

# Effects and mechanism of RhoC downregulation in suppressing ovarian cancer stem cell proliferation, drug resistance, invasion and metastasis

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**Abstract.** Cancer stem cells are considered to be the root cause of tumor initiation, metastasis, recurrence and therapeutic resistance. Recent studies have reported that RhoC plays a critical role in regulating cancer stem cells; however, its function in ovarian cancer stem cells (OCSCs) remains unknown. The ovarian cancer cell line A2780, and the paclitaxel-resistant A2780 cell line (A2780-PTX) were obtained. A2780 cells were used to isolate and identify the highly invasive A2780-PM cells, and A2780-PTX cells were used to isolate and identify the highly drug-resistant and highly invasive A2780-PTX-PM cells by Transwell assay. MTT, Transwell and wound healing assays were used to compare the differences in cell proliferation, invasion and migration ability among the four cell lines. Immunofluorescence was used to detect the expression of stem cell markers CD117 and CD133. OCSCs were sorted by flow cytometry. Following si-RhoC transfection of the OCSCs, cell proliferation, drug resistance, invasion and migration ability and RhoC, CD117 and CD133 expression levels were assayed. RT-PCR was used to assess RhoC, CD117, CD133 and matrix metalloproteinase 9 (MMP9) mRNA expression levels. A2780-PM and A2780-PTX-PM cells exhibited higher cell proliferation, drug resistance, and invasion and migration ability than the A2780 and A2780-PTX cell lines. Furthermore, CD133 and CD117 expression levels were higher in the A2780-PM and A2780-PTX-PM cells than levels in the A2780 and A2780-PTX cells. Transfection of si-RhoC in OCSCs suppressed the proliferation, drug resistance, invasion, migration and CD117 and CD133

expression levels. Furthermore, the expression levels of RhoC, CD117, CD133, MDR1, and MMP9 mRNA were downregulated in the transfected population. Taken together, our results demonstrated that RhoC downregulation may inhibit the proliferation, drug resistance, invasion and migration of OCSCs, and RhoC may play an important role in the formation of OCSCs.

## Introduction

Epithelial ovarian cancer (referred to as ovarian cancer) is a common malignancy of the female reproductive system and has a 5-year survival rate of less than 44% (1). Recurrence and drug resistance are the main reasons for its poor prognosis (2). Cancer stem cells are a group of cells capable of self-renewal and unlimited proliferation, and they can also be drug-resistant and highly invasive (3,4). According to the theory of cancer stem cells, although tumor cells contain extremely few cancer stem cells, they are the root cause of tumor initiation, metastasis, recurrence and therapeutic resistance (5-9). Therefore, it is extremely important to discover new approaches to regulate ovarian cancer stem cells (OCSCs).

Ras homolog gene family member C (RhoC) is a small G protein and is one of the three members of the Rho subfamily of GTPases (10). Upregulation of RhoC is closely linked to the growth, metastasis, invasion and progression or adverse prognosis of various malignancies (11,12). More importantly, RhoC plays a critical role in regulating cancer stem cells, including head and neck squamous cell carcinoma and breast cancer (13,14). Our previous studies showed that RhoC over-expression in ovarian cancer may promote cancer invasion and metastasis, and may be an independent prognostic factor for ovarian cancer (15,16). A previous study reported that the RhoC homolog RhoA is closely linked to the Wnt pathway, which has been implicated in stem cell activity (17). Therefore, in the present study we aimed to explore the role of RhoC in the proliferation, cisplatin resistance and invasion ability of OCSCs.

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## Materials and methods

**Cell culture.** The OC cell lines A2780, A2780-PM and A2780-PTX were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. The cells were harvested by centrifugation, rinsed with phosphate-buffered saline (PBS), and were then used for extraction of total RNA.

**Cell selection.** The resistant ovarian cancer cell line A2780-PTX was cultured in the top chambers of Matrigel<sup>TM</sup>-coated Transwell<sup>®</sup> inserts (Beckon-Dickinson Biosciences) containing 1 ml of DMEM containing 10% FBS in the lower chamber. A2780-PTX cells that were highly invasive were able to pass through the membrane to the lower chamber. These cells have high resistance and high invasiveness, and were termed A2780-PTX-PM. Flow cytometry was used to determine the OCSCs from this population. Briefly, the cells were trypsinized to prepare a single cell suspension, adjusting the cell concentration to 5x10<sup>5</sup>/tube cells. Then, 2 ml of diluted CD117 antibody was added to each tube containing the cell suspension, the mixture was incubated at room temperature for 30 min, washed thrice with PBS, and centrifuged at 1,000 rpm for 5 min. The cells were then incubated with fluorescein isothiocyanate (FITC)-labeled secondary antibodies at room temperature for 30 min, washed thrice with PBS, centrifuged at 1,000 rpm for 5 min, and resuspended in 2 ml PBS, prior to detection of fluorescence intensity and cell sorting. The sorted cells were cultured after the completion of the relevant experiments.

**Proliferation assay.** Cell proliferation was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were directly seeded into 96-well plates (5x10<sup>3</sup> cells/well) and allowed to adhere. At different time points (0, 24, 48 and 72 h) after seeding, 20 µl of 5 mg/ml of MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to the cells, and the cells were incubated at 37°C for 4 h. The supernatants were removed, and 150 µl of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to each well. The absorbance of each well was measured at 490 nm.

**Assessment of wound healing.** Cells were seeded at 1x10<sup>6</sup> cells/well in 6-well culture plates and cultured to confluence. The cell monolayer was then scratched with a pipette tip (200 µl). Cells were washed thrice with PBS and cultured in an FBS-free medium. The cells were photographed at 0, 24 and 48 h, and the scratched areas were measured using ImageJ software. The wound healing rate was calculated using the following formula: Wound healing rate = (area of original wound - area of actual wound at different times)/area of original wound x 100%.

**Assessment of cell invasion.** First, 5x10<sup>4</sup> cells were resuspended in FBS-free DMEM and seeded into the top chambers of Matrigel<sup>TM</sup>-coated Transwell<sup>®</sup> inserts. The lower compartment of the chamber contained 10% FBS as

a chemoattractant. After incubation for 48 h at 37°C in a 5% CO<sub>2</sub> atmosphere, the cells on the upper surface of the membrane were wiped away. Cells on the lower surface of the membrane were washed with PBS, fixed in 4% methanol and stained with crystal violet dye to quantify the extent of invasion.

**Real-time reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was extracted from OC cell lines using TRIzol reagent (Takara Bio, Japan). Real-time RT-PCR was carried out with 2 µg of total RNA using AMV reverse transcriptase and random primers (Takara Bio). PCR primers were designed according to the sequences in GenBank. cDNA amplification was carried out with a SYBR<sup>TM</sup> Premix Ex Taq II kit (Takara), using glyceraldehyde 3-phosphate dehydrogenase (18s) as an internal control, according to the manufacturer's instructions. Briefly, cDNA amplification for each primer was carried out in a final volume of 20 µl containing 10 µl SYBR Premix Ex Taq (2X), 0.08 µl of each primer, 0.4 µl of ROX reference dye, and 1 µl of template cDNA (50 µg/µl). The PCR protocol was as follows: initial incubation at 95°C for 30 sec followed by 40 cycles of denaturation at 95°C for 5 sec and annealing at 60°C for 34 sec. The relative gene expression levels (amount of the target gene normalized to that of the endogenous control gene) were calculated using the comparative Ct method (2<sup>-ΔΔCt</sup>).

**Immunofluorescence.** For the immunofluorescence experiment, the cells were cultured on glass coverslips, fixed with PBS containing 4% formaldehyde for 10 min, and permeabilized with 0.2% Triton X-100 in PBS for 30 min at room temperature. After washing with PBS, the cells were incubated overnight at 4°C with antibodies to RhoC, CD117 and CD133 (1:50; Proteintech, Shanghai, China). Then, the cells were washed three times with PBS and treated with the secondary antibody for 2 h at room temperature in a dark and humidified chamber. After washing thrice with PBS, the immunostained cells were mounted in mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) for 5 min, and washed thrice with PBS. The cells were then visualized under a fluorescence microscope equipped with a camera.

**Statistical analyses.** Ranked data were analyzed using Spearman's rank correlation coefficient. The Mann-Whitney U test was used to differentiate between the mean values of different groups. A P-value of <0.05 was considered to indicate a statistically significant result. SPSS v.17.0 (IBM, Armonk, NY, USA) was employed to analyze data.

## Results

**Expression of stem cell markers and proliferation, migration and invasion ability of ovarian cancer cells.** A2780-PM and A2780-PTX-PM cells showed higher cell proliferation (Fig. 1A), drug resistance (Fig. 1B and C) and invasion and migration ability (Fig. 2) than these parameters in the A2780 and A2780-PTX cell lines. Additionally, CD133 and CD117 expression levels were higher in the A2780-PM and A2780-PTX-PM cells than these levels in the A2780 and A2780-PTX cells (Fig. 3A and B).

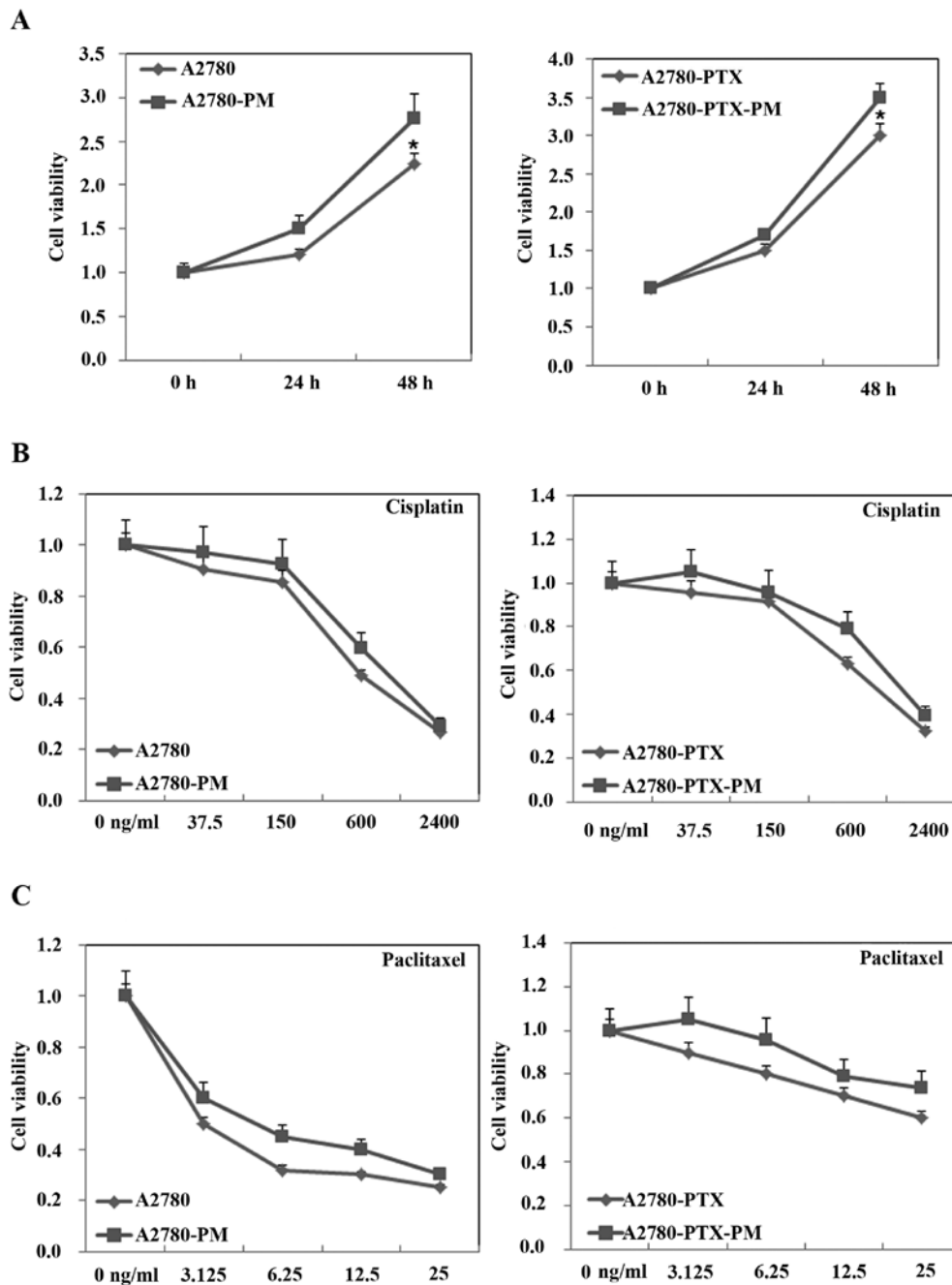


Figure 1. Cell proliferation and drug resistance in four ovarian cancer cell lines. A2780-PM and A2780-PTX-PM cells showed higher (A) cell proliferation, (B) cisplatin and (C) paclitaxel resistance when compared to A2780 and A2780-PTX cell lines. Results are representative of three separate experiments; data are expressed as the mean  $\pm$  standard deviation; \* $P < 0.05$ .

**Selection of OCSCs.** Cells were sorted through flow cytometric analysis to select ovarian cancer cells with stem cell-like characteristics (Fig. 4).

**Effects of si-RhoC on OCSCs.** OCSCs showed induced cell proliferation, drug resistance and migration, and invasion ability. si-RhoC transfection in OCSCs reduced cell proliferation (Fig. 5A), drug resistance (Fig. 5B and C), and migration and invasion ability (Fig. 6). Results of the immunofluorescence analysis revealed that the expression of RhoC, CD133 and CD117 were also induced in the OCSCs,

while transfection with si-RhoC reduced the expression of RhoC, CD133 and CD117 (Fig. 3C-E).

**Expression of the mRNA of phenotype-related molecules in ovarian cancer cells.** We assessed the mRNA expression levels of RhoC, CD133, CD117, MDR1 and MMP9 in A2780-PTX-PM cells, OCSCs and si-RhoC-transfected OCSCs. The results revealed that the expression levels of all of these markers were higher in OCSCs than levels in the A2780-PTX-PM cells, but the expression levels were lower in the si-RhoC-transfected OCSCs than levels in the OCSCs (Fig. 7).

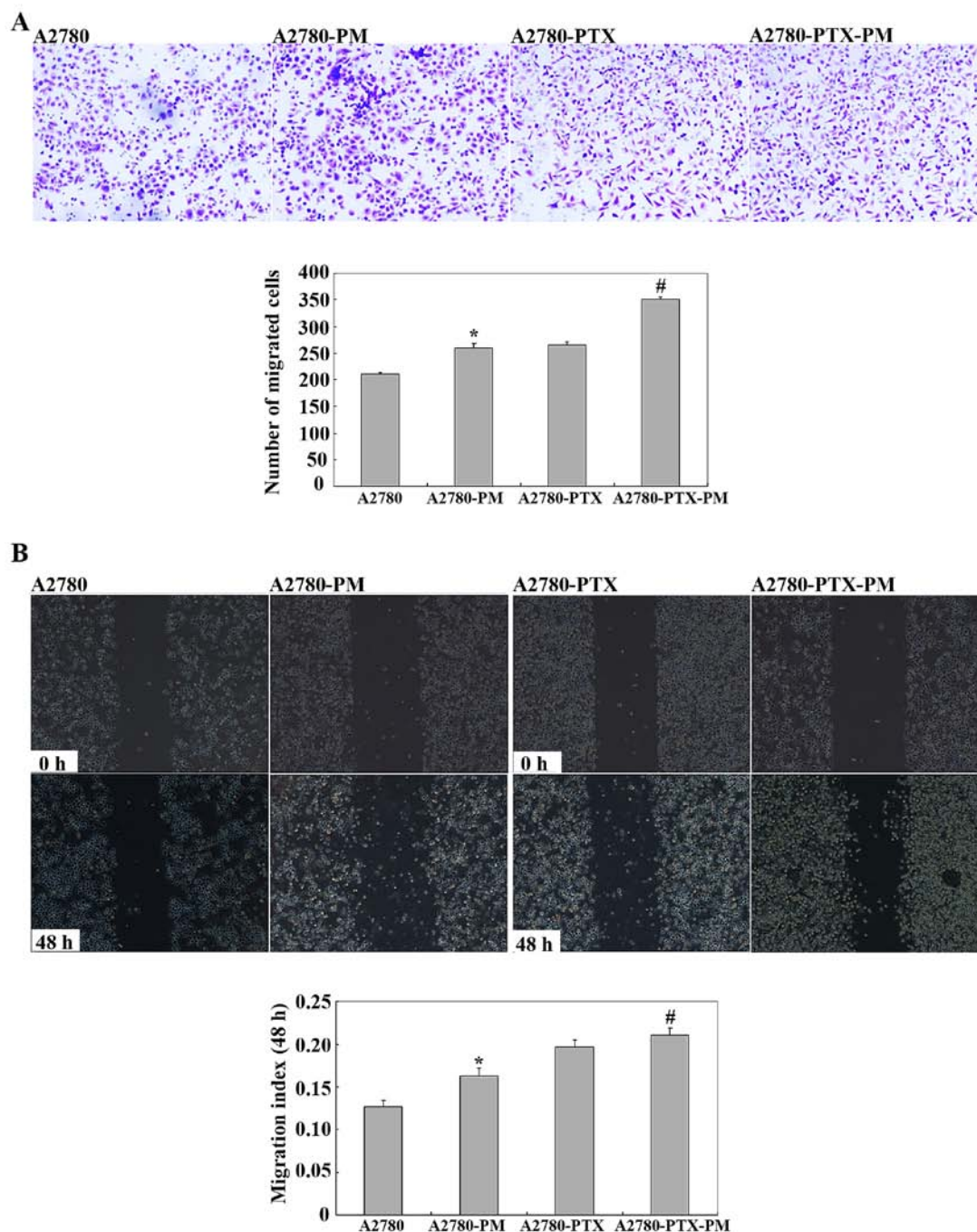


Figure 2. Cell migration and invasion ability of ovarian cancer cells. A2780-PM and A2780-PTX-PM cells showed higher (A) invasion and (B) migration ability when compared to A2780 and A2780-PTX cell lines. Results are representative of three separate experiments; data are expressed as the mean  $\pm$  standard deviation. \*P<0.05. #P<0.05.

## Discussion

Given that stem cells have the ability of self-renewal, unlimited proliferation, drug resistance, high invasion and express high levels of CD117 and CD133 (18,19), we identified highly invasive populations of A2780 and A2780-PTX cells through Transwell assays. We screened the drug-resistant ovarian cancer cell line A2780-PTX for a highly drug-resistant ovarian cancer cell population with high invasiveness, and designated this population A2780-PTX-PM. Flow cytometric analysis was then applied to sort ovarian cancer cells bearing stem

cell-like characteristics, ovarian cancer stem cells (OCSCs) expressing high levels of CD117 and CD133.

*RhoC* is known to be involved in the entire process of tumor progression and resistance in a variety of tumors. The *RhoC* protein regulates cell growth and proliferation mainly by affecting the cell cycle. Xie *et al* studied hepatoma cells *in vitro* and reported that *RhoC* expression regulated growth and apoptosis, and si-*RhoC*-transfected cells exhibited reduced cell proliferation and cell growth and a significantly reduced S-G2/M phase cell population (20). *RhoC* can affect cells in a variety of ways by altering the morphology and cell polarity,

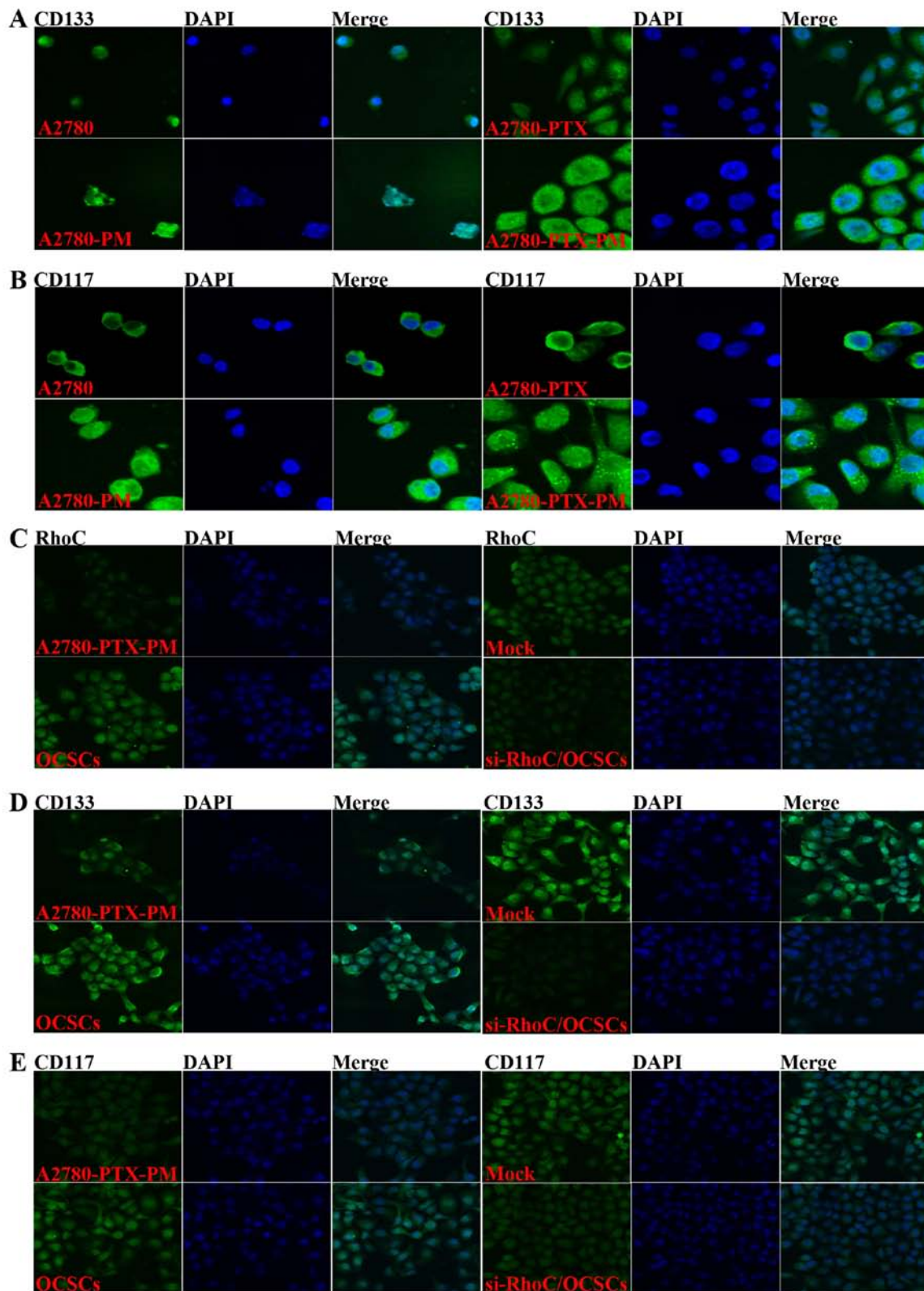


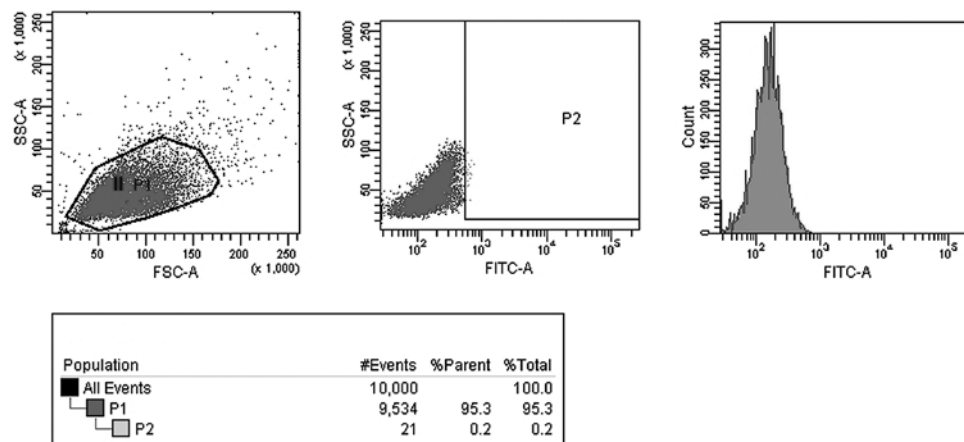
Figure 3. Expression of stem cell marker CD133 in ovarian cancer cells. (A) CD133 expression levels were higher in A2780-PM and A2780-PTX-PM cells than levels in A2780 and A2780-PTX cells. (B) CD117 expression levels were higher in A2780-PM and A2780-PTX-PM cells than levels in A2780 and A2780-PTX cells. (C) RhoC expression level was higher in OCSCs than that in A2780-PTX-PM cells, while si-RhoC transfection reduced RhoC expression. (D) CD133 expression level was higher in OCSCs than that in A2780-PTX-PM cells, while si-RhoC transfection reduced CD117 expression. (E) CD117 expression level was higher in OCSCs than that in A2780-PTX-PM cells, while si-RhoC transfection reduced CD117 expression. OCSCs, ovarian cancer stem cells.

forming blood vessels, and is involved in tumor invasion and metastasis. Studies have shown that RhoGEF TEM4 (an

activator of Rho family GTPases) regulates the cell migration of endothelial cells. TEM4 regulates cellular migration by



## Mock



## CD117

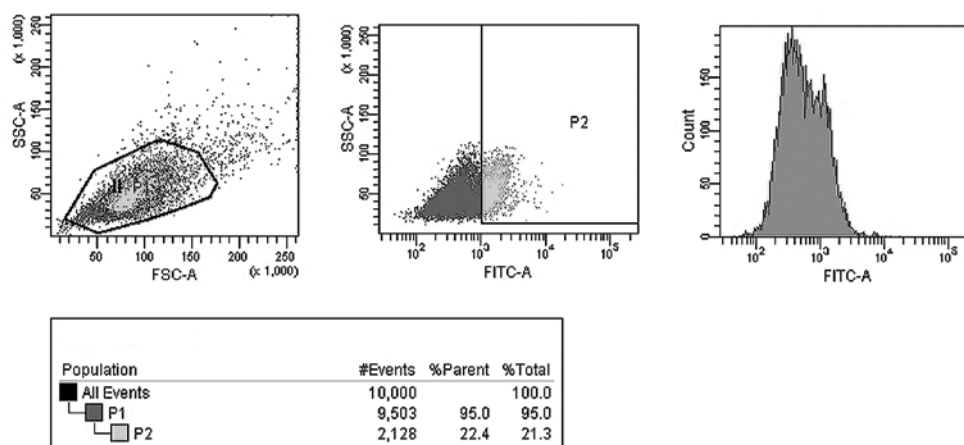


Figure 4. Selection of ovarian cancer stem cells (OCSCs). Cells were sorted by flow cytometric analysis to select ovarian cancer cells with stem cell-like characteristics.

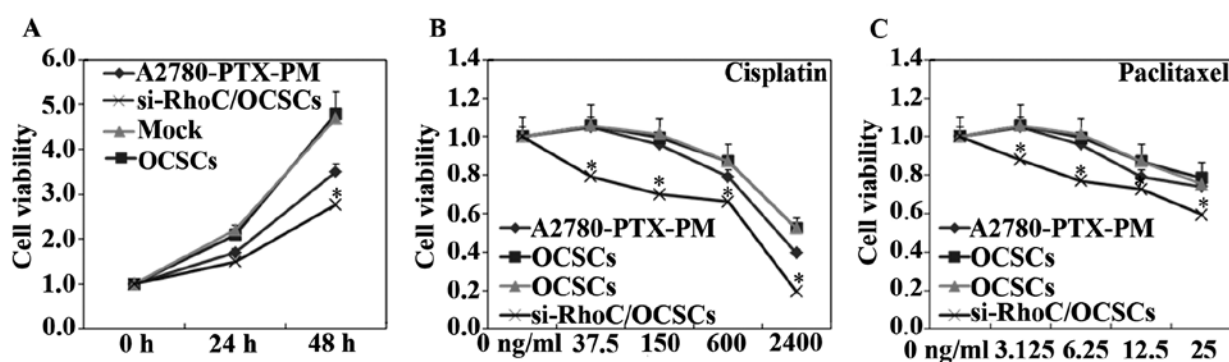


Figure 5. Effects of si-RhoC transfection on OCSC proliferation. OCSCs showed induced (A) cell proliferation, (B and C) drug resistance, while si-RhoC transfection in OCSCs reduced cell proliferation and drug resistance. Results are representative of three separate experiments; data are expressed as the mean  $\pm$  standard deviation; \* $P < 0.05$ . OCSCs, ovarian cancer stem cells.

signaling to RhoC as suppression of its expression recapitulated the loss of TEM4 phenotypes, and RhoC activation was impaired in TEM4-depleted cells (21). In addition, the destruction of the extracellular cell transfer mechanism is an important condition that is essential to the secretion of matrix

metalloproteinases (MMPs). Various researchers have found associations between MMPs and the *RhoC* gene and reported that the *RhoC* gene promotes the expression of the *MMP2* and *MMP9* genes (20). Additionally, we found that RhoC was expressed at a higher level in epithelial ovarian carcinomas

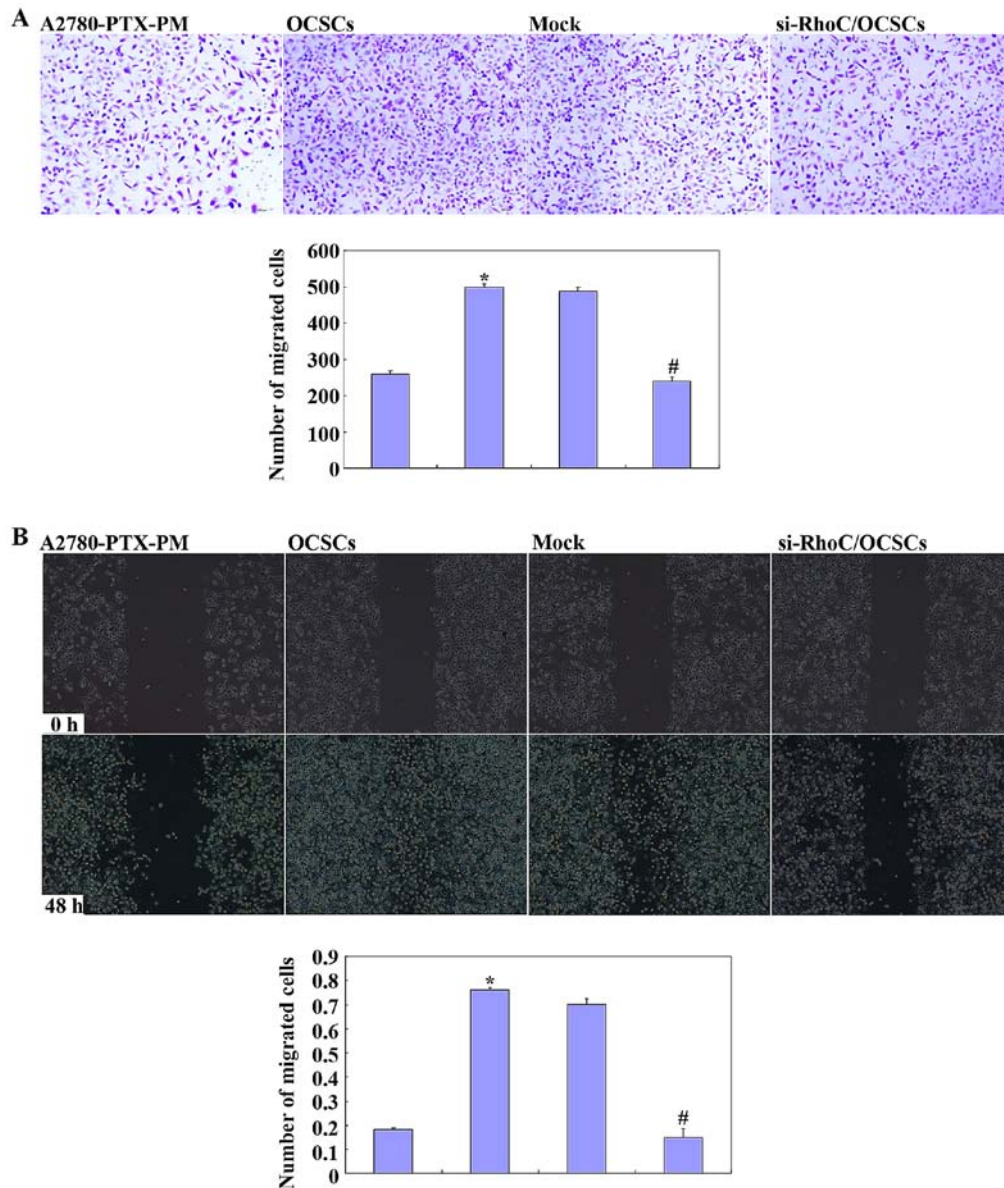


Figure 6. Effects of si-RhoC transfection on OCSC cell invasion and migration ability. OCSCs exhibited increased (A) cell invasion and (B) migration ability, while si-RhoC transfection in OCSCs reduced cell invasion and migration ability. Results are representative of three separate experiments; data are expressed as the mean  $\pm$  standard deviation; \* $P < 0.05$ . OCSCs, ovarian cancer stem cells. # $P < 0.05$ .

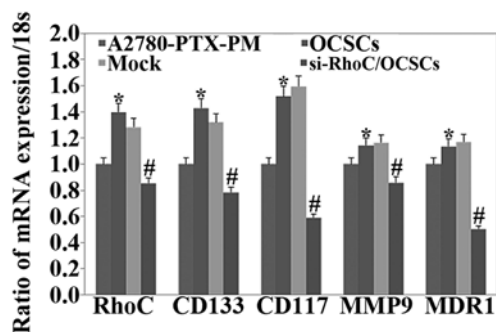


Figure 7. mRNA expression of phenotype-related molecules in ovarian cancer cells. The expression levels of RhoC, CD133, CD117, MDR1 and MMP9 were higher in OCSCs than levels in the A2780-PTX-PM cells, while expression levels were lower in si-RhoC-transfected OCSCs than levels in OCSCs. Results are representative of three separate experiments; data are expressed as the mean  $\pm$  standard deviation; \* $P < 0.05$ . OCSCs, ovarian cancer stem cells. # $P < 0.05$ .

than this level in normal ovarian and benign ovarian tumor tissues. The *RhoC* expression level was positively correlated with ovarian cancer staging and differentiation. In addition, functional studies have shown that overexpression of *RhoC* promoted the invasion and metastasis of ovarian cancer through expression of VEGF, MMP9 and Rho-associated kinase (ROCK). After si-RhoC transfection in OCSCs, the proliferation, cisplatin resistance, invasion and stemness were decreased; the expression of associated proteins MDR1 and MMP9 were also decreased; and the positive expression of CD117 and CD133 was decreased. Therefore, based on our findings, we conclude that *RhoC* is an important oncogene that is required for the maintenance and propagation of OCSCs in ovarian cancer.

This is the first study to investigate the role and molecular mechanisms of *RhoC* in OCSCs, *RhoC* is an important gene that can be used for the treatment of OCSCs. We believe that

the findings of the present study can shed insight into the root cause of ovarian cancer recurrence.

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