mode of tumor cell cycle regulation, examining the important issue of targeted drug therapy, has become central to research in the field of tumor-associated diseases.

The aim of studying the effects of the protein pPeOp from *O. lapidescens* Schroet on the migration and cell cycle distribution of the human gastric cancer cell line MC-4 was to elucidate the underlying molecular mechanisms through which pPeOp functions. pPeOp was demonstrated to significantly inhibit the proliferation of MC-4 cells; the proliferation of MC-4 cells was arrested in the S phase and led to abnormal distribution of G₁ and G₂ phase cells compared with the conventional chemotherapy drug 5-FU. Results from RT-qPCR analysis of the mRNA expression levels of cell cycle-associated genes and from the cell cycle analysis identified that pPeOp induced upregulation of cyclin A and CDK4, which arrested cells in S phase. Previous studies have suggested that cyclins and CDKs are essential regulatory proteins in the cell cycle, and arrest of the cell cycle at the G₀/G₁ or G₂/M phases is one of the mechanisms caused by anticancer therapies (33-35). The results of the present study demonstrated that pPeOp is able to cause an abnormal G₀/G₁ and G₂/M phase distribution of cells via downregulating the expression of cyclin D1, cyclin B, CDK1, and CDK2 genes in a concentration-dependent manner. In order to further clarify the role of pPeOp, the alteration in cell cycle-associated protein expression was examined, which revealed similar results to RT-qPCR analysis. Expression of cyclin B protein was notably downregulated and no marked expression was identified in the pPeOp-treated cells, indicating

that pPeOp induces MC-4 cell apoptosis, which makes detection of the already low level of the corresponding expression of cyclin D1 difficult. Furthermore, the results of the wound healing assay demonstrated that pPeOp decreased the migratory rate of MC-4 cells in a concentration-dependent manner. In addition, pPeOp had a marked lethal effect on MC-4 cells, which is consistent with Chen *et al* (14). However, the effect of pPeOp on cell migration was not investigated by Chen *et al*. In the present study, pPeOp induced apoptosis in the majority of the cells and induced cell migration. Additionally, the secretion of MMP-2 and MMP-9 decreased as shown by western blotting results. Cell migration was inhibited by the expression of MMP-2 and MMP-9. Concomitant with an increase in the concentration of pPeOp, the expression levels of MMP-2 and MMP-9 protein were decreased.

The downregulation of cyclin D1, cyclin B, CDK1 and CDK2, and upregulation of cyclin A and CDK4 by pPeOp arrested MC-4 cells in the S phase of the cell cycle and led to an abnormal distribution of G₀/G₁ and G₂/M phase cells. Furthermore, by downregulating MMP-2 and MMP-9 expression, pPeOp inhibited the migration of MC-4 cells. These results indicate that pPeOp serves a role in cell cycle arrest and the inhibition of migration of MC-4 gastric tumor cells. The identification and determination of the expression of other proteins that may be involved in the underlying molecular mechanism of action of pPeOp is warranted by further study.

Acknowledgements

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