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Original Article

MiR181c inhibits ovarian cancer metastasis and progression by targeting PRKCD expression

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Abstract: MicroRNAs (miRNAs) regulate many important cancer related gene expression in the posttranscriptional process. Dysregulated expression of miRNAs has been observed in numerous human cancers including ovarian cancer. In this study, we found that the expression of the miR-181c was significantly decreased in ovarian cancer tissue and in tissues with lymph node metastasis when compared with their control samples, respectively. Moreover, among pathological stages, the expression of miR-181c was significantly decreased in the tissues with IV stage compared with other stages. In vitro, miR-181c significantly inhibited the proliferation, metastasis of A2780 cell line, and induced G1 phase arrest. Through bioinformatics prediction, protein kinase C delta (PRKCD) was identified as a target gene of miR-181c. Western blot results showed that PRKCD was increased in ovarian cancer tissue, in tissues with lymph node metastasis and IV stage of ovarian cancer pathological samples. After knocking down PRKCD, the cell cycle of A2780 cells was also arrested in G1 phase. The proliferation and the metastasis of A2780 cells were reduced. The dual luciferase reporter experiments showed that miR-181c regulated the expression of PRKCD by combining with its 3'UTR. These results indicate that miR-181c inhibits ovarian cancer metastasis and progression by targeting PRKCD expression.

Keywords: Ovarian cancer, miR-181c, invasion and metastasis

Introduction

Ovarian cancer is one of the most common gynecologic malignancies. Its incidence ranks sixth in malignant gynecological tumors and its mortality rate is the highest, seriously threatening women's health [1]. Because of the poor diagnosis in early stage without significant symptom, most patients are in mid or late stage when diagnosed. The five-year survival rate is around 30% [2]. It is indicated that the degree of malignancy in ovarian cancer is usually higher and the chance of the abdominal metastasis and the recurrence is higher [3]. The main reason of the poor prognosis in ovarian cancer is invasion and metastasis [4]. Actually the mechanisms of the metastasis are not understood clearly. It is valuable to identify the biomarkers of diagnosis and prognosis, and to further explore the molecular mechanisms in the processes of ovarian cancer development.

MicroRNAs (miRNAs) are a class of 18-25nt small non-coding RNA molecules, which play important roles in the post-regulation of transcription [5]. Through complementary binding with the target 3'UTR, mature miRNAs can lead to mRNA degradation or translational repression [6, 7]. Previous studies indicate that the expression profiles of miRNAs are usually changed significantly during the development of tumors [8, 9], such as miR-21, let-7, miR-16, which play essential roles in the growth, invasion, and differentiation of tumor cells [10-12]. MiR-181c belongs to the miR181 family that is identified as novel tumor-associated miRNAs in recent years. However, the reported functions of miR-181 family in the development of cancer are controversial. MiR-181a can inhibit the invasion and metastasis of the tumor cells, while miR-181b is reported to improve the processes of metastasis [13, 14]. For the miR-181c, the report about its molecular function in tumor is rare, and its roles in ovarian cancer are yet to be elucidated.

In this study, we explored the biological functions of miR-181c in ovarian cancer by quantitative real-time PCR (qRT-PCR), western blot, siRNA inference, and transwell assay. Combined

with bioinformatics prediction, the dual luciferase assay and western blot was used to validate that PRKCD was the target gene of miR-181c.

Materials and methods

Tissue sample collection

Fifty four primary ovarian cancer tissues and associated contralateral ovarian tissues were collected after surgeries between September 2013 and September 2014 in the department of gynaecology and obstetrics from Binzhou Medical University Hospital. All the cancer tissues were confirmed as epithelial ovarian cancer by pathology and the contralateral ovarian tissues were all normal. Among 54 tissues, 14 were stage I, 10 were stage II, 21 were stage III, and 9 were stage IV by FIGO 2000 [15]. And 21 patients were found with metastasis, while 32 had no metastasis. All patients were not treated by chemotherapy, and other adjuvant therapies before surgeries. Patients had no complications and other history of cancer. The ages were 32-63 years with an average age of 41.5 years. Prior written and informed consent were obtained from every patient and the study was approved by the ethics review board of Binzhou Medical College.

Cell culture

The Ovarian cancer cell lines A2780 were cultured in RPMI1640 (GIBCO, Grand Island, USA) supplemented with 10% fetal bovine serum and were kept in a humidified incubator with 5% CO_2 at 37°C. The trypsin digestion was used when the cell confluence reached 90%. The 3-5 generation cells were used in this study.

qRT-PCR

Total RNA of ovarian cancer tissues and associated controls were isolated using Trizol (Invitrogen, California, USA) according to the manufacture's protocol. The purity of RNA was detected by Spectrophotometer 260/280. Then 1 ug of RNA was reverse transcribed to cDNA. For the quantitative PCR of miRNA, the reaction system included 10 μl qRT-PCR-Mix, 0.5 μl forward primer and 0.5 μl reverse primer, 1 μl cDNA and 8 μl ddH_2O. Three replicates for each sample were run under the following conditions: 95°C for 10 min followed by 40 cycles of amplification at 95°C for 1 min, 60°C for 30 s.

A2780 cell transfection

Logarithmic growth A2780 cells were seeded in 24-well plates (3×10^5) in medium without antibiotic. The miR-181c mimics and PRKCD siRNA were transfected using Lipofectamine™ 2000 reagent (Invitrogen, California, USA) according to the manufacturer's instructions. After 48 h, the transfected cells were collected to detect the protein change of PRKCD. The cell transfected with miR181 mimics was regarded as miR-181c mimics group, and the cell transfected with random synthetic sequence was regarded as NC group. The control group was the normal growth cells.

Western blot analysis

For protein isolation, each 100 mg tissue was ground into powder, and lysed with RIPA (containing 1% PMSF) lysis buffer. Proteins from transfected A2780 cells were collected at 48 h after transfection. The isolated protein was loaded into SDS-PAGE and then transferred to PVDF membrane. The primary antibody was mouse anti-human PRKCD (1:1000) and rabbit anti-human GAPDH antibody (1:10000). The second antibody was HRP-conjugated goat anti-mouse and goat anti-rabbit IgG (1:10000). All the antibodies were brought from the Bioworld Company (Minnesota, USA). Finally, the membrane was developed by enhanced chemiluminescence plus reagent. The developed film was scanned using the AlphaImager gel imaging systems (AlphaImager, Santa Clara, California, USA). And the Western blot images were analyzed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). GAPDH was used as an internal control.

CCK-8 assay

The cells from control, NC and miR-181c mimics groups were seeded into 96-well plate at the density of 2×10^3/well. Each well had three replicate wells. The CCK-8 was added at 24 h, 48 h, and 72 h of incubation, and then incubated at 37°C for 1 h. The absorbance of each well was measured at 490 nm wavelength. The cell proliferation curve was plotted.

Cell cycle analysis by flow cytometry

The changes of cell cycle of cells from control, NC and miR-181c mimics groups were detected according to the instruction of BD Cell Cycle/

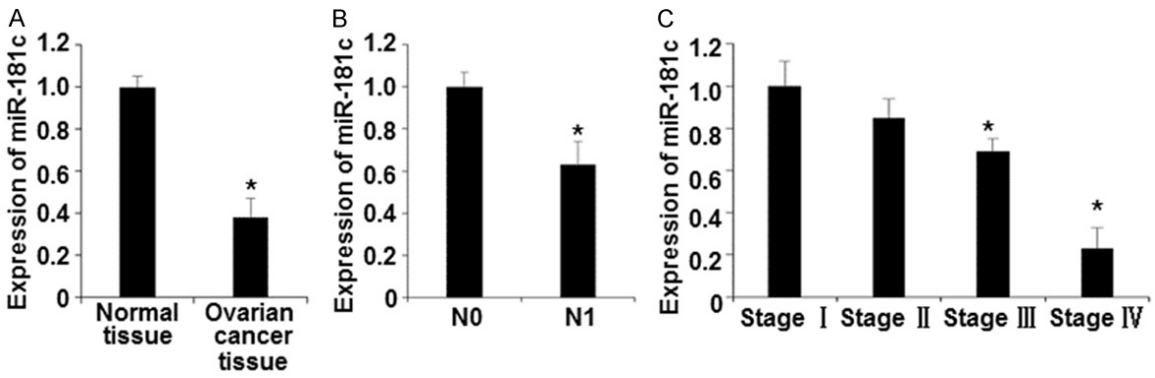

Figure 1. Expression of miR-181c in ovarian cancer samples. The expression of miR-181c was detected using qRT-PCR. A. MiR-181c expression was decreased in ovarian cancer tissues compared with normal tissues ($P < 0.05$); B. MiR-181c also decreased in tissues with lymph node metastasis (N1), compared with tissues without lymph node metastasis (N0) ($P < 0.05$); C. MiR-181c decreased significantly in tissues of III or IV stages compared with I or II stages ($P < 0.05$).

DNA Kits and Templates (BD Company, New Jersey, USA). After transfected 24 h, the cell ratio of G1, S, and G2/M was analyzed by Modfit software.

Cell scratch test

After transfection for 24 h, A2780 cells were seeded into the 6-well plates and plated in 37°C incubators with 5% CO_2. A scratch was made on a single layer of cells using ultra-high temperature sterilized toothpicks, then the cells were washed three times with PBS. At 24 h after scratch, cell migration near the scratch was observed using the inverted fluorescence microscope.

Transwell migration and invasion assay

The migration and invasion assays were performed using the Transwell chamber (Millipore, Billerica, USA). The transfected cells were seeded into the upper chambers (1×10^5 cell/well) that were incubated by Matrigel in 200 μl of serum free DMEM medium, while the bottom of the chamber was incubated with 500 μl of medium containing 10% fetal bovine serum. After culture for 48 h, cells were fixed by formaldehyde, washed by PBS, and stained by GIMSA. Finally, pictures of the cells were taken under a microscope with 5 random views. The number of invaded cells was counted to detect the invasion of cells.

Dual luciferase assay

The wild-type 3'UTR and the mutant 3'UTR of PRKCD were synthesized in vitro and were cloned into the downstream of pMIR-REPORT luciferase vector by Spe-1 and Hind III enzyme. A2780 cells were co-transfected with miR-181c mimic and wild-type PRKCD 3'UTR or the mutant 3'UTR. After transfection for 24 h, cells were lysed and luciferase intensity was measured by GloMax 20/20 luminometer (Promega, Wisconsin, USA). The intensity of *Renilla* was used as control, and all step followed by the protocol of the luciferase kit (Sigma, Saint Louis, USA).

Statistical analysis

All the data were shown as the mean ± SD, and difference were determined by two-tailed Student's t-test of SPSS. $P < 0.05$ was considered as statistically significant.

Results

MiR-181c is down-regulated in ovarian cancer tissues

To determine the roles of miR-181c in ovarian cancer, the expression level of miR-181c was detected by qRT-PCR. As shown in **Figure 1**, it was found that miR-181c expression was significantly decreased in ovarian cancer tissues, compared with normal tissues ($P < 0.05$) (**Figure 1A**). And miR-181c also significantly decreased in tissues with lymph node metastasis, compared with tissues without lymph node metastasis ($P < 0.05$) (**Figure 1B**). The expression of miR-181c decreased significantly in tissues of III or IV stages compared with I or II stages ($P < 0.05$) (**Figure 1C**). These results indicate that miR-181c might be associated

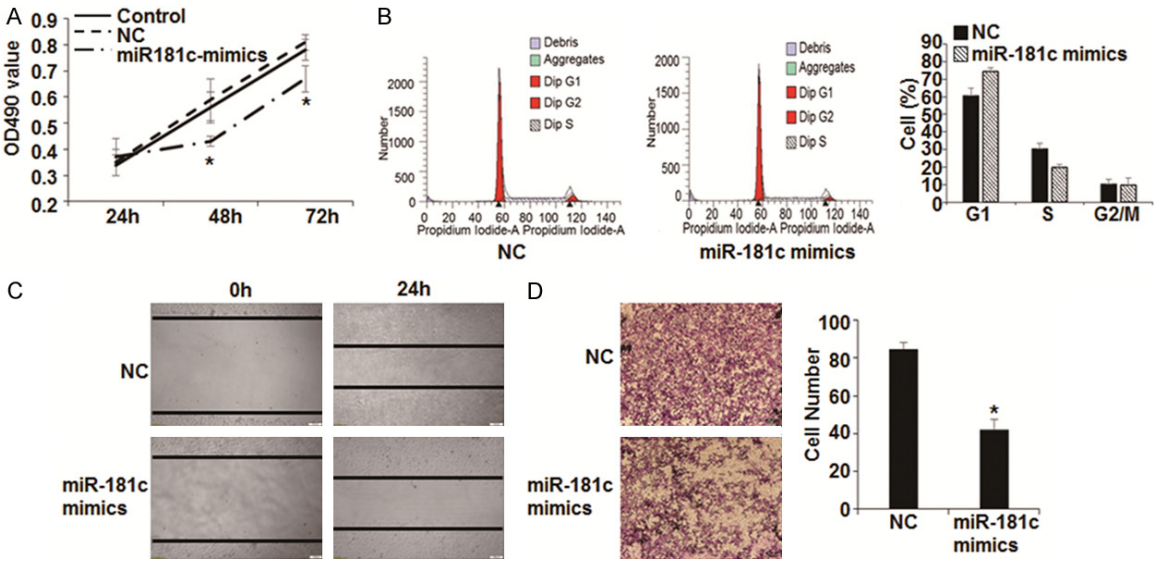

Figure 2. MiR-181c inhibits the proliferation and invasive behaviors of A2780 cells. A. The proliferation of A2780 cells was detected with CCK-8 assay; B. Flow cytometry analysis was used to detect the cell cycle of A2780 cells; C. The scratch test was performed to analyze of migration of A2780 cells; D. Transwell assay was conduct to analyze the invasion of A2780 cells.

with ovarian cancer development and progression.

MiR-181c inhibits the proliferation, migration and invasion of A2780 cells

To investigate whether miR-181c is related to tumor proliferation and metastasis, we detected the effect of miR-181c on cell proliferation, migration and invasion. After transfection with miR-181c mimics, the proliferation of A2780 cells was inhibited significantly, as detected by CCK-8 assay ($P < 0.05$) (**Figure 2A**). Similarly, through flow cytometry analysis, we found that about 74.30 ± 2.31 A2780 cells with miR-181c overexpression were arrested at G1 phase (**Figure 2B**). And, the difference in G1 phase was significant ($P < 0.05$). The scratch test results showed that the high expression of miR-181c inhibited the metastasis of A2780 cells (**Figure 2C**). From Transwell migration and invasion analysis, we found that miR-181c overexpression significantly reduced the migration and invasion of A2780 cells ($P < 0.05$) (**Figure 2D**). Overall, these results suggestion that miR-181c may participate in A2780 proliferation and metastasis.

PRKCD is a direct target gene of miR-181c

To identify the target genes of miR-181c, we predicted candidate targets of miR-181c using bioinformatics method (miRwalk). Considering the potential roles in cancers, we selected PRKCD as our candidate target for further study. To determine whether PRKCD was regulated directly by miR-181c, we detected the GFP intensity by dual luciferase assay. As shown in **Figure 3**, miR-181c seed sequence and the binding sites on PRKCD 3'UTR and several base mutation were generated (**Figure 3A**). A2780 cells were co-transfected with miR-181c and wild-type or mutated PRKCD 3'UTR respectively. We found that miR-181c reduced the GFP intensity of PRKCD 3'UTR, while no effect on the controlled by mutant PRKCD 3'UTR ($P < 0.05$) (**Figure 3B**).

To explore whether miR-181c participates the progression of ovarian cancer through targeting PRKCD, we detected the expression of PRKCD after transfection of miR-181c mimics in A2780. As **Figure 3C** shown, the PRKCD expression was significantly lower than control ($P < 0.05$). These results indicated that PRKCD is the target of miR-181c.

PRKCD is up-regulated in ovarian cancer tissues

To explore whether PRKCD is associated with the ovarian cancer development, we detected the expression of PRKCD by western blot. As shown in **Figure 4**, the protein expression of PRKCD was significantly increased in ovarian

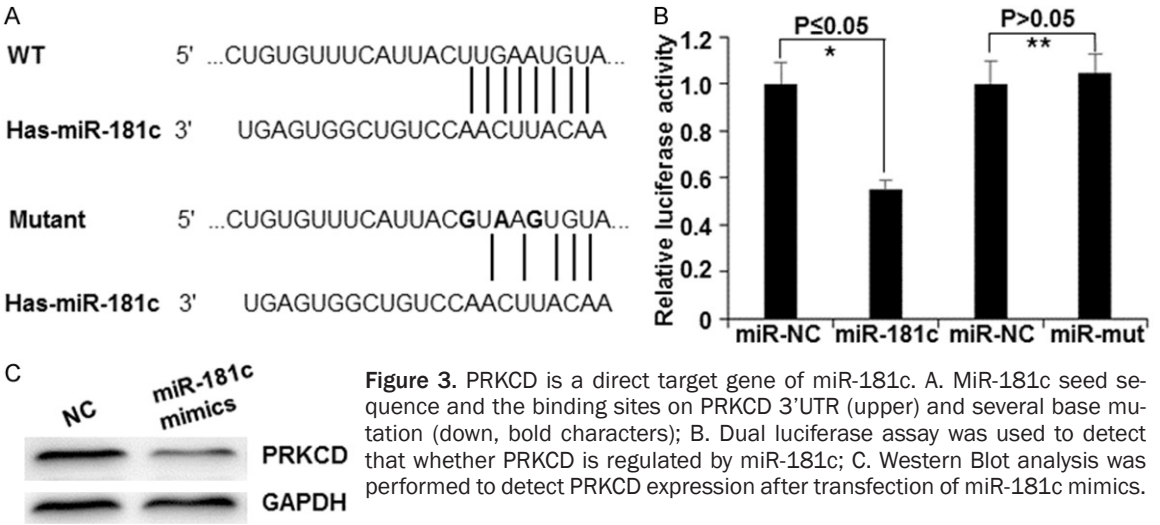

Figure 3. PRKCD is a direct target gene of miR-181c. A. MiR-181c seed sequence and the binding sites on PRKCD 3'UTR (upper) and several base mutation (down, bold characters); B. Dual luciferase assay was used to detect that whether PRKCD is regulated by miR-181c; C. Western Blot analysis was performed to detect PRKCD expression after transfection of miR-181c mimics.

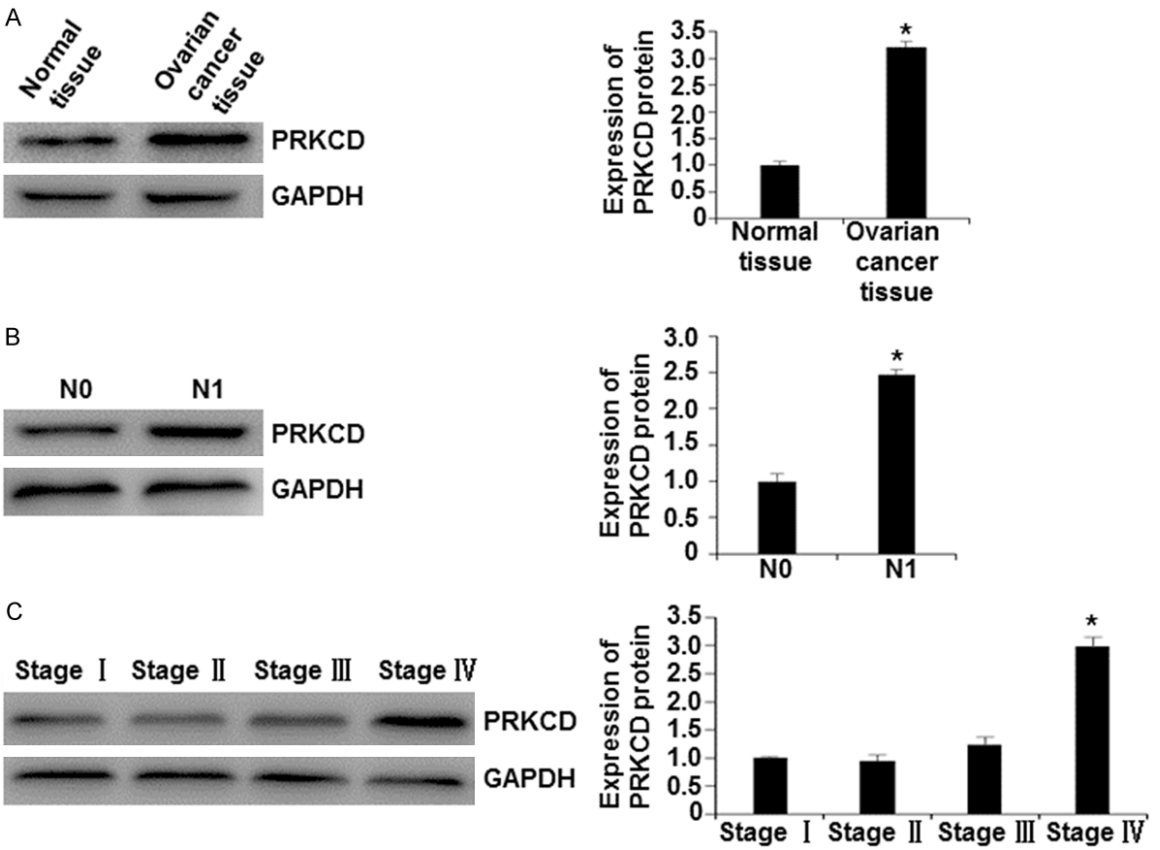

Figure 4. The expression of PRKCD in ovarian cancer samples. Western Blot analysis was performed to detect PRKCD expression. A. PRKCD was increased in ovarian cancer tissues compared with normal tissues ($P < 0.05$); B. PRKCD was increased in tissues with lymph node metastasis (N1), compared with tissues without lymph node metastasis (N0) ($P < 0.05$); C. PRKCD was increased significantly in tissues of IV stages compared with I or II stages ($P < 0.05$).

cancer tissues compared with the normal control ($P < 0.05$) (**Figure 4A**). The PRKCD also significantly increased in tissues with lymph node metastasis (**Figure 4B**) and in tissues of stage IV ($P < 0.05$) (**Figure 4C**), compared with tissues without lymph node metastasis and tissues of

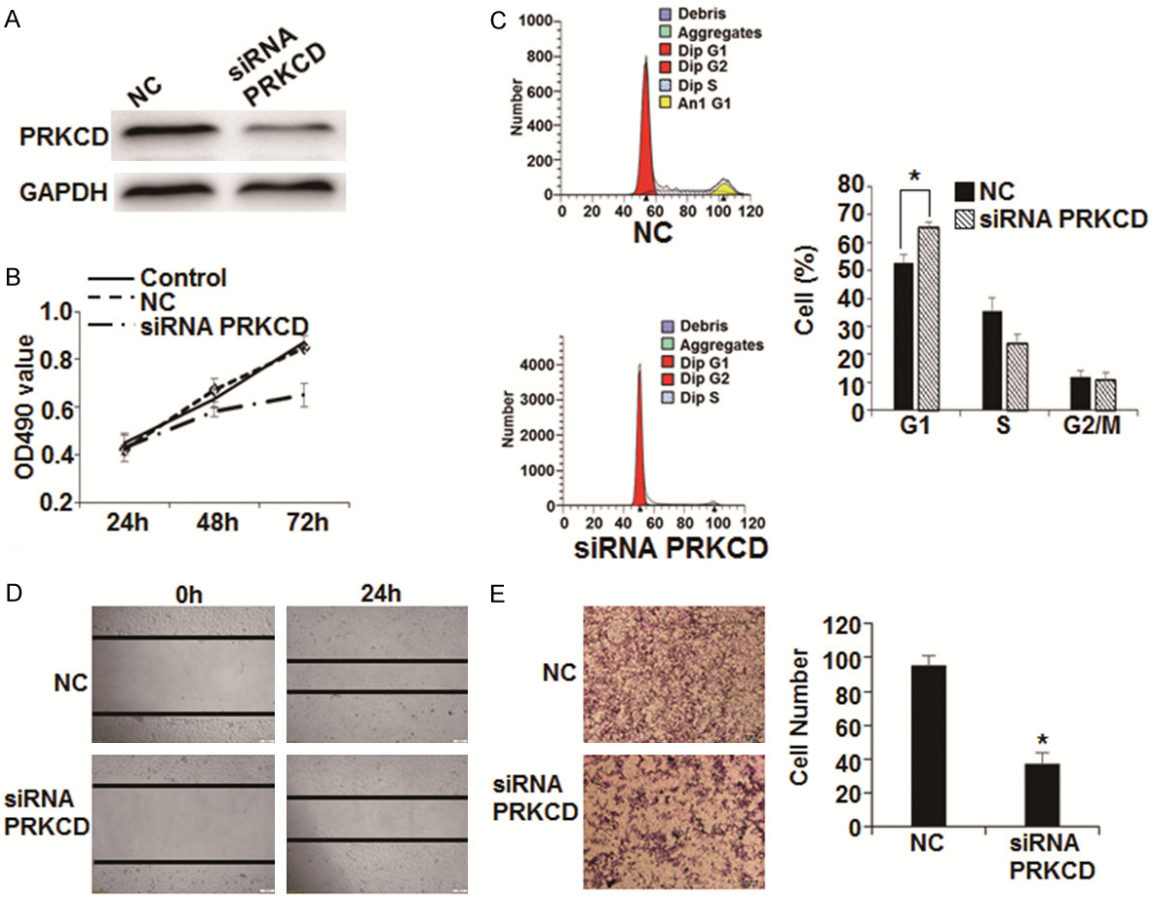

Figure 5. The changes of cellular characters of A2780 after PRKCD RNA interference. A. Expression of PRKCD by Western Blot analysis; B. CCK-8 assay was used to measure the proliferation of A2780 cells; C. Flow cytometry analysis was performed to detect the cell cycle of A2780 cells; D. The scratch test was conducted to analyze of migration of A2780 cells; E. Transwell assay was used to analyze invasion of A2780 cells.

stage I, II, and III respectively. Overall, these results suggest that PRKCD expression is highly correlated with the malignant degree of ovarian cancer.

The changes of cellular characters of A2780 after PRKCD siRNA interference

To investigate whether knockdown of PRKCD has effects on A2780 cells, we performed CCK-8 assay, flow cytometry, scratch test and Transwell assay. After PRKCD siRNA transfection for 48 h, the protein expression of PRKCD was decreased (**Figure 5A**), indicating that PRKCD expression was successfully knocked down. Compared with control group, the proliferation of A2780 was significantly inhibited after PRKCD knockdown ($P < 0.05$) (**Figure 5B**). As shown in **Figure 5C**, the cell cycle of A2780 cells transfected with PRKCD siRNA was arrested at G1 phase. And, the difference in percentage of cells in G1 phase was significant ($P < 0.05$). In addition, the migration and invasion of A2780 was also decreased significantly after PRKCD knockdown ($P < 0.05$) (**Figure 5D** and **5E**). All these results indicate that down-regulation of PRKCD can inhibit the proliferation, invasion and metastasis of A2780 cells.

Discussion

MiRNAs play important roles in cancer development and progression of cancers through regulating the expression of target genes [16]. MiR-181 family contains miR-181a, miR-181b, miR-181c and miR181d. This family is reported to be highly involved in different cancers [17-19]. The miR-181a has high sequence homology with miR-181c, while miR-181b has high identity with miR-181d. Different studies reported that miR-181 family plays important roles in different cancers, either as oncogene or suppres-

sor gene [20, 21]. The down-regulation of miR-181a is related to the prognosis of non-small cell lung cancer [22], while miR-181b is up-regulated in acute myeloid leukemia (AML) to promote cancer cell proliferation and inhibit cell apoptosis [23]. For the miR-181c in ovarian cancer, it is still unclear about how miR-181c participates in the biological processes of ovarian cancer.

In this study, we showed that miR-181c was down-regulated in ovarian cancer tissues and functioned as a tumor suppressor. Then PRKCD was identified as a direct target gene of miR-181c. We found that knockdown of PRKCD inhibited cell proliferation, migration and invasion. Our results propose a novel mechanism of miR-181c in ovarian cancer cells and indicate that miR-181c might play an important role in ovarian cancer progression through targeting PPKCD.

In conclusion, miR-181c can inhibit the proliferation, invasion and metastasis of ovarian cancer cells through regulating the expression of PRKCD, and the expression of miR-181c is highly correlated with the pathological stages and metastasis. We present a novel mechanism that miR-181c plays a role in the progression of ovarian cancer as a suppressor gene. The miR-181c may be a potential biomarker and target in clinical treatment.

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Disclosure of conflict of interest

None.

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