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Original Article

Polo-like kinase 1 controls cell proliferation regulated by miR-296-5p in ovarian cancer

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Abstract: Polo-like kinase 1 (PLK1) is overexpressed in various human cancers. However, the biological functions of PLK1 regulated by microRNAs in ovarian cancer are still unknown. The study is to determine whether PLK1 can be a target of ovarian cancer therapy, and to identify a microRNA targeting PLK1. We found that miR-296-5p expression was lower in ovarian cancer tissues compared with their normal controls, so did the ovarian cancer cells. miR-296-5p restoration in ovarian cancer cells induced G2/M arrest in cell cycle assay, and reduced cell proliferation and tumor formation ability in vivo. Luciferase assay analysis identified PLK1 as a direct target gene of miR-296-5p. miR-296-5p inhibited ovarian cancer progression via PLK1. Collectively, our findings suggested that miR-296-5p acts as a tumor negative regulator in ovarian cancer by targeting PLK1.

Keywords: Ovarian cancer, proliferation, cell cycle, PLK1, miR-296-5p

Introduction

Ovarian cancer is one of the most common tumor in females with a high incidence and mortality. Many new cases are diagnosed as ovarian cancer every year in the world. With the development of molecular biology and cell biology, there are emerging many new potential target molecules and the five-year survival rate is higher than before, however, there are some problems needed to solve such as cancer metastasis and drug resistance. Therefore, it is important to further investigate the molecular mechanism of ovarian cancer to find new therapeutic targets.

MicroRNAs (miRNAs) are noncoding small mRNA with about 22-nucleotides which act as important regulators of gene expression [4, 5]. miRNAs suppress gene expression by specifically binding and cleaving mRNAs or inhibiting their translation. Recently, there has been reported many miRNAs that involved in human ovarian cancer development. miRNAs are aberrantly over-expressed or down-regulated during its progression, including miR-211, miR-93-5p, miR-569, miR-214 and etc [6-10]. miR-296-5p is reported as a tumor suppressor or a tumor promoter in cancer [11-13], but there are no reports on the role and tumorigenesis of miR-296-5p in ovarian cancer.

In this study, the purpose is to explore the role of miR-296-5p in ovarian cancer. miR-296-5p expression in human ovarian cancer cells and tissues were examined and the celluar function such as cell growth, cell-cycle distribution and colony formation were analyzed. We also investigated the role of miR-296-5p on ovarian tumorigenesis in vivo. By bioinformatics, we predicted PLK1 was a potential target gene of miR-296-5p. We identified that miR-296-5p was a tumor suppressor negatively regulated PLK1 in ovarian cancer.

Material and methods

Ovarian cancer tissues

Ovarian cancer tissues and their normal controls were obtained from the First Affiliated Hospital of Zhengzhou University e (Henan, China). Before we collected the ovarian cancer tissues from the patients for the study, informed consent was obtained from each patient. The methods to collect and store the samples were

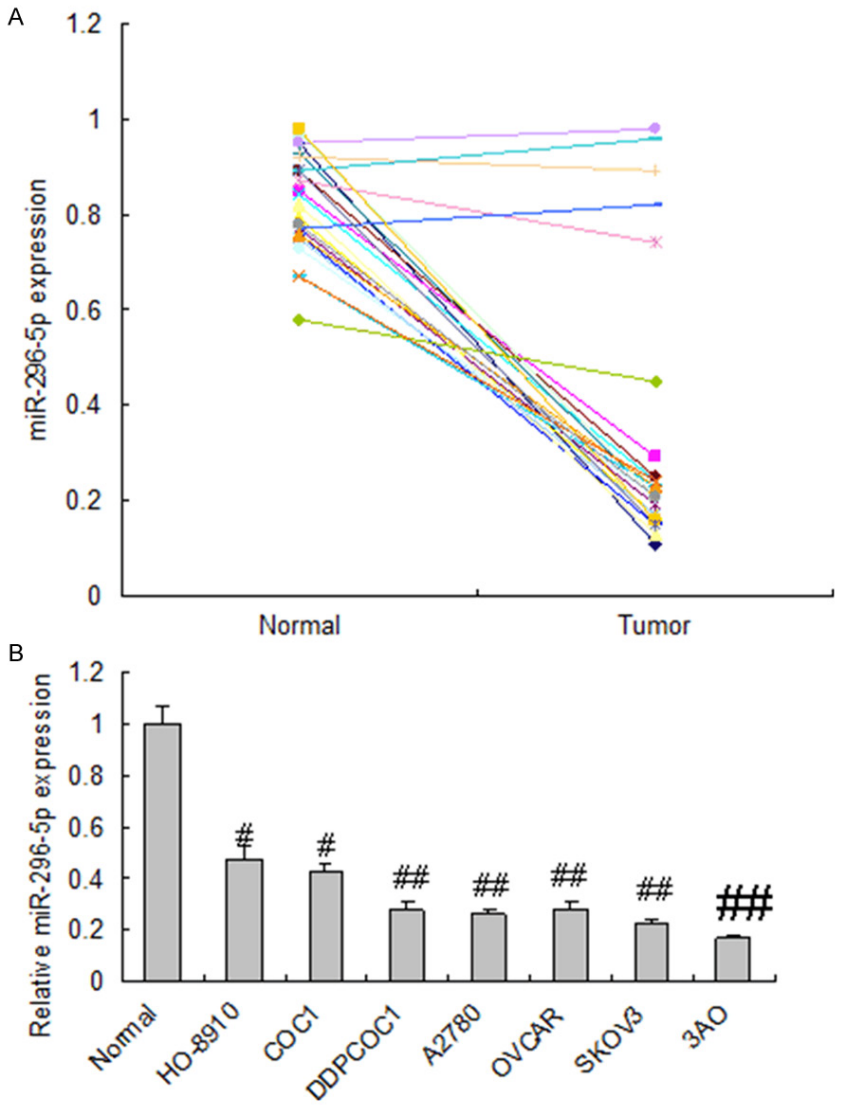

Figure 1. Low expression of miR-296-5p in human ovarian cancer. A. miR-296-5p expression levels in human ovarian cancer specimens. miR-296-5p was assessed by real time RT-PCR and normalized to U6 RNA. B. miR-296-5p expression was analyzed in SKOV3, OVCAR3, 3AO, and ES-2 ovarian cancer cells and normal cells by real time PCR.

approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (China).

Cell lines and cell culture

Normal ovarian epithelial cells (HUM-CELL-0088) were stored in our lab. Ovarian cancer cell lines HO-8910, COC1, DDPCOC1, A2780, OVCAR, SKOV3 and 3AO cell lines were purchased from the ATCC (Manassas, VA, USA). The cells were maintained in the suggested medium according to the protocols supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin/streptomycin (Invitrogen).

miR-296-5p lentivirus vector and its transduction

Lentivirus vectors mediated miR-296-5p or its control (miR-control) was constructed according to the protocol from Invitrogen. Cells were lentivirally transfected with either the miR-296-5p recombined vector or empty vector. Oligonucleotide transfection or lentivirus construction was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Lentivirus-mediated silencing of miR-296-5p was verified by qRT-PCR.

Plasmid constructs for miRNA functional assay PLK1

Plasmid vectors used in the luciferase reporter assays for miRNA post-transcriptional regulation were constructed as described previously. PLK1 3'-untranslated region (UTR) was amplified and the downstream of the luciferase gene in pGL4.13 vector (Promega, Madison, WI). All the constructs were verified by sequencing.

Real-time RT-PCR

Total RNA, was isolated from the cells using Trizol reagent (Invitrogen) following the manufacturer's instructions. After that, 2 μg of RNA was taken and treated with DNase to remove contaminating DNA prior to the reverse transcription to cDNA using SYBR® PCR Kit (Takara, Japan). To measure mRNA expression, real-ti-

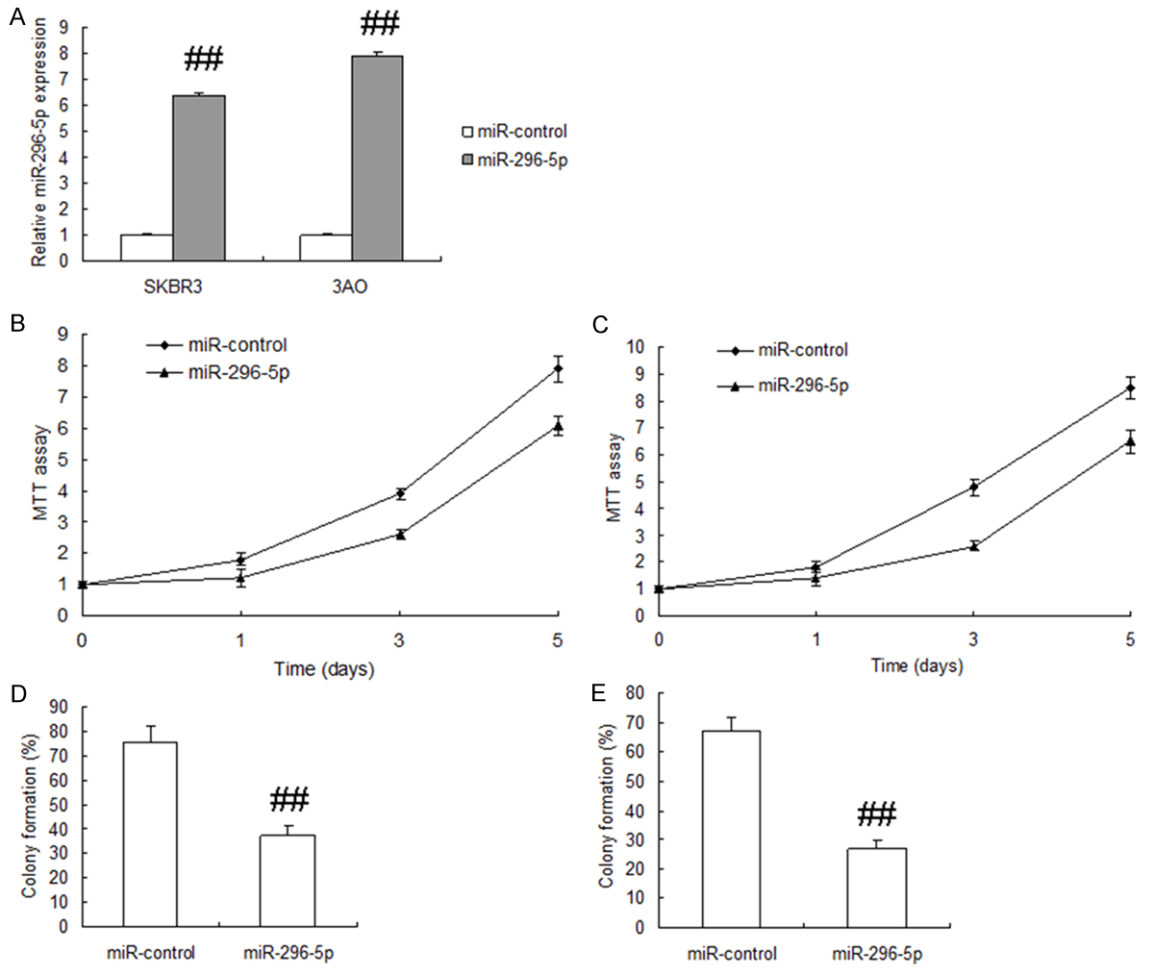

Figure 2. miR-296-5p induced growth inhibition in ovarian cancer cells. A. SKOV3 and 3AO cells were transfected with miR-296-5p and its expression levels were enhanced. miR-296-5p was assessed by real time RT-PCR and normalized to U6 RNA. B and C. miR-296-5p inhibited cell proliferation measured by MTT assay after miR-296-5p transfection in SKOV3 and 3AO cells. D and E. miR-296-5p inhibited cell proliferation measured by colony formation assay after miR-296-5p transfection in SKOV3 and 3AO cells.

me RT-PCR was performed using a sequence detector (ABI-Prism, Applied Biosystems). Primers were purchased from Invitrogen. The relative expression levels were calculated by comparing Ct values of the samples with those of the reference, all data normalized to the internal control GAPDH.

MTT assay

MTT assay was employed to detect the growth of ovarian cancer cells and the growth curve was delineated. Briefly, 2.5 × 10^3 cells/well were seeded to a 96-well plate and allowed to adhere. At different time points, 20 μl of the MTT solution was added to each well (5 mg/ml, 0.5% MTT) and the cells were continued to culture for 4 h. After the incubation, the supernatant was discarded and 150 μl dimethyl sulfoxide was added to each well, and the culture plate was shaked at low speed for 10 min until crystal dissolved completely. The ELISA reader was used to measure the absorbance.

Western blot analysis

Cells were harvested and lysed using Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) and separated by 10% SDS-polyacrylamide gel (SDS-PAGE) and blotted to polyvinylidene fluoride (PVDF) membranes (Millipore, Darmstadt, Germany). After blocking with 5% non-fat milk in TBST (1‰), the PVDF membranes were incubated overnight at 4°C with primary antibodies such as PLK1 and then with a horseradish peroxidase-conjugated secondary antibody for 1 hours at room temperature. Protein bands were detected using

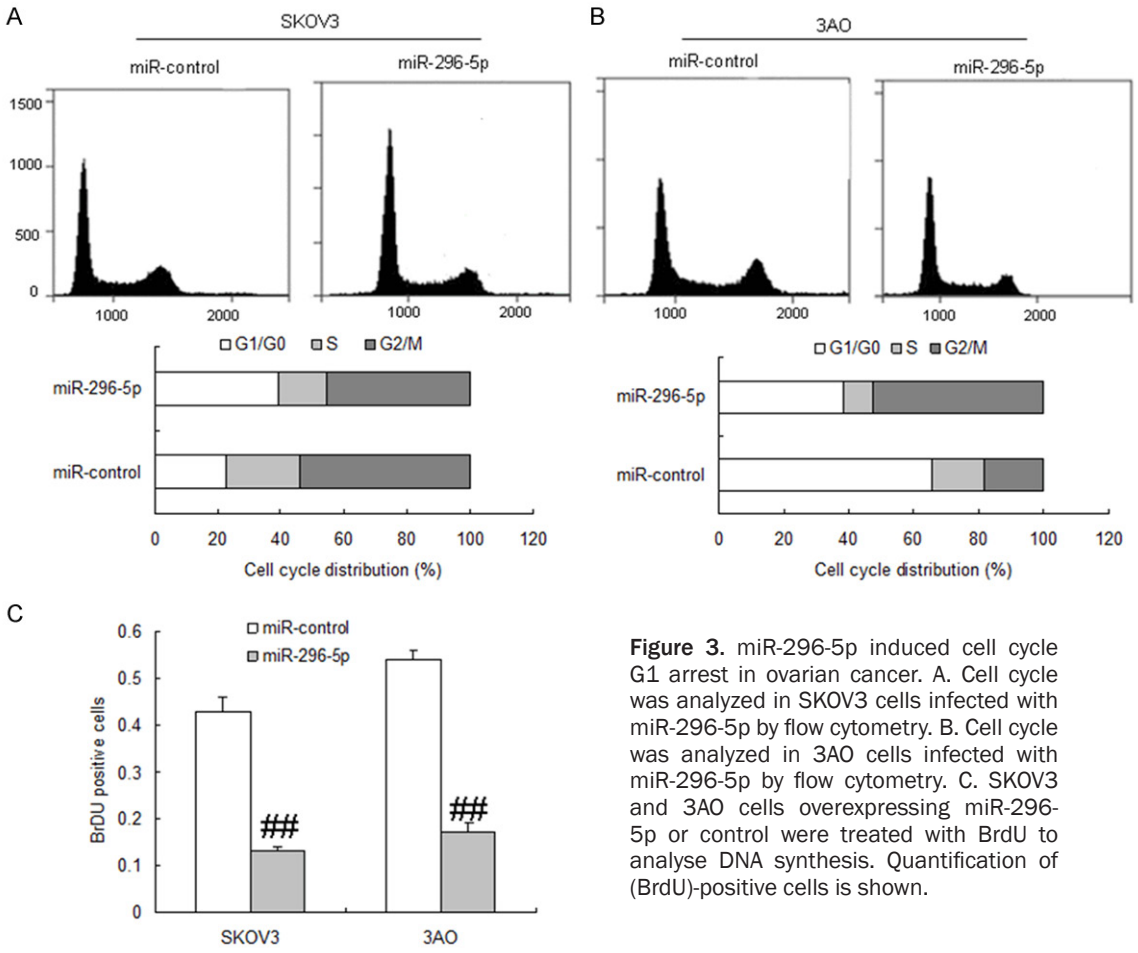

Figure 3. miR-296-5p induced cell cycle G1 arrest in ovarian cancer. A. Cell cycle was analyzed in SKOV3 cells infected with miR-296-5p by flow cytometry. B. Cell cycle was analyzed in 3AO cells infected with miR-296-5p by flow cytometry. C. SKOV3 and 3AO cells overexpressing miR-296-5p or control were treated with BrdU to analyse DNA synthesis. Quantification of (BrdU)-positive cells is shown.

Chemiluminescent Western Blot Scanner (Gene Company, HongKong, China). The β-actin band intensity served as the control.

Statistical analysis

All statistical analyses were carried out using the SPSS 15.0 statistical software package. Continuous variables were expressed as mean ± SEM. Differences between groups were calculated with Student's t test. A two-tailed *P* value test was used with a *P* value of < 0.05 considered statistically significant.

Results

Decreased expression of miR-296-5p in human ovarian cancer

miR-296-5p expression in 24 human ovarian cancer tissues was examined by real time RT-PCR and it was lower in cancer samples than those in normal samples (**Figure 1A**). miR-296-5p expression in ovarian cancer cell lines was lower in cancer cells compared with normal cells (**Figure 1B**). The data suggested that miR-296-5p may play a suppressing miRNA in the development of human ovarian cancer.

miR-296-5p inhibits proliferation of ovarian cancer

Usually, as a tumor suppressor, miR-296-5p may inhibit cell proliferation. To investigate it, SKOV3 and 3AO cells were transfected with miR-296-5p or the control respectively, and miR-296-5p expression was restored in ovarian cancer cells (**Figure 2A**). The survival rates measured by MTT assay in SKOV3 and 3AO cells were significantly inhibited (**Figure 2B** and **2C**). The results from colony formation rates were also inhibited (**Figure 2D** and **2E**).

miR-296-5p changes cell cycle distribution of ovarian cancer

We next observed the effect of miR-296-5p on cell cycle distribution in ovarian cancer cells. In

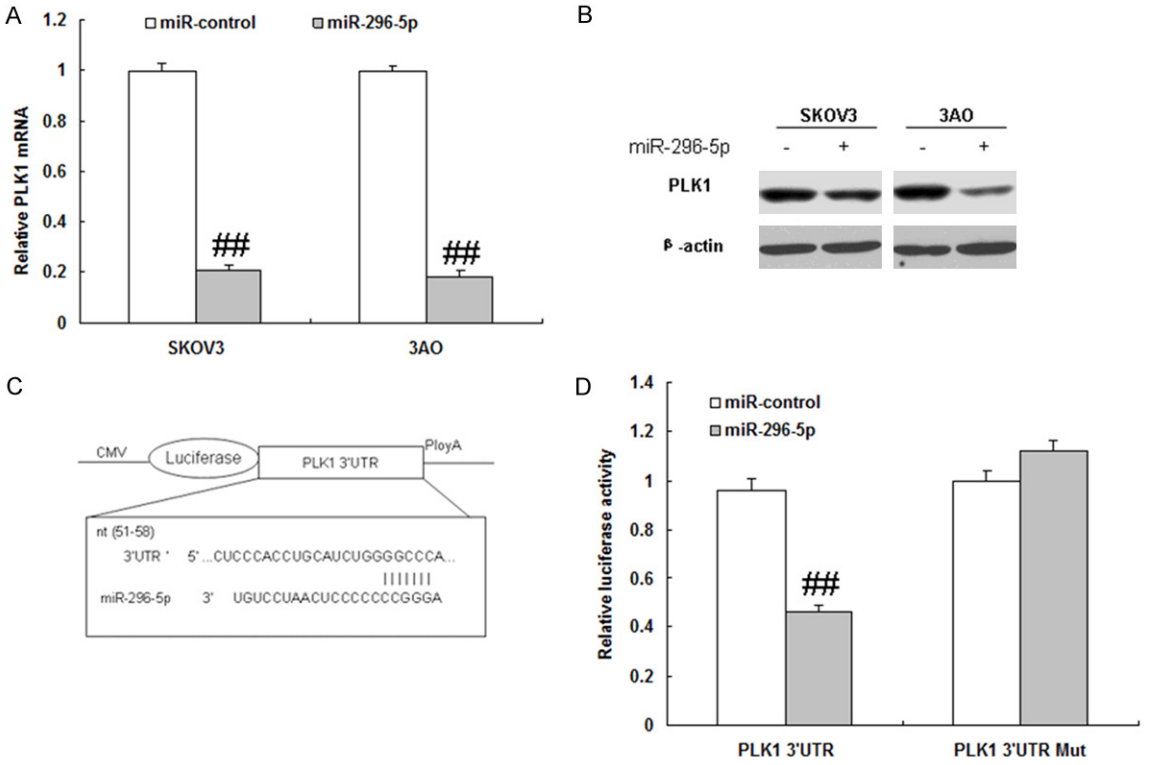

Figure 4. miR-296-5p down-regulates PLK1 expression. A. miR-296-5p down-regulated PLK1 mRNA in ovarian cancer cells. Cells were transfected with miR-296-5p or control for 48 hours, and RNA was extracted for real-time PCR. B. miR-296-5p down-regulated PLK1 protein in ovarian cancer cells. Cells were transfected with miR-296-5p or miR control for 48 hours, and total protein was isolated for western blot analysis. C. The 3'-UTR of the PLK1 gene contains binding sites for miR-296-5p according to bioinformatic analysis. D. miR-296-5p suppressed the expression of a luciferase reporter gene harbouring the 3'-UTR of PLK1. The pGL4 plasmid was modified by adding the human 3'-UTR or the 3'-UTR with mutations in regions complementary to miR-296-5p seed regions behind the firefly luciferase gene. The data presented are shown as means ± s.d. collected from three independent experiments.

SKOV3 and 3AO cells with miR-296-5p expression restoration, there was a significant increase in G1 phase accompanied by an increase compared to control cells (**Figure 3A** and **3B**). These results indicated that the growth-suppressive effect of miR-296-5p in ovarian cancer cells was partly due to a G1-phase arrest. The 5-bromo-20-deoxyuridine (BrdU) incorporation assay confirmed that miR-296-5p over-expressing SKOV3 and 3AO cells are less proliferating than control cells (**Figure 3C**).

PLK1 is down-regulated by miR-296-5p in ovarian cancer cells

miRNAs inhibits gene expression by targeting their 3'UTR. To further explore the molecular mechanisms that *miR-296-5p* inhibits ovarian cancer progression. PLK1 was predicted as the target gene of miR-296-5p with high possibility according to the predicted result (**Figure 4A**). The wide type (W) and mutation type (M) of vectors with PLK1 3'UTR were constructed based on pGL4 and the vectors were co-transfected with *miR-296-5p and miR-control, the* luciferase activity of wide type was much lower than in control cells and mutation type was rescued in SKOV3 cells (**Figure 4B**). *miR-296-5p* could regulate endogenous PLK1 mRNA in SKOV3 and 3AO cells (**Figure 4C**). PLK1 protein decreased in the cells with miR-296-5p (**Figure 4D**).

miR-296-5p inhibits ovarian cancer by targeting PLK1

To know whether miR-296-5p regulates proliferation in ovarian cancer cells with over-expression of PLK1. The result showed that *miR-296-5p* in SKOV3 and 3AO cells inhibited proliferation of the cells with PLK1 overexpression (**Figure 5A** and **5B**). In order to investigate miR-296-5p

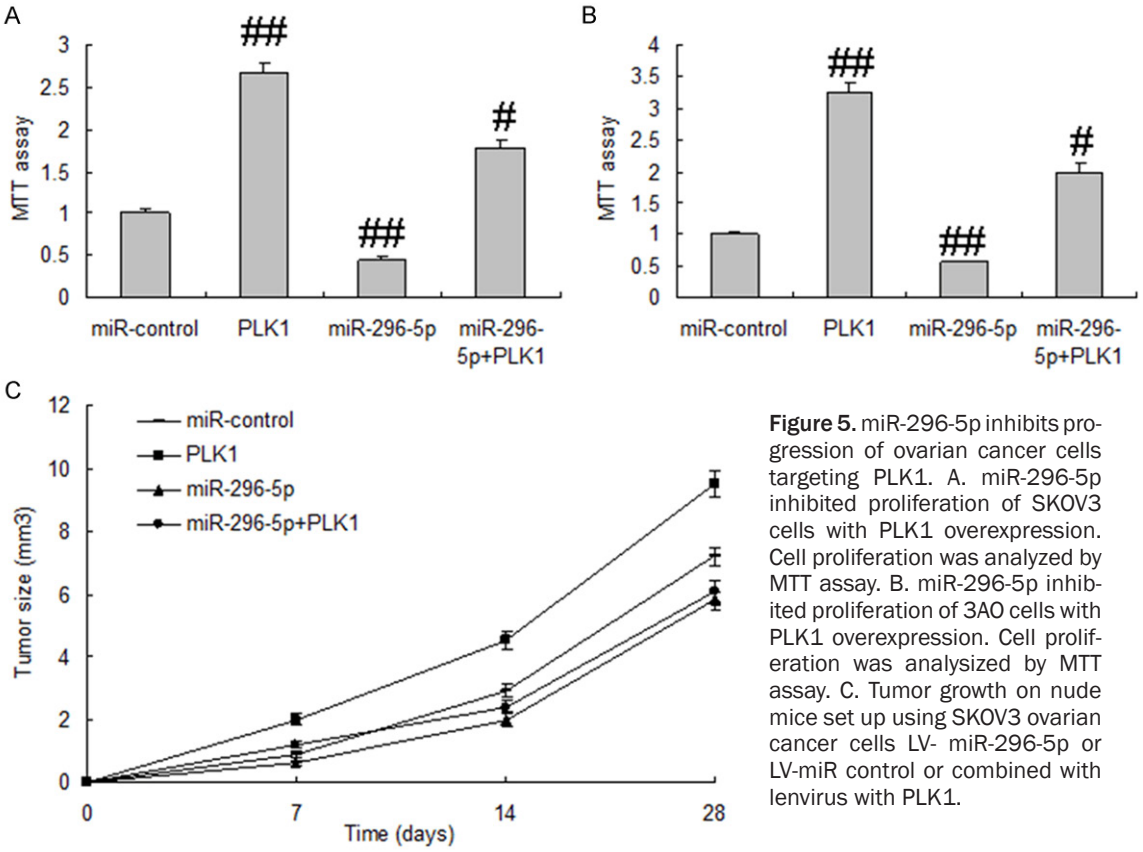

Figure 5. miR-296-5p inhibits progression of ovarian cancer cells targeting PLK1. A. miR-296-5p inhibited proliferation of SKOV3 cells with PLK1 overexpression. Cell proliferation was analyzed by MTT assay. B. miR-296-5p inhibited proliferation of 3AO cells with PLK1 overexpression. Cell proliferation was analysized by MTT assay. C. Tumor growth on nude mice set up using SKOV3 ovarian cancer cells LV- miR-296-5p or LV-miR control or combined with lenvirus with PLK1.

mediating growth inhibition in vivo, ovarian cancer nude mice were set up using SKOV3-miR-296-5p and SKOV3-miR control; the results showed that miR-296-5p suppressed ovarian cancer growth (**Figure 5C**).

Discussion

The previous data indicated that miR-296-5p may play as an oncogene or tumor suppressor gene. We found that the expression of miR-296-5p was significantly downregulated in ovarian cancer tissues compared with that of normal tissues. Our data demonstrated that miR-296-5p inhibited ovarian cancer progression by targeting PLK1.

miR-296-5p was expressed low in cancer tissues, which are consistent with previous reports [11-13]. We also found that miR-296-5p induced G1 arrest of ovarian cancer cells and inhibited cell proliferation. In addition, miR-296-5p overexpression in SKOV3 cells suppressed ovarian tumorigenesis in nude mice, which suggested that miR-296-5p functions as a tumor suppressor in ovarian cancer. To find the potential target genes of miR-296-5p, we used bioinformatics to search potential target genes and found that PLK1 was a novel target gene of miR-296-5p.

Polo-like kinases (PLK) are a family of serine-threonine kinases with a kinase domain, which includes five members PLK1 to PLK5. PLK1 is the best well-known member and its characters are relatively clear with a diverse range of biological functions involving cell cycle, DNA damage, cell proliferation and others. Overexpressed PLK1 overrides mitotic checkpoints, and lead to immature cell division without proper chromosome alignment and segregation, resulting in chromosomal instability and aneuploidy, a feature of cancer [14, 15]. PLK1 functions as an oncogene. PLK1 is overexpressed in various types of cancers, such as colon [16], breast [17], stomach [18], pancreas [19], head and neck [20], and ovarian cancers [21]. In phase I-II clinical trials, several PLK inhibitors have been used for study in many types of tumor, however not including ovarian cancer. Our study demonstrated that PLK1 was a potential target of ovarian cancer therapy [22]. So, there has great significance to elucidate the

molecular mechanism of PLK1 regulation in ovarian cancer. PLK1 is regulated by miR-100 [23], miR-10b*[24] and miR-593*[25], we verified that miR-296-5p regulates PLK1 expression and its function in cell proliferation and cell cycle.

In a summary, we identified miR-296-5p to be a tumor suppressor miRNA in ovarian cancer, and low miR-296-5p expression in ovarian cancer cells is related to cell proliferation and cell cycle change. miR-296-5p partially influences human ovarian cancer through the regulation of PLK1. These results suggest that miR-296-5p is a potential target for treating ovarian cancer and the critical roles of miR-296-5p in ovarian cancer tumorigenesis are needed to further research.

Disclosure of conflict of interest

None.

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