Abstract. Matrix metalloproteinase 9 (MMP-9) is upregulated in various types of malignancy, including human ovarian carcinomas. It promotes invasion, metastasis, growth and the survival of malignant cells. However, relatively little is known about the role of MMP-9 in epithelial ovarian carcinoma. Therefore, the aim of the present study was to determine the effects of targeting this molecule on ovarian carcinoma progression. A plasmid, psi-MMP-9, carrying a short hairpin RNA against MMP-9 gene expression was constructed and transfected into the human ovarian cancer cell line SKOV3 using a human U6 promoter-driven DNA template approach to determine the effect of MMP-9 gene RNA interference (RNAi) on the proliferation, apoptosis, migration, invasion and tumorigenicity of the human ovarian carcinoma cells. The results demonstrated that siRNA-mediated knockdown of MMP-9 in the human ovarian cancer cell line SKOV3 inhibited cell proliferation, migration and invasion in vitro. The results also demonstrated that downregulation of MMP-9 led to cell apoptosis in SKOV3 cells, inhibited the expression of anti-apoptotic molecules, including B cell lymphoma-2, survivin and X-linked inhibitor of apoptosis protein, and enhanced the activity of caspase-3 and caspase-8. In addition, knockdown of MMP-9 inhibited tumorigenicity in nude mice. Taken together, MMP-9 gene RNAi in ovarian carcinoma cells inhibited proliferation, migration and invasion, induced cell apoptosis in vitro and suppressed tumor growth in nude mice. These results suggest that MMP-9 is an ovarian cancer-associated gene and is a potential target for therapeutic anti-cancer drugs.

Introduction

Ovarian epithelial carcinoma, which accounts for 90% of cases of ovarian cancer, continues to be the leading cause of mortality and has the highest mortality rate among gynecological malignancies worldwide (1). Despite the improved combination of surgery, radiation and chemotherapy, the 5-year survival rates of ovarian cancer patients remain poor due to the difficulty in removing the highly invasive ovarian tumor and the short- and long-term adverse effects of conventional post-surgical adjuvant therapies (2,3). The development of new therapeutics or drugs to combat this disease is therefore essential to improve the survival rate of patients with ovarian epithelial carcinoma.

Tumor cells acquire invasive and metastatic characteristics mainly due to their ability to produce and activate proteolytic enzymes, including serine, metallo- and cysteine proteinases, which are able to degrade extracellular matrix (ECM) components and break down natural barriers, thereby aiding tumor invasion and metastasis (4). Matrix metalloproteinases (MMPs) are an important proteolytic enzyme family with zinc in the activated region. It has been reported that MMPs are able to degrade ECM and basal membrane components (5,6) and are important in tissue repair, tumor invasion and metastasis (7). The generation and analysis of transgenic and knockout mice for MMPs and tissue inhibitors of MMPs have revealed that MMPs are also important in the process of carcinogenesis (8). MMP-9, a principal member of the MMP family, has been found to be upregulated in various types of cancer, including ovarian cancer (9-11). Several studies have demonstrated that MMP-9 stimulates tumor cell growth, migration, invasion and metastasis and regulates tumor angiogenesis by releasing vascular endothelial growth factor (VEGF)-A from an extracellular reservoir (12-14). A previous study demonstrated that downregulation of MMP-9 by RNA interference (RNAi) inhibited cancer cell invasion and metastasis (15). Taken together, these studies suggest that MMP-9 is important in tumorigenesis and metastasis.

However, relatively little is known about the role of MMP-9 in ovarian epithelial carcinoma cells. Thus, to improve understanding of the role of MMP-9 in ovarian epithelial carcinoma.
Materials and methods

Construction of plasmids. To inhibit the expression of MMP-9, a specific shRNA targeting the MMP-9 transcript was designed using siRNA Target Designer software, version 1.5 (Promega, Madison, WI, USA). The synthesized oligonucleotides, which contain a specific target sequence, a loop, the reverse complement of the target sequence, a stop codon for the U6 promoter and two sticky ends were annealed and ligated into the BamHI and HindIII sites of the linearized pSilencer™ 2.1-U6 neo shRNA expression vector (Ambion, Austin, TX, USA) for each target site. The target sequence in the oligonucleotide for suppressing MMP-9 was: 5'-GACTACGATAAGGACGGCAAA-3' (sense) and the non-specific siRNA sequence, which does not target any gene product, was: 5'-AATCTTCCGAACGTGTACCTG-3' (sense). The plasmid encoding MMP-9 siRNA was referred to as psi-MMP-9 and the plasmid encoding non-specific siRNA, which was used as a negative control, was referred to as the psi-NC vector.

Cell culture and transfection. Cells from the human ovarian cancer cell line SKOV3 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in modified RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA). Cells were maintained in a humidified 37°C incubator with 5% CO2. The medium was renewed every 3 days. Transfection was performed using Lipofectamine 2000 (Ambion) according to the manufacturer's instructions. The ratio of the plasmids and transfection reagent was 1 mg:2 ml. The nonspecific siRNA transfected cells (pSilencer 2.1-NC) and the untransfected cells were used as controls. SKOV3 cells stably expressing shRNA were selected with medium containing 800 mg/ml G418 (Sigma-Aldrich) after 48 h. The stably transfected cells were termed SKOV3-MMP-9, SKOV3-NC and SKOV3-neo, respectively.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR for MMP-9 transcripts in SKOV3 cells was performed using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA using a Primerscript™ RT reagent kit according to the manufacturer's instructions (Takara Bio, Inc., Shiga, Japan). RT-qPCR was conducted using the SYBR Green I fluorescent dye method and a Rotor Gene 3000 real-time PCR apparatus (Applied Biosystems, Foster City, CA, USA). MMP-9 gene-specific amplification was confirmed by qPCR with the following specific primers: MMP-9, sense 5'-ACAGCCAACATATGACCG-3' and antisense 5'-TGCCACCAGGAACAGG-3' and subjected to melting curve analysis. β-actin was used as an internal control for standardization. The primer sequences used were as follows: β-actin, forward 5'-GATCATTGCTCCTCCTGAGC-3' and reverse 5'-ACTCCTGTTGCTGATCCAC-3'. The PCR conditions were as follows: Pre-denaturing at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 20 sec and annealing/extension at 51°C for 30 sec. All RT-qPCR tests were performed in triplicate and were performed after the third day of plasmid transfection. The data were analyzed using the comparative Ct method.

Western blot analysis. SKOV3 cells were collected and lysed by incubation on ice for 30 min in lysis buffer [25 mM Tris-HCl (pH 8.0), 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 125 mM NaCl] containing the Complete Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Cell lysates were harvested by centrifugation at 10,000 x g for 15 min and protein concentrations were determined using Bradford reagent (Sigma-Aldrich). Equal quantities of protein (15 µg/ lane) from the cell lysates were separated on an 8-15% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The membrane was incubated for 2 h in phosphate-buffered saline (PBS; Sigma-Aldrich) plus 0.1% Tween-20 and 5% non-fat skim milk to block nonspecific binding. Subsequently, the membranes were incubated overnight at 4°C with the following mouse monoclonal anti-human primary antibodies: MMP-9 (1:1,000; sc-21733; Santa Cruz Biotechnology, Inc.), survivin (1:1,000; sc-8808; Santa Cruz Biotechnology, Inc.), B cell lymphoma-2 (Bcl-2; 1:3,000; sc-70418; Santa Cruz Biotechnology, Inc.), XIAP; 1:2,000; sc-55551; Santa Cruz Biotechnology, Inc.), VEGF (1:3,000; sc-57496; Santa Cruz Biotechnology, Inc.) and β-actin (1:5,000; cat. no. 1025; Cell Signaling Technology, Inc., Beverley, MA, USA). Following washing, proteins were visualized using an ECL detection kit with the rabbit anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (1:8,000; sc-358923; Santa Cruz Biotechnology, Inc.). All the assays were performed after the third day of plasmid psi-MMP-9 and psi-NC transfection into SKOV3 cells.

Proliferation assays. To measure the effect of RNAi-mediated MMP-9 silencing on cell proliferation, an MTT assay was conducted. Cells without any treatment and cells treated with plasmid psi-MMP-9 and psi-NC were seeded in 96-well plates and kept at 37°C for 48 h. The cells were then washed with PBS and incubated in 50 ml of 0.5 mg/ml MTT in culture medium at 37°C for 4 h. Following the addition of 100 µl of isopropanol, the absorbance was read at 595 nm using an ELISA plate reader (SpectraMax M5; Molecular Devices Corp., Sunnyvale, CA, USA). The mean proliferation of cells without any treatment was expressed as 100%. Each assay was performed in triplicate.

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay. To measure the effect of RNAi-mediated MMP-9 on cell apoptosis, a TUNEL assay was performed. In brief, after SKOV3 cells were treated with the indicated plasmid for 24 h, cellular DNA fragmentation was measured using the ApoTag Red in situ Apoptosis detection kit (Chemicon International, Temecula, CA, USA) according to the manufacturer's instructions. To quantify the
apoptotic cells, TUNEL-positive cells were counted using confocal microscopy (FV1000-IX81; Olympus, Tokyo, Japan).

In addition, at the molecular level, anti-apoptotic molecules were detected, including survivin, XIAP and Bcl-2 protein expression by western blotting as an additional indicator of apoptosis.

**Caspase activity.** The activity of caspase-3 and caspase-8 were measured using caspase colorimetric protease assay kits (Millipore Corporation, Billerica, MA, USA) according to the manufacturer's instructions. In brief, SKOV3 cells were treated with the indicated plasmid for 24 h. Following treatment, cells were washed twice with ice-cold PBS (pH 7.2) and harvested by centrifugation at 700 x g for 5 min. The cell pellets were then lysed in 150 µl buffer provided in the kit (Millipore Corporation). An aliquot of lysates (80 µl) was incubated with 10 µl substrate of each caspase at 37°C for 2 h. Samples were analyzed at 405 nm using a microplate reader (Multiskan MK3; Thermo Fisher Scientific Inc., Waltham, MA, USA). The relative caspase activity of the control group was taken as 100%. Each assay was performed in triplicate.

**Wound healing assay.** To assess the effect of RNAi-mediated knockdown of MMP-9 on cell migration, a wound-healing assay was performed. In brief, 1x10⁵ SKOV3 cells were plated in 12-well plates in RPMI-1640 medium containing 10% FBS. After 24 h, a scratch was made through the confluent cell monolayer and then the cells were treated with the indicated plasmid in 3 ml of complete medium. After 48 h treatment, cells were stained with hematoxylin and eosin (H&E). The images were compared between the three treatments, including cells without any treatment, cells treated with psi-NC and cells transfected with psi-MMP-9. Cells invading the wound line were observed under an inverted phase-contrast microscope (CKX41; Olympus). Images were analyzed using ImageJ software (version, 1.44; National, Institutes of Health, Bethesda, MD, USA) and the formula for calculating the % of wound closure was used as described previously (16). All experiments were performed in triplicate.

**Matrigel invasion assay.** To determine the effect of RNAi-mediated MMP-9 on cell invasion, cellular invasion assays were performed using BioCoat Matrigel Invasion Chambers (Becton Dickinson, Bedford, MA, USA) with 8-mm pores in 6-well plates according to the manufacturer's instructions. In brief, 2x10⁵ SKOV3 cells in RPMI-1640 medium containing 0.5% FBS were added to the upper chamber containing 8 mm pore polycarbonate coated with 1 mg/ml Matrigel; the lower chamber was filled with RPMI-1640 media containing 10% FBS. The indicated plasmid was added to the upper chambers, respectively. Cells without any treatment were used as the control. After 24 h incubation, cells invading the bottom surface of the filter were fixed and stained with 0.1% crystal violet in 20% methanol. Invasiveness was determined by counting the penetrating cells under a CKX41 phase-contrast microscope and counted in >10 fields of view at x200 magnification. The invasion of cells without any treatment was determined as 100%.

**Measurement of VEGF level.** Protein levels of VEGF in the cell supernatant were determined using a Human VEGF ELISA kit (Yanyu, Shanghai, China) according to the manufacturer's instructions when SKOV3 cells were treated with the indicated plasmid for 24 h.

**Tumorigenicity in nude mice.** All animal experiments were performed in accordance with institutional guidelines, following a protocol approved by the Ethics Committees of the Disease Model Research Center, Jilin University (Changchun, China). In total, 30 female BALB/c nude mice aged 6-7 weeks were purchased from the Institute of Laboratory Animal Science, Jilin University (Changchun, China) and were maintained under specific pathogen-free conditions and provided with food and water at the Laboratory Animal Center of Jilin University. Stable cell clone SKOV3-MMP-9, SKOV3-NC and SKOV3-neo (5x10⁵/mouse) were subcutaneously injected into the left flank of mice, respectively. Tumor volume was continuously measured in a blinded manner by using calipers every 5 days until mice were sacrificed under anesthesia. Each tumor was excised and weighed when mice were sacrificed on day 21. The tumorigenicity inhibition rate was calculated using the following formula: Tumorigenicity inhibition rate = [(tumor weight (control) - tumor weight (shRNA) / tumor weight (control)) x 100%. Sections of each tumor tissue were wax embedded for H&E staining to investigate cell apoptosis in vivo by TUNEL. In addition, spleen tissue was collected and cultured for a splenocyte surveillance study as described previously (17). All animal experiments were performed in accordance with institutional guidelines.

**Statistical analysis.** For all assays, each experiment was repeated three times in triplicate (independent experiments). Average values are expressed as the mean ± standard deviation. Data were analyzed by one-way analysis of variance followed by a Tukey's post hoc test using the SPSS® statistical package, version 19.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism software version 5.01 (GraphPad Software, San Diego, CA, USA) for Windows®. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Silencing MMP-9 causes effective downregulation of MMP-9 expression.** To evaluate the silencing capacity of psi-MMP-9 in SKOV3 cells, RT-qPCR and western blot analysis were performed 72 h after treatment with the indicated plasmid. RT-qPCR results demonstrated that no significant inhibition of MMP-9 mRNA expression was observed in the control group and psi-NC group. Compared with the psi-NC group and control group, MMP-9 mRNA expression in the psi-MMP-9 group was significantly decreased (Fig. 1A; P<0.01). At the protein level, no significant inhibition in MMP-9 protein expression was identified in the psi-NC group and control group (P>0.05; Fig. 1B), while the band density markedly decreased in the psi-MMP-9 group compared with the psi-NC group and control group (P<0.05; Fig. 1B). These results demonstrated that silencing MMP-9 significantly decreased MMP-9 expression in the epithelial ovarian cancer cell line.

**Silencing MMP-9 inhibits cell proliferation and induces cell apoptosis.** To examine the effects of MMP-9 silencing on the cell proliferation of SKOV3 cells, MTT assays were performed...
Silencing MMP-9 inhibits cell proliferation and induces cell apoptosis in SKOV3 tumor cells. (A) MTT assay of SKOV3 cell viability. (B) Terminal deoxynucleotidyl transferase-mediated nick end labeling assay of cell apoptosis in SKOV3 cells. *P<0.05 vs. control.
measured by ELISA. It was found that silencing MMP-9 significantly decreased the level of VEGF compared with the control group and psi-NC group (P<0.05; Fig. 4C).

Silencing MMP-9 suppresses tumor growth in vivo. The in vivo therapeutic efficacy of downregulating MMP-9 by siRNA was assessed in female BALB/c mice bearing SKOV3 tumor cells. It was observed that the tumor weight of the psi-MMP-9 group was significantly lower than those of the control group and psi-NC group (P<0.05; Fig. 5A). In addition, the results also demonstrated that the tumor volume was significantly decreased in the psi-MMP-9 group compared with the control group and psi-NC group on different days (P<0.05; Fig. 5B).

To assess the efficacy of silencing MMP-9 in modulating splenocyte proliferation, an MTT assay was performed. As shown in Fig. 5C, the inhibitory rates of the psi-MMP-9 group were higher than those of the control group and psi-NC groups (P<0.05; Fig. 5C). In addition, tumor tissue cell apoptosis in vivo was also determined by TUNEL. The results demonstrated that the apoptotic ratio of the psi-MMP-9 group in vivo was significantly increased compared with the control group and psi-NC group (P<0.05; Fig. 5D). Taken together, these results demonstrated that silencing MMP-9 suppressed the tumor growth of ovarian epithelial carcinoma in vivo.

Discussion

Ovarian cancer is the seventh most common cancer in females and has the highest mortality rate among all types of gynecological malignancy worldwide (18). Although there have
been advances in diagnostic and therapeutic methodologies, the 5-year survival rate of ovarian cancer patients remains poor due to the development of drug resistance to cytotoxic chemotherapeutic agents and the high metastatic potential of cancer cells. Therefore, a more effective therapeutic modality is urgently required for inhibiting cancer cell metastasis of ovarian cancer.

Previous studies have focused on the functions of genes and on designing gene therapeutic technologies for various types of cancer (19,20). Double-stranded RNA-mediated interference is able to specifically silence specific genes and has been used as a powerful tool in genetic therapy for various types of carcinoma, including ovarian cancer by post-transcriptional silencing of essential genes for tumor progression, which has generated significant interest for the treatment of cancer (21‑24). For example, Lin et al found that the vector expressing three siRNAs targeting the cyclooxygenase-2 (COX-2) gene in tandem was able to silence the expression of COX-2 in ovarian cancer cells and COX-2 knockdown resulted in a decrease in cell proliferation and inhibition of the cell cycle in ovarian tumor cells (25). In the present study, eukaryotic expression plasmids (psi-MMP-9) capable of inhibiting MMP-9 expression were constructed and successfully transfected into the epithelial ovarian cancer cell line SKOV3. The mRNA and protein expression of MMP-9 was measured and the results demonstrated that the plasmid, psi-MMP-9, had an inhibitory effect on the mRNA and protein expression of MMP-9. These results indicated that vector-based MMP-9-specific shRNA could silence the expression of MMP-9 effectively in SKOV3 cells. The present study also assessed the effect of silencing MMP-9 on cell proliferation, apoptosis, migration and invasion of SKOV3 cells and found that downregulation of MMP-9 by specific shRNA could inhibit cell proliferation, migration and invasion and induce cell apoptosis in vitro. These results imply that silencing MMP-9 is an effective method for the treatment of ovarian cancer.

MMP-9 is one of the most important metalloproteases and is critical in regulating ECM dissolution, activating growth factors as well as initiating intracellular signaling leading to tumor progression and metastasis (26-29). Several studies have demonstrated that inhibiting the activities of MMP-9 by RNAi resulted in apoptosis in melanoma cells (15), malignant meningioma cells (27) and medulloblastoma cells (28). Knocking down the expression of MMP-9 significantly inhibited MMP-9 levels and altered downstream signaling molecules, thereby leading to transcriptional inhibition of anti-apoptotic molecules and directing the cells towards apoptosis (28-30). Kotipatruni et al reported that siRNA-mediated knockdown of MMP-9 led to cell apoptosis and that downregulation of MMP-9 inhibited the expression of anti-apoptotic molecules, including Bcl-2, Bcl-xL, survivin, XIAP and cIAPI, activated BID cleavage, enhanced the expression of Bak, translocated cytochrome C to the cytosol and increased the activity of caspase-3 and caspase-9 (30). Consistent with this study, the results from the present study demonstrated that downregulation of MMP-9 could induce cell apoptosis in SKOV3 cells, suppress the expression of anti-apoptotic molecules, including Bcl-2, survivin, XIAP as well as enhance the activity of caspase-3 and caspase-8. These studies and our results suggested that specifically targeting MMP-9 with a single
drug or in combination with other drugs or targeting gene expression could have implications for the treatment of cancer.

In conclusion, in the present study, the results demonstrated that silencing MMP-9 could significantly suppress the proliferation, migration and invasion of SKOV3 cells, induce cell apoptosis in vitro and inhibit tumor growth of ovarian cancer in vivo. Taken together, these results suggest that shRNA targeting MMP-9 appears to have therapeutic potential for the treatment of ovarian epithelial carcinoma.

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References