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MiR-203 inhibits the malignant behavior of prostate cancer cells by targeting RGS17

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Abstract. – OBJECTIVE: The aim of this study was to explore the role of microRNA-203 (miR-203) in Prostate Cancer (PCa), and to further verify its influence in PCa cell function.

PATIENTS AND METHODS: The expression level of miR-203 in 55 clinical PCa cases and cell lines was detected by qRT-PCR. Then, the target gene of miR-203 in PCa cells was predicted and verified by online prediction software and Luciferase reporter gene assay, respectively. Furthermore, the role of miR-203 in PCa cell proliferation, colony formation, cell cycle and metastasis capacities was detected through a series of *in vitro* experiments.

RESULTS: The expression of miR-203 in PCa tissues and cells was significantly reduced when compared with that of normal tissues and cells. In searching for potential downstream targets of miR-203, a regulator of G-protein signaling 17 (RGS17) entered our sight due to its active role in a variety of malignant tumors. More importantly, the negative regulation of RGS17 by miR-203 was verified by Luciferase reporter gene assay. Functional experiments demonstrated that low expression of RGS17 in PCa cells induced by up-regulation of miR-203 could significantly restrain the proliferation, invasion and migration capacities of PCa cells.

CONCLUSIONS: MiR-203 served as a tumor suppressor gene in PCa. Through targeting RGS17, miR-203 significantly controlled the malignant behavior of PCa cells. Our findings revealed that miR-203/RGS17 axis might be a potential therapeutic target for the treatment of PCa.

Key Words:
Prostate cancer (PCa), MicroRNA-203 (miR-203), Regulator of G-protein signaling 17 (RGS17), Proliferation.

Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer among men in Europe and the United States, which is also the second leading cause of cancer deaths next to lung cancer. The prevalence of PCa is relatively low. However, its growth rate has become quite high in China and overdeveloped countries, such as Europe and America. According to statistics, the incidence of PCa in China has increased more than tenfold in the past two decades, ranking sixth in male malignant tumors. It undoubtedly poses a challenge to Chinese urologists[1]. In terms of PCa therapy, the treatment methods have been continuously improved in recent years. However, there is still a difficulty in the treatment of metastatic PCa at present. According to the data from the US Surveillance, Epidemiology and End Results (SEER) database, the survival rate of metastatic PCa is only 28%[2]. It is noteworthy that localized and metastatic PCa cases account for 85%[3] and 15% of new PCa cases in the United States, respectively[2]. In contrast, the proportion of localized PCa in Japan is close to 50%[4]. However, domestic multi-center research data has shown that this proportion exceeds 50% in China. Meanwhile, a large number of patients have lost the opportunity of radical cure due to the late stage when initially diagnosed. Therefore, it is believed that early detection of PCa and assessment of its metastatic risk, as well as targeted treatment are of extreme importance to PCa therapy.

Micro-ribonucleic acids (miRNAs) are a class of non-coding single-stranded small ribonucleic acid (RNA) molecules with approximately 20-24 nucleotides in length. Its physiological function is mainly to regulate the expression of post-transcriptional genes in organisms. Currently, more than 28,000 miRNA molecules have been discovered[5]. Genomics has found that miRNA regulates nearly one-third of human genes. MiRNA can reduce the biological stability of the target

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gene messenger RNA (mRNA). Meanwhile, it indirectly degrades target gene mRNA, or directly suppresses the translation of target gene mRNA by binding to a specific target of 3'-untranslated region (UTR). Therefore, they may further inhibit target gene from translating into related proteins, eventually leading to conduction abnormalities in the corresponding signaling pathways[6]. MiRNAs have been proved to be involved in the mammalian development and homeostasis regulation. Recently, many studies[7-9] have found that miRNAs play a crucial role in the initiation, development, metastasis and recurrence of cancer.

MiR-203 is a small molecule first identified in skin cells. Sonkoly et al[10] have shown that miR-203 is correlated with skin inflammation. It is also a specific miRNA of skin, mainly distributed in mature epithelial cells. The expression level of miR-203 in psoriasis and allergic eczema is significantly higher than that of healthy normal skin cells. However, researchers have found that the expression level of miR-203 is significantly decreased in esophageal cancer tissues, which is distinguished from normal esophageal tissues. With the development of the tumor, the expression level of miR-203 molecule gradually decreases[11,12]. Subsequently, significant changes in the expression level of miR-203 are also observed in ovarian cancer[13], oral cancer[14] and lung cancer[15,16].

In our work, we detected the expression of miR-203 in PCa tissue specimens and cell lines. Furthermore, we explored its role in PCa cells through cell experiments.

Patients and Methods

PCa Tissues and Cells

From July 2015 to September 2017, 55 PCa patients aged 45-65 who underwent treatment in Taizhou People's Hospital and Xinghua People's Hospital enrolled in this study. Complete data were collected from PCa patients. All specimens were immediately stored in liquid nitrogen after surgical resection. Signed informed consent was obtained from each patient before the study, that has been approved by the Ethics Committee of Taizhou People's Hospital and Xinghua People's Hospital.

Human PCa cell line (DU-145) and human prostatic epithelial cell line (RWPE-1) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). DU-145 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% newborn fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in an incubator with 5% CO_2 at 37°C. After fusion, the cells were digested with 0.25% trypsin and passaged. Subsequently, the cells in the logarithmic growth phase were collected for the following experiments.

Target Prediction and Luciferase Reporter Gene Assay

Bioinformatics online sites (miRBase, TargetScan, and PicTar) predicted the targeted binding of miR-203 to the regulator of G-protein signaling 17 (RGS17). On this basis, the 3'-UTR sequence of RGS17 was amplified in DU-145 cell genomic DNA *via* Polymerase Chain Reaction (PCR). The Luciferase reporter vector pGL3 (pGL3-RSG17-WT) was inserted. Meanwhile, mutant vector (pGL3-RSG17-MUT) was constructed as well. The above plasmids were co-transfected with miR-203 mimics into the DU-145 cell for 48 h. Finally, the Luciferase activity was measured according to the instructions of Dual-Luciferase kits (Promega, Madison, WI, USA).

Cell Transfection

DU-145 cells were first inoculated into 96-well plates at a density of approximately 1×10^6/well. 0.18 mL of DMEM was added to each well, followed by incubation for another 24 h. After DU-145 cell adherence, cell transfection was performed. MiR-203 mimics (40 nmol/L) or LV-RSG17 (20 nmol/L) was transfected into cells according to the instructions of Lipofectamine^{TM}2000 (Invitrogen, Carlsbad, CA, USA). Relevant indicators were detected after 48 h.

Three groups were established: NC group (negative control), miR-203 mimics (DU-145 cell transfected with miR-203 mimics) and mimics + RGS17 (DU-145 cell transfected with miR-203 mimics and si-RGS17).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The extracted RNA was reversely transcribed into complementary DNA (cDNA). QRT-PCR was carried out using Roach480 fluorescence qRT-PCR instrument. Specific reaction

conditions were as follows: pre-denaturation at 95°C for 10 min, denaturation at 95°C for 30 s, renaturation at 55°C for 30 s, and extension at 72°C for 2 min, for a total of 35 cycles. The expression levels of the target genes (miR-203 and RGS17) were quantified by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: RGS17, F: 5'-CCAGGAACCCCTCCTTACTC-3', R: 5'-TGTGTCCGAAGGACTAGGGA-3'; miR-203, F: 5'-AGGGGTGTAACATCCTCGACTG-3', R: 5'-AGTGTCGTGGAGTTTGCCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot (WB) Analysis

Proteins were extracted from DU-145 cells using protein lysate. The concentration of extracted protein was quantified *via* the bicinchoninic acid (BCA) assay (Pierce, Waltham, MA, USA). Extracted proteins were loaded, electrophoresed and transfected onto membranes. Then, the membranes were incubated with primary antibodies of rabbit anti-human RGS17 (1:1000) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:2000) at 4°C overnight. After being washed, the membranes were incubated again with the secondary antibody of horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (IgG) (1:2500) at 37°C for 1 h. After washing with Tris-Buffered Saline and Tween-20 (TBST), the color was developed using the enhanced chemiluminescence (ECL) method (Thermo Fisher Scientific, Waltham, MA, USA).

Cell Proliferation and Cell Cycle

48 h after transfection, the cells were digested with trypsin, centrifuged and collected. Then the cells were inoculated into 96-well plates (1×10^3/well). 5 repeated wells were set in each group. Approximately 200 μL of phosphate-buffered saline (PBS) was added to each well around the 96-well plate to avoid evaporation. The optical density (OD) value at the wavelength of 490 nm was detected according to the instructions of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) kits (Sigma-Aldrich, St. Louis, MO, USA) at 1, 2, 3, 4 and 5 d after transfection, respectively. Cell viability was finally analyzed.

For the colony formation assay, the cells were digested, collected and inoculated into new 6-well plates (1×10^3/well) after transfection, followed by culture for another 7 d. After the culture medium was discarded, the cells were washed with PBS three times and fixed with 1 mL methanol for 15-20 min. After the removal of methanol, the cells were fixed with 0.1% crystal violet solution for 20-30 min. The residual crystal violet solution was washed away slowly, and the plate was dried. Finally, the cells were photographed, counted and analyzed.

For cell cycle, the transfected cells were collected and washed with PBS three times. Then the cells were incubated with 70% ethanol solution at 4°C overnight. After washing twice with PBS, the supernatant was removed and the cells were re-suspended with about 100 μL RNase, followed by incubation *via* water bath at 37°C for about 30 min. 100 μL propidium iodide (PI) (Solarbio, Beijing, China) was added for incubation at 4°C for about 30 min in the dark. Flow cytometry was used to detect red fluorescence at an excitation wavelength of 488 nm, and the data were collected for analysis.

Cell Invasion and Migration Assays

24 h after transfection, the cells were digested, centrifuged and counted. Then they were diluted to 1.5×10^5 cells/mL cell suspension with serum-free medium. The cell suspension was added to the transwell upper chamber (200 μL/well); meanwhile, 600 μL of complete medium was added to the transwell lower chamber. After culturing in an incubator for 24 h, the cells fixed with formaldehyde for 15 min and stained with crystal violet for 15 min. The cells on the inner membrane were wiped gently with a cotton swab. The number of cells passing through the filter membrane was counted. 4 high-power fields (×200) were randomly selected for each sample.

The transwell chamber membrane was evenly coated with 50 μ Matrigel gel (l0.2 μg/μL) and incubated at 37°C for 15 min, so that the Matrigel gel was coagulated. The subsequent procedure was the same as the transwell migration assay.

Statistical Analysis

Statistical analysis was performed with Student's *t*-test or *F*-test. All *p*-values were two-sided, and $p<0.05$ was considered statistically significant. Prism 6.02 software (La Jolla, CA, USA) was used for all statistical analyses.

Results

MiR-203 Expression in PCa Tissues and Cells

The expression of miR-203 in PCa tissues and cells was detected by qRT-PCR. The results demonstrated that the expressions of miR-203 in PCa tissues and cells were both significantly lower than those of normal tissues ($p<0.001$) and cells ($p<0.01$) (Figure 1A, 1B).

RGS17 Was a Direct Target of MiR-203 in PCa Cell

Online database predicted that there was a miR-203 binding site in RSG17 3'-UTR. Luciferase reporter gene assay indicated that miR-203 overexpression significantly decreased the activity of wild-type RGS17 Luciferase ($p<0.001$). However, no statistically significant difference was found in the activity of mutant-type Luciferase ($p>0.05$) (Figure 2A). The above results further revealed that RGS17 was a target gene of miR-203.

The transfection efficiency was detected and confirmed by qRT-PCR assay ($p<0.001$) (Figure 2B). Furthermore, the regulation of RGS17 by miR-203 was also reflected in WB assay results. Compared with the control group, the protein expression of RGS17 in DU-145 cells of the miR-203 mimics group was significantly repressed ($p<0.01$) (Figure 2C).

MiR-203 Suppressed Proliferation of PCa Cells

In MTT experiment, statistically different proliferation curves were found between the miR-203 mimics group and the miR-NC group. On the fifth day, the absorbance at 490 nm in DU-145 cells of the miR-203 mimics group was significantly lower than that of the miR-NC group ($p<0.001$) (Figure 3A). This indicated that the proliferative capacity of PCa cells was hindered by miR-203 intervention.

Corresponding to this result, the number of colonies in the miR-203 mimics group was significantly less than that of the control group ($p<0.01$) (Figure 3C, 3D). The above results suggested that miR-203 could markedly reduce the colony formation capability of PCa cells. Meanwhile, our findings showed the effect of miR-203 on the tumorigenic ability of PCa cells. Thus, the effect of miR-203 on cell cycle of PCa cells was also detected.

At the same time, flow cytometry demonstrated that the cell cycle of DU-145 cells transfected with miR-203 mimics was arrested in the G0/G1 phase (Figure 3B). The proportion of PCa cells in G0/G1 phase was significantly increased ($p<0.05$). However, the proportion of cells in the S and G2/M phase was remarkably decreased ($p<0.05$). The data explained why the proliferation of DU145 cells in the miR-203 mimics group was significantly restricted in MTT assay.

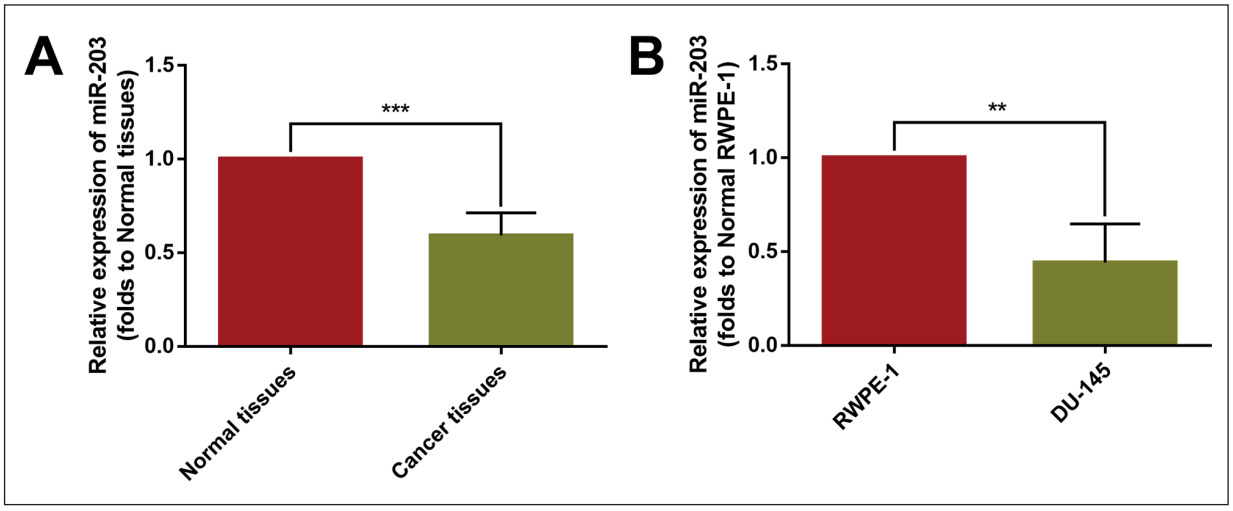

Figure 1. The expressions of miR-203 in PCa tissue samples and the corresponding adjacent normal tissues, as well as PCa cells and human prostatic epithelial cells (RWPE-1). ***A,*** Difference in the expression of miR-203 between PCa tissues and the corresponding adjacent normal tissues (***$p<0.001$ compared with adjacent normal tissue). ***B,*** Difference in the expression of miR-203 between DU-145 cells and control RWPE-1 cells (**$p<0.01$ compared with RWPE-1 cells).

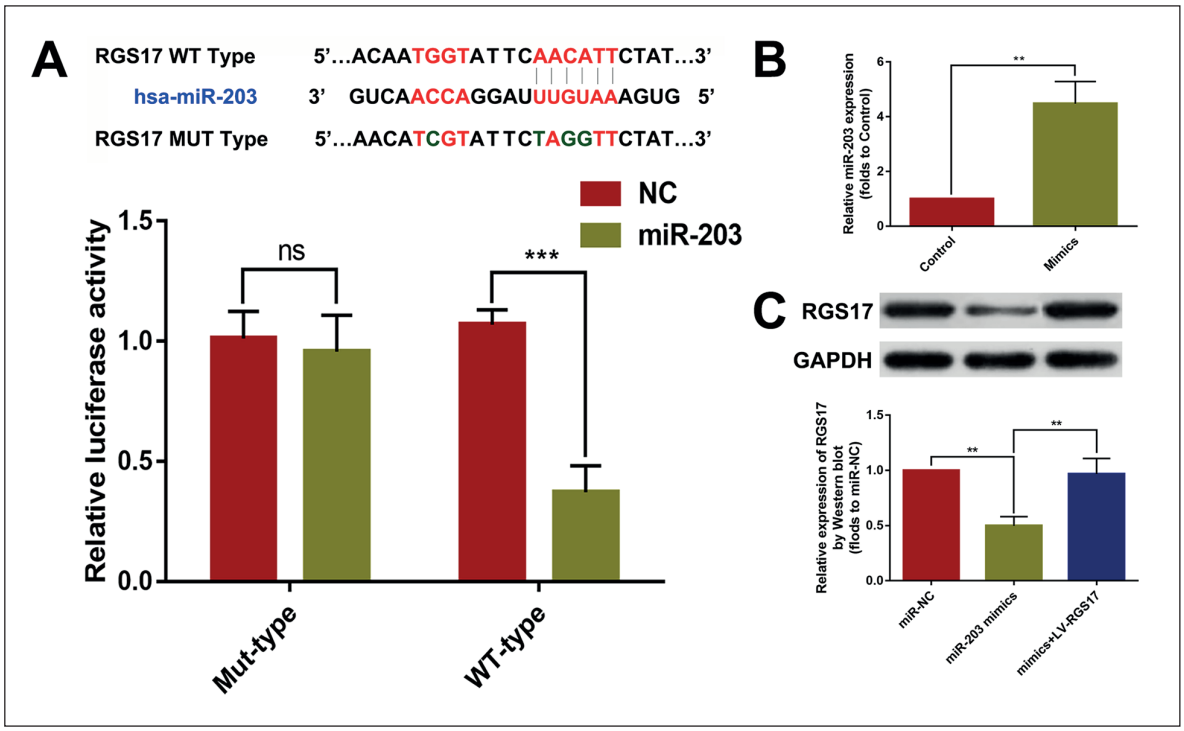

Figure 2. RGS17 was a direct and functional target of miR-203. ***A,*** Diagram of putative miR-203 binding sites of RGS17 and relative activities of Luciferase reporters. ***B,*** Transfection efficiency detected by qRT-PCR (ns $p>0.05$, $^{**}p<0.01$). ***C,*** The protein expressions of RGS17 in PCa cell lines after transfection. Data were presented as means ± standard deviations ($^{**}p<0.01$).

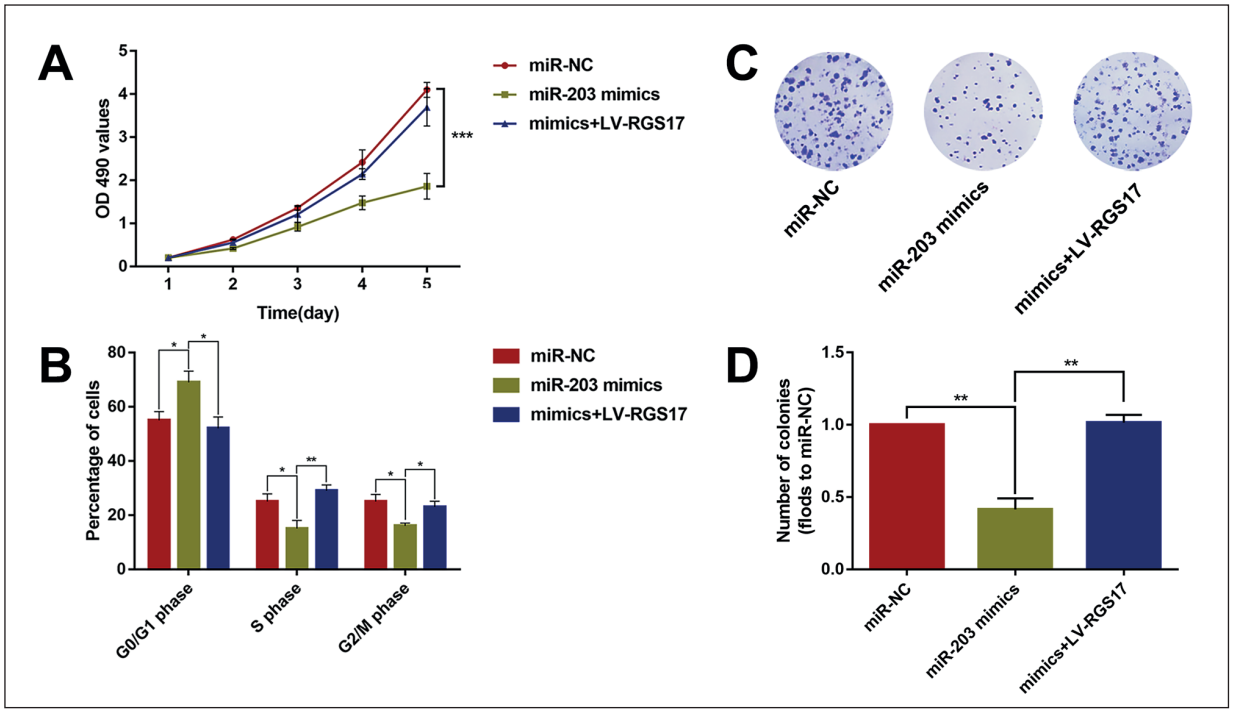

Figure 3. ***A-B,*** MiR-203 decreased cell proliferation ($^{***}p<0.001$) and arrested cell cycle in G0/G1 phase ($^{*}p<0.05$, $^{**}p<0.01$). ***C-D,*** Assessment of colony formation. Data were presented as means ± standard deviations ($^{**}p<0.01$).

MiR-203 Inhibited Invasion and Migration of PCa Cells

The effect of miR-203 on PCa cells was also found in the control of its metastatic ability. The invasion and migration ability was regarded as a criterion for cell metastatic ability in many experiments. In our study, transwell results indicated that the number of invasive and migrating cells in the miR-203 mimics group was significantly less than that of the miR-NC group ($p<0.01$, $p<0.05$) (Figure 4).

Performance of RGS17 in PCa Cells After Reduction

To rigorously verify the role of RGS17 in PCa cells, we additionally added RGS17 exogenous intervention group in miR-203 overexpressing cells in each experimental session. The results showed that after the expression of RGS17 was restored, the abnormal proliferation and metastatic ability reappeared in PCa cells (Figure 3, 4). These experiments confirmed the role of MIR-203/RGS17 in PCa cell competence.

Discussion

Some studies[17-19] demonstrated that miRNAs can serve as an important molecular marker for early diagnosis and prognosis evaluation of PCa. Meanwhile, miRNA dysregulation is closely related to the occurrence and development of PCa. It has been confirmed that miR-203 involves in the progression of various malignant tumors. MiR-203 can promote malignant biological behavior of tumor cells by interacting with multiple target genes[20-22]. In this study, the expression levels of miR-203 in PCa tissues and corresponding tumor-adjacent tissues were detected using qRT-PCR. The results revealed that the expression level of miR-203 in PCa tissues was significantly higher than that of tumor-adjacent tissues. Such a phenomenon was also observed in PCa cell lines.

MiRNAs play a corresponding biological role only by binding to target mRNA 3'-UTR. Therefore, the discovery and identification of the target genes are of great significance to study the function of miRNAs. The binding targets of miR-203 and RGS17 in 3'-UTR were found through an online prediction software. Luciferase reporter gene assay, as a gold standard for the detection of miRNA target, can detect the gene expression in an extremely sensitive and efficient mode. In our study, the Luciferase reporter gene assay indicated that miR-203 could significantly decrease the activity of wild-type RGS17 Luciferase rather than the mutant-type Luciferase. This showed that RGS17 was a definitive target of miR-203 in PCa cells.

As a regulator of the G-protein signaling pathway, RGS has become a research focus in recent years. Located on the inner surface of cell membrane, G-protein is a bridge between receptors on the surface of the cell membrane (G-protein coupled receptors, GPCRs) and intracellular effectors[23]. G-protein regulates the activity of downstream effectors, such as adenylate cyclase (AC), phospholipase C and mitogen-activated protein kinase (MAPK) signaling pathways[24]. RGS can regulate the frequency and duration of the G-protein signaling transduction. The distribution and activity of intracellular RGS proteins determine the efficiency of the G-protein signaling transduction as well as the balance regulation among differ-

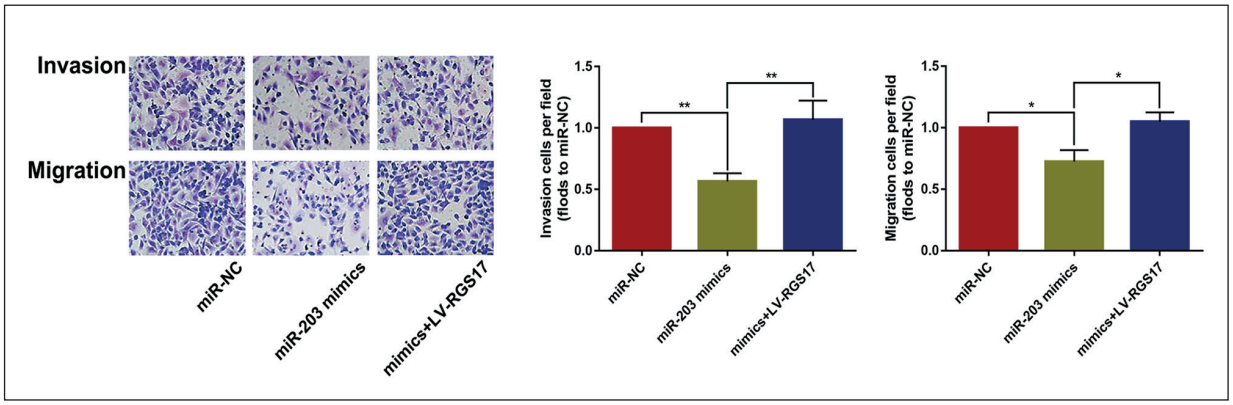

Figure 4. MiR-203/RGS17 axis inhibited the invasion and migration of PCa cells. RGS17 overexpression attenuated the suppressive effect of miR-203 on DU-145 cells (*$p<0.05$, **$p<0.01$) (Magnification: 40×).

ent G-protein signaling pathways[25]. Currently, approximately more than 20 RGS homologs have been identified. The main function of RGS17 is to accelerate hydrolysis of guanosine triphosphate (GTP) and inactivate G-protein. James et al[26] have found that the expression levels of RGS17 in lung cancer and PCa are significantly increased. RGS17 maintains tumor cell proliferation through the induction of cAMP signaling and CREB phosphorylation. Meanwhile, RGS17 has also been shown to play an important role in the maintenance of cell proliferation in malignant tumors[26,27]. Therefore, some scholars believe that researching RGS17 is of important significance for the study on tumor-targeted drugs.

In this work, we demonstrated that miR-203 served as a tumor suppressor. Meanwhile, its expression was significantly decreased in PCa tissues and cells than that of normal controls. Targeted regulation of miR-203 on RGS17 was confirmed by Luciferase reporter gene assay. Western blot indicated that the protein expression of RGS17 was markedly down-regulated after miR-203 overexpression in PCa cells. Furthermore, the proliferation, invasion, metastasis and colony capabilities of PCa cells were also significantly inhibited. The above findings laid the foundation for further exploring the role of miR-203 in downstream effectors of specific pathways in PCa. In addition, it played a vanguard role in the development of drug targets in the future.

Unfortunately, this work did not have the opportunity to obtain better confirmation from *in vivo* experiments. This would be our next research direction.

Conclusions

MiR-203 served as a tumor suppressor gene in PCa in this study. Through targeting RGS17, miR-203 significantly controlled the malignant behavior of PCa cells. Our findings revealed that miR-203/RGS17 axis might be a potential therapeutic target for the treatment of PCa.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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