

miR-593-3p Promotes Proliferation and Invasion in Prostate Cancer Cells by Targeting ADIPOR1

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Background: Accumulating evidence has indicated that dysregulation of microRNAs (miRNAs) contributes to the tumorigenesis of prostate cancer (PCa). Nevertheless, the role of miR-593-3p in the development of PCa remains unclear. The objective of this study was to investigate the role and mechanisms of miR-593-3p in PCa cells.

Methods: RT-PCR was used to detect the expression levels of miR-593-3p. CCK-8, colony formation, spheroid formation and transwell assays were performed to examine the proliferation, migration and invasion of C4-2, DU145 and RWPE-1 cells. And then, transcriptome sequencing, dual-luciferase reporter assay and Western blot were taken to identify the target gene and downstream mechanisms of miR-593-3p.

Results: Here, we found that miR-593-3p promoted PCa cell proliferation, colony formation, spheroid formation, migration and invasion. Further mechanistic studies revealed that miR-593-3p possessed binding sites of ADIPOR1 3'-UTR and played an important role in 5'-AMP-activated protein kinase (AMPK) signaling pathway and epithelial-mesenchymal transition (EMT) process. In addition, the transfection of si-ADIPOR1 also enhanced the PCa cell proliferation and invasion.

Conclusion: Our study provides an empirical investigation of miR-593-3p, which exerts oncogenic function through targeting ADIPOR1 in PCa cells.

Keywords: miR-593-3p, ADIPOR1, PCa, proliferation, invasion

Introduction

Prostate cancer (PCa) is one of the most commonly diagnosed malignant tumors in males worldwide.^{1,2} It accounted for 13.5% of all cancer incidences and 6.7% of cancer-related deaths in males, in 2018³. Although the mortality rate of PCa has greatly decreased over the past decades due to significant advances in early diagnosis and therapy, the development of androgen resistance and distant metastasis cause most PCa to progress to an aggressive state.⁴⁻⁶ Therefore, it is crucial to investigate the molecular mechanisms of PCa progression and metastasis.

MicroRNAs (miRNAs) are a class of short non-coding RNAs, composed of 18–25 nucleotides, which regulate gene function by binding to the 3'-UTR of target mRNAs.^{7,8} It has been reported that miRNAs influence the cell cycle, differentiation and apoptosis.⁹⁻¹¹ Furthermore, emerging investigations have shown that dysregulation of miRNAs contributes to the progression or suppression of PCa.¹²⁻¹⁴

Increasing evidence suggests that miR-593-3p participates in different biological processes, in multiple malignancies. It has been reported that miR-593-3p suppresses tumor growth and inhibits epithelial-to-mesenchymal transition (EMT) phenotype in gastric cancer cells.¹⁵ Another study suggests that hsa_circ_0136666

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initiates osteosarcoma tumorigenesis via the miR-593-3p/ZEB2 pathway.¹⁶ In non-small cell lung cancer, the miR-593-3p/CCND2 axis contributes to the promotion of cell growth and invasion.¹⁷ In addition, high expression of miR-593-3p in peritoneal lavage fluid predicts poor prognosis of patients with pancreatic cancer.¹⁸ However, the relationship between miR-593-3p and tumor progression in PCa remains unknown.

Adiponectin receptor 1 (ADIPOR1) is a receptor for adiponectin, which regulates fatty acid catabolism and glucose levels.^{19,20} Previous studies have demonstrated that inhibition of ADIPOR1 expression promotes tumorigenesis and metastasis in hepatocellular carcinoma, glioblastoma and breast cancer.^{21–23} Research on the underlying mechanism has confirmed that binding of adiponectin to encoded protein results in activation of the 5'-AMP-activated protein kinase (AMPK) signaling pathway.²¹ Another study demonstrated that adiponectin is a potential therapeutic target for PCa,²⁴ and the role of adiponectin receptor in regulation of angiogenesis in PCa has also been suggested.²⁵ Although the role of ADIPOR1 has been reported in some cancers, the interaction of ADIPOR1 and miR-593-3p needs to be further studied.

In this study, we found that miR-593-3p promoted proliferation, colony formation, migration and invasion of C4-2 and DU145 cells. There were no significant biological effects in prostate normal epithelial cells transfected with miR-593-3p. Mechanistically, ADIPOR1 was confirmed to bind to miR-593-3p by bioinformatics and luciferase reporter assay. Moreover, miR-593-3p also affected downstream AMPK signaling pathway and EMT process. Further study revealed that the inhibition of ADIPOR1 in PCa cells has the same effects as the over-expression of miR-593-3p. These findings indicate that miR-593-3p promotes cell growth and invasion via ADIPOR1 in PCa.

Materials and Methods

Cell Culture and Transfection

C4-2 and DU145 human PCa cell lines were obtained from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI1640 (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA). Human normal prostate epithelial cells, RWPE-1, were grown in Keratinocyte serum-free medium (Gibco, Carlsbad, CA, USA) supplemented with epidermal growth factor and

bovine pituitary extract (KGM). All cells were maintained in a 5% CO₂ atmosphere. After cultured in 6-well plates for 24 h, cells were transfected with miR-593-3p mimics and mimics negative control (20 nM, GenePharma, Shanghai, China) by RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used to transfect si-Control and si-ADIPOR1 (20 nM, GenePharma, Shanghai, China), and transfection efficiency was detected after 2 days. The RNA oligo sequences were listed in [Table S1](#).

Dual-Luciferase Reporter Assay

To investigate whether the 3'-UTR region of ADIPOR1 interacts with miR-593-3p, the binding sites of miR-593-3p to ADIPOR1 were predicted via TargetScan website. Wild-type and mutant-type reporter plasmid of ADIPOR1 (ADIPOR1-WT and ADIPOR1-MUT) were designed and synthesized (Genechem, Shanghai, China). Then C4-2 cells (1×10⁵ cells/well) were seeded into 24-well plates and co-transfected with ADIPOR1-WT/ADIPOR1-MUT plasmids (0.3 µg/mL), both miR-593-3p mimics as well as mimics negative control, separately, via Lipofectamine 3000. After transfection for 24 h, luciferase activity was measured by dual-luciferase reporter system (Promega, Madison, WI, USA).

Western Blot

Cell lysates were harvested from 6-well plates by radio-immunoprecipitation assay (RIPA; Beyotime, China) lysis buffer containing protease inhibitors, and centrifuged at 12,000×g for 15 min. Proteins were separated by 10% polyacrylamide gels and transferred to polyvinylidene fluoride membranes (PVDF; Millipore, Bedford, MA, USA). After blocking in 5% skim milk for 1 h, membranes were incubated with primary antibodies at 4 °C overnight. Thereafter, membranes were washed with TBST solution and incubated with HRP-conjugated secondary antibodies for 1 h. The film was developed and the protein bands were analyzed. ImageJ software was used to determine protein levels by densitometry. The primary antibodies used were: anti-p-AMPK (CST, Danvers, MA, USA; 2535; 1:1000), anti-AMPK (CST; 5831; 1:1000), anti-E-cadherin (CST; 3195; 1:1000), anti-ADIPOR1 (Abcam, Cambridge, UK; ab126611; 1:3000), and anti-GAPDH (CST; 2118; 1:5000).

Quantitative Real-Time PCR (RT-PCR)

After transfection of miR-593-3p mimics and mimics negative control for 2 days, cells were collected and total RNA was isolated by RNA-Quick Purification kit (Yishan Biotech, Shanghai, China). Reverse transcription was performed utilizing PrimeScript™ RT reagent kit (Takara, Dalian, China). Real-Time PCR was performed using SYBR Green gene expression assay (Takara, Dalian, China) on the ABI fast real-time PCR system according to the manufacturer's instructions. miR-593-3p expression levels were normalized to U6, and results were analyzed by $2^{-\Delta\Delta Ct}$ method. The primer sequence of miR-593-3p was: 5'-TGTCTCTGCTGGGGTTTCT-3'. The mRQ 3' primer, U6 forward primer and U6 reverse primer were provided within the Takara reagent kit. The vascular endothelial growth factor (VEGF) expression levels were normalized with GAPDH. The primers were: VEGF-A (forward) 5'-AGCCTTG CCTTGCTGCTCTA-3', VEGF-A (reverse) 5'-GTGCTGGC CTTGGTGAGG-3', VEGF-D (forward) 5'-TCCCATCGG TCCACTAGGTTT-3', and VEGF-D (reverse) 5'-AGGGCT GCACTGAGTTCTTTG-3'.

Cell Proliferation and Colony Formation Assay

Cell proliferation (5×10^3 cells/well) was determined by real-time cell analysis (RTCA) assay using xCELLigence system (ACEA Biosciences, San Diego, CA, USA). The CCK-8 assay was used to quantify cell viability. Briefly, 5×10^3 cells were inoculated into 96-well plates after transfection. Subsequently, 10 μ L CCK-8 reagents (Beyotime, Shanghai, China) were added and absorbance was measured at 490 nm after incubation for 4 h. For colony formation assay, cells (500/well) were cultured in 6-well plates. When clones were observed clearly under the microscope, cells were rinsed twice with phosphate buffered saline, and fixed with methanol for 15 min, followed by Coomassie brilliant blue (Beyotime, Shanghai, China) staining for 10 min. The number of cell clones was scanned and statistically analyzed.

Spheroid Formation Assay

PCa cells were suspended in 12-well low adherent plate at a density of 4×10^3 cells in 1 mL RPMI1640 medium supplemented with B27 (1: 50, Gibco, Carlsbad, CA, USA), EGF (20 ng/mL, Gibco, Carlsbad, CA, USA), FGF (20 ng/mL, Gibco, Carlsbad, CA, USA), and 100 IU/mL penicillin-streptomycin (Gibco, Carlsbad, CA,

USA).^{26,27} 100 μ L medium was supplemented every two days. The sphere density and diameter were monitored every day. Finally, the total number of spheroids were counted and analyzed on day 9.

Transwell Assay

After digestion and centrifugation, cells were resuscitated in serum-free medium and centrifuged again. 2×10^5 cells (200 μ L) were added to the upper layer of migration chamber (Corning, NY, USA), while 800 μ L complete medium was added to the lower layer. After incubation for 24 h, the chamber was taken out, and the cells were fixed with methanol and stained with Coomassie brilliant blue. Non-migrating cells were gently wiped with cotton swabs. Finally, cells were photographed and counted under an optical microscope. The migration ability of PCa cells was detected by Transwell without matrigel, and the invasion ability of PCa cells was determined by Transwell with matrigel gel.

Wound Healing Assay

The two-well culture insert (Ibidi GmbH, Germany) was placed in a 6-well plate. Cell suspension was prepared and adjusted to 7×10^5 cells/mL with complete medium, and 70 μ L cell suspension was pipetted into each well. After incubation for 24 h, the culture insert was gently extracted by using sterile tweezers, and 2 mL complete medium was added for further 24 h incubation. Wound closure was monitored using an optical microscope, over a period of 24 h.

Statistical Analysis

All data obtained from three independent experiments were analyzed by GraphPad Prism 8.0 software (GraphPad Software, La Jolla, CA, USA). One-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple-comparison test and *t*-test were performed for statistical comparisons. All data are presented as the mean \pm standard deviation (SD). $P < 0.05$ was considered to be statistically significant.

Results

miR-593-3p Promotes PCa Cell Proliferation, Colony Formation and Spheroid Formation

To investigate the functions of miR-593-3p in PCa, miR-593-3p was overexpressed in C4-2 and DU145 cells by transfection with miR-593-3p mimics and its expression levels quantified by RT-PCR. The results showed that miR-593-3p levels were

higher in the overexpressed miR-593-3p mimics group compared with the control group (Figure 1A). miR-593-3p overexpression obviously enhanced the cell proliferation abilities of C4-2 and DU145 cells (Figures 1B and C). However, miR-593-3p overexpression had no significant effects on normal prostate cell proliferation (Figure S1A). Colony formation and spheroid formation assays showed that overexpression of miR-593-3p resulted in more colonies and spheroids, compared with negative control in PCa cells after 9 days of incubation (Figures 1D and S1B).

miR-593-3p Promotes PCa Cell Migration and Invasion

Although miR-593-3p has been reported to participate in EMT process in several tumors, its role in PCa remains unclear. Therefore, migration, invasion and wound healing assays were performed to evaluate cell motility. Overexpression of miR-593-3p enhanced PCa cell migration and invasion abilities (Figure 2A and B), and promoted wound healing (Figure S2B). However, the invasiveness of normal prostate cells did not change after transfection with miR-593-3p mimics (Figure S2A).

Downstream Expression Profiling of miR-593-3p Overexpression

Next, Gene expression profiling of miR-593-3p overexpression in C4-2 cells was subsequently evaluated. The differentially expressed genes were presented using volcano plot. There were 114 upregulated and 65 downregulated genes with fold change >1.5 and $P < 0.05$ (Figure 3A). To identify the potential target genes, a heatmap showing the top 15 genes with the highest differential expression was constructed (Figure 3B). Then Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were used to explain the differential expression (Figures S3A and S3B, 3C and D). It was observed that these genes were involved in previously reported pathways such as metabolism, adherens junction, and AMPK signaling pathway.

miR-593-3p Directly Targets ADIPOR1 and Affects the AMPK Signaling Pathway

To elucidate the downstream mechanisms of miR-593-3p, the TargetScan website was used to identify target genes and predict binding sites. The transcriptome sequencing results showed that ADIPOR1 was one of the most

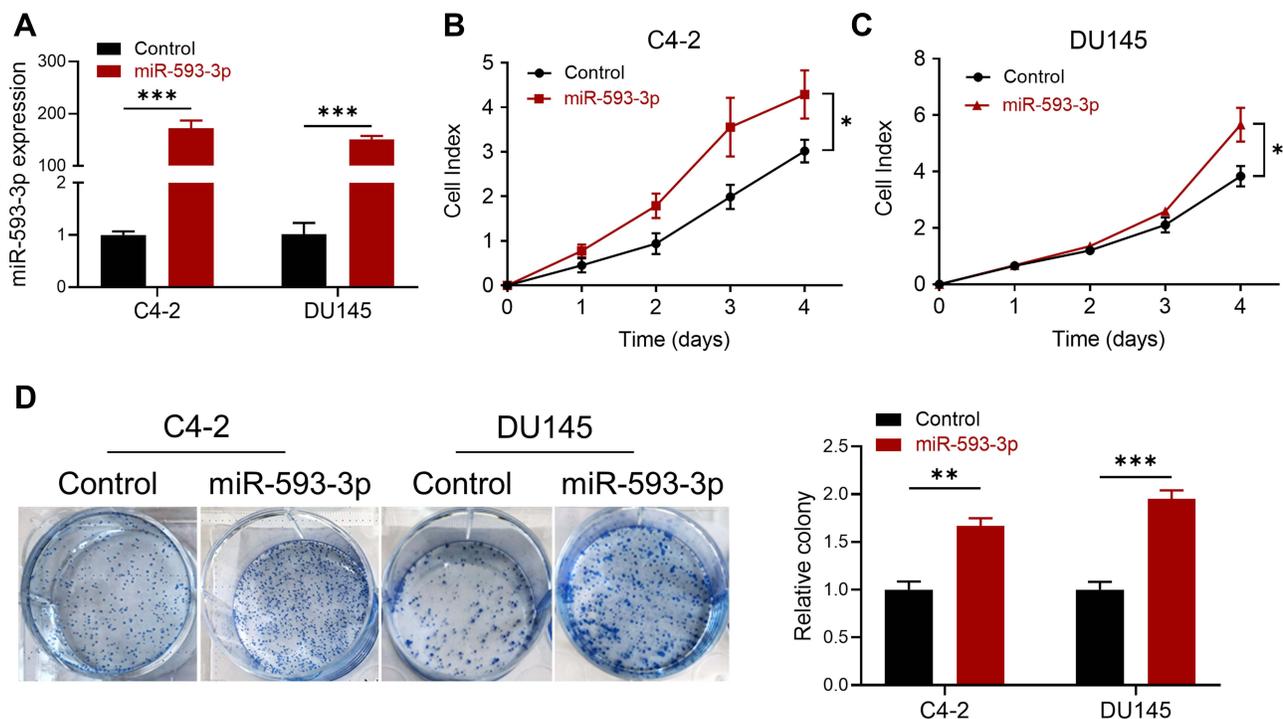


Figure 1 miR-593-3p promotes cell proliferation and colony formation in PCa cells. (A) RT-PCR was used to detect the relative expression of miR-593-3p in C4-2 and DU145 cells transfected with miR-593-3p mimics and negative control mimics. (B and C) Evaluation of the effect of miR-593-3p on PCa cell proliferation, via the RTCA assay. (D) Colony formation assay was performed to determine PCa cell growth. (Data are represented as the mean \pm SD; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

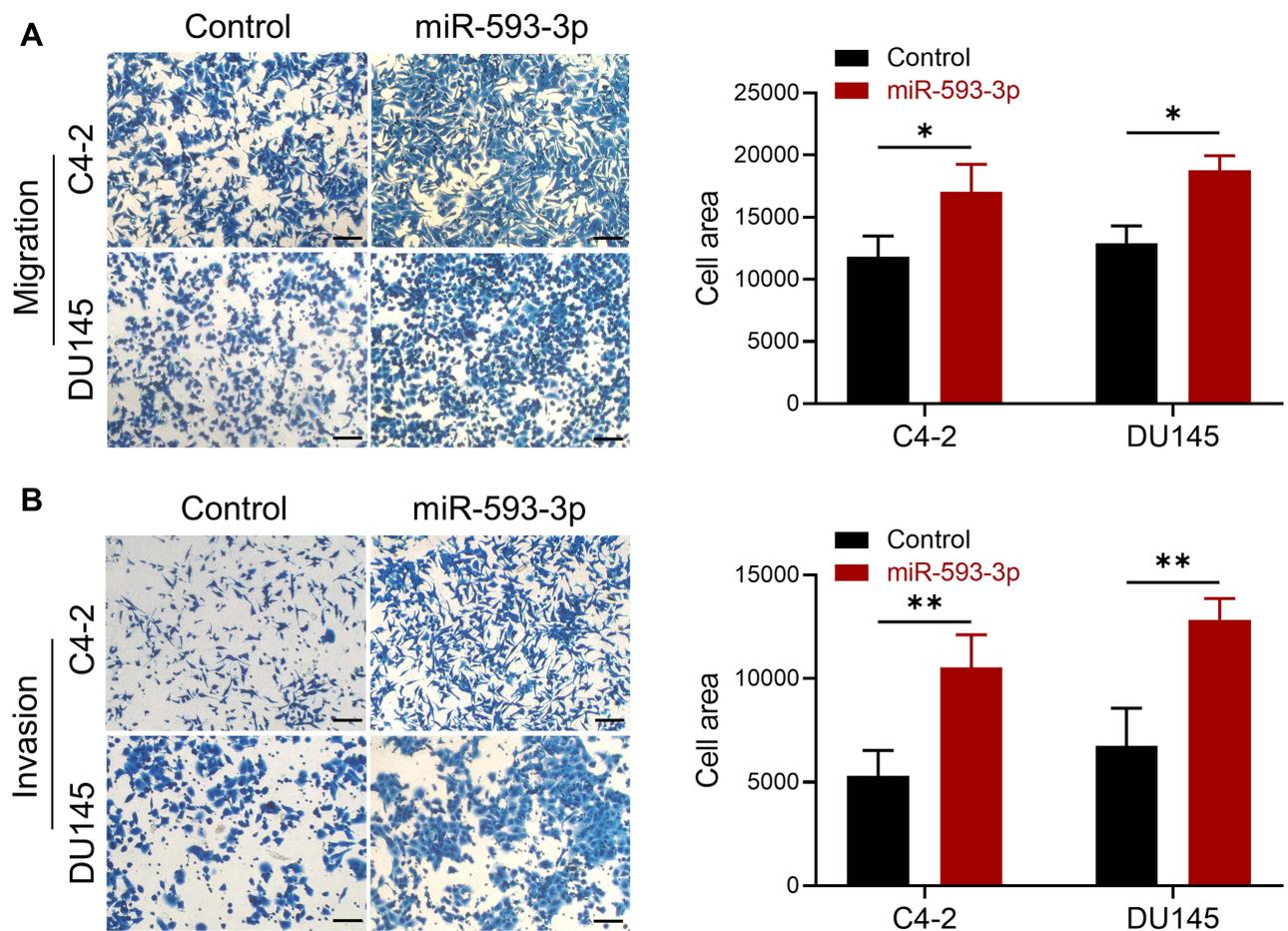


Figure 2 miR-593-3p promotes cell migration and invasion in PCa cells. (A) Cell migration results of PCa cells transfected with miR-593-3p mimics and negative control mimics. Scale bar=100 μ m. (B) Cell invasion results of PCa cells transfected with miR-593-3p mimics and negative control mimics. Scale bar=100 μ m. (Data are represented as the mean \pm SD; * P < 0.05, ** P < 0.01).

downregulated genes in miR-593-3p-overexpressing cells. Wild-type and mutant-type ADIPOR1 vectors were then constructed to perform dual-luciferase reporter analysis. miR-593-3p significantly reduced the relative luciferase activity in PCa cells transfected with the wild-type vector, but not in the mutant (Figure 4A). Suppression of ADIPOR1 in protein level was observed after transfection of miR-593-3p mimics (Figure 4B). The constitutive expression of ADIPOR1 was also detected in normal prostate epithelial cell line and PCa cell lines (Figure S4A). These findings demonstrated that ADIPOR1 was a target gene of miR-593-3p. In addition, Survival analysis showed that high ADIPOR1 expression might be associated with good overall survival and disease-free survival in PCa patients. Regrettably, the difference was not statistically significant (Figure S4B and C). Furthermore, it has been reported that ADIPOR1 plays a key role in vascularization,²⁵ therefore, the VEGF levels in PCa cells

were determined. PCR results showed that miR-593-3p overexpression significantly increased the expression of VEGF-A and VEGF-D in C4-2 cells (Figure S4D and E). KEGG pathway analysis showed the enrichment of adherens junction and AMPK signaling pathway, in line with previous studies that suggested an association between ADIPOR1 and AMPK signaling pathway or EMT process.^{21,22} As cellular energy receptors, activated AMPK regulates cell energy supplementation and cell proliferation.²⁸ Meanwhile EMT is identified as a crucial step in the early stages of PCa cells metastasis.²⁹ Given the key role of AMPK signaling pathway and EMT process, the protein levels of AMPK, p-AMPK, and E-cadherin were evaluated. As expected, overexpression of miR-593-3p inhibited the expression of p-AMPK and E-cadherin in C4-2 cells, but not the overall expression of AMPK. However, only E-cadherin expression was

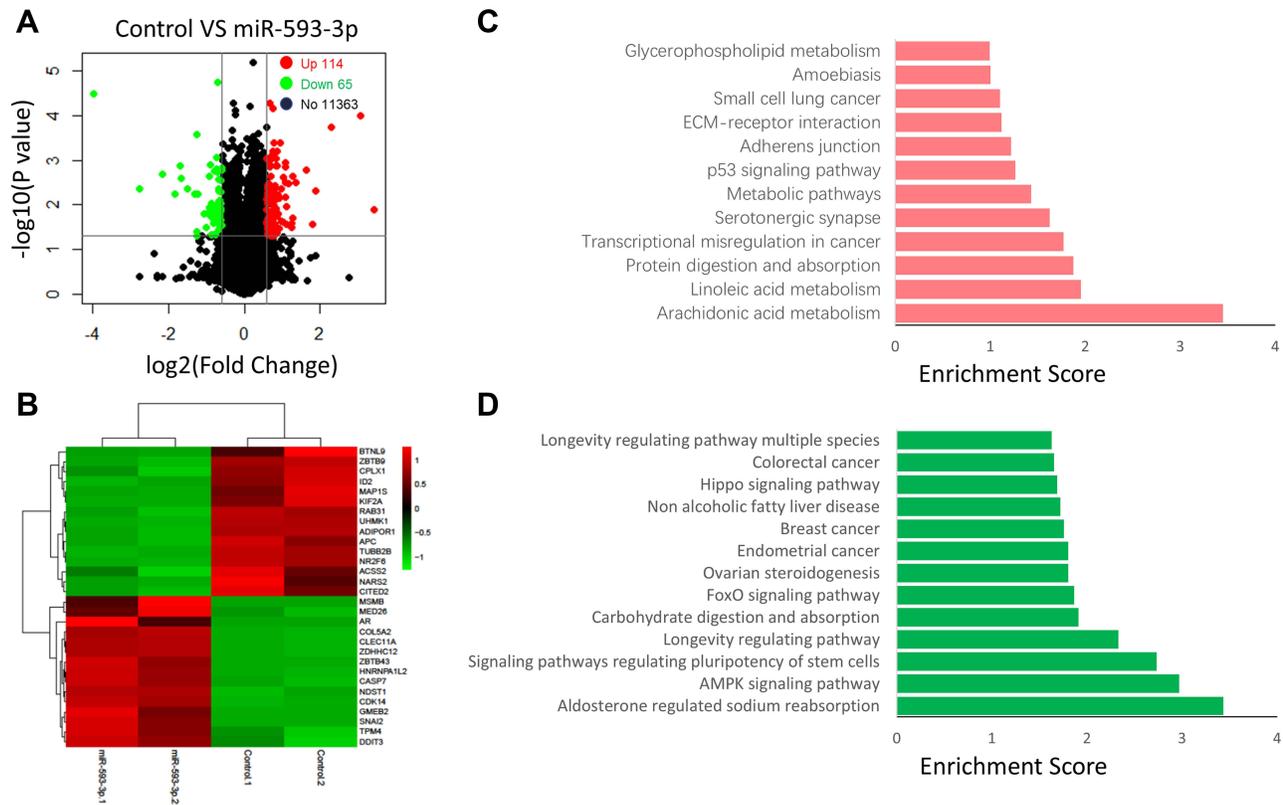


Figure 3 The transcriptome sequencing results of miR-593-3p overexpression. **(A)** The gene expression profile of PCa cells with miR-593-3p overexpression. **(B)** Heatmap of top 15 genes with the highest differential expression. **(C and D)** KEGG pathway analysis of genes with significant upregulation and downregulation.

downregulated in DU145 cells, while the variation in p-AMPK levels was not significant. (Figure 4C).

Depletion of ADIPOR1 Promotes PCa Cell Growth and Invasion

si-ADIPOR1 was then transfected to clarify the effect of suppressing ADIPOR1 in PCa cells (Figure 5A). The CCK-8, colony formation and transwell assays confirmed that depletion of ADIPOR1 enhanced PCa cell proliferation (Figures 5B and C), colony formation (Figure 5D), and invasiveness (Figure 5E). These data suggested that miR-593-3p could promote PCa cell growth and invasion by negatively regulating ADIPOR1.

Discussion

miRNAs have been reported to regulate nearly 30% of human genes and affect different biological processes in tumors, including growth, metastasis and drug resistance.^{30,31} Due to the heterogeneity of tumors, miR-593-3p plays different roles in multiple malignancies. In the present study, it was observed that miR-593-3p

promoted PCa cell proliferation, colony formation, and spheroid formation. In addition, miR-593-3p overexpression enhanced PCa cell migration and invasiveness.

Although the function and target gene of miR-593-3p were revealed, the mechanism of action remains unknown. AMPK, as a major regulator of lipid and glucose metabolism, plays a key role in regulating cellular homeostasis.³² Recent studies revealed that AMPK activation promotes nutrient scavenging and anabolism in PCa cells.³³ AMPK represents a link between energy homeostasis and cancer, which physiologically integrates nutritional and hormonal signals, and also regulates cell growth-related metabolic pathways to maintain intracellular ATP levels.³⁴ The application of AMPK activators may represent a promising therapeutic strategy for PCa. In this study, transcriptome sequencing, GO and KEGG pathway analysis were performed to explore biological changes in PCa cells induced by miR-593-3p. The analysis revealed that miR-593-3p regulated the AMPK signaling pathway and linoleic acid and arachidonic pathways in PCa cells. Interestingly, the variation in AMPK signaling pathway was confirmed by Western blot, while the variation of

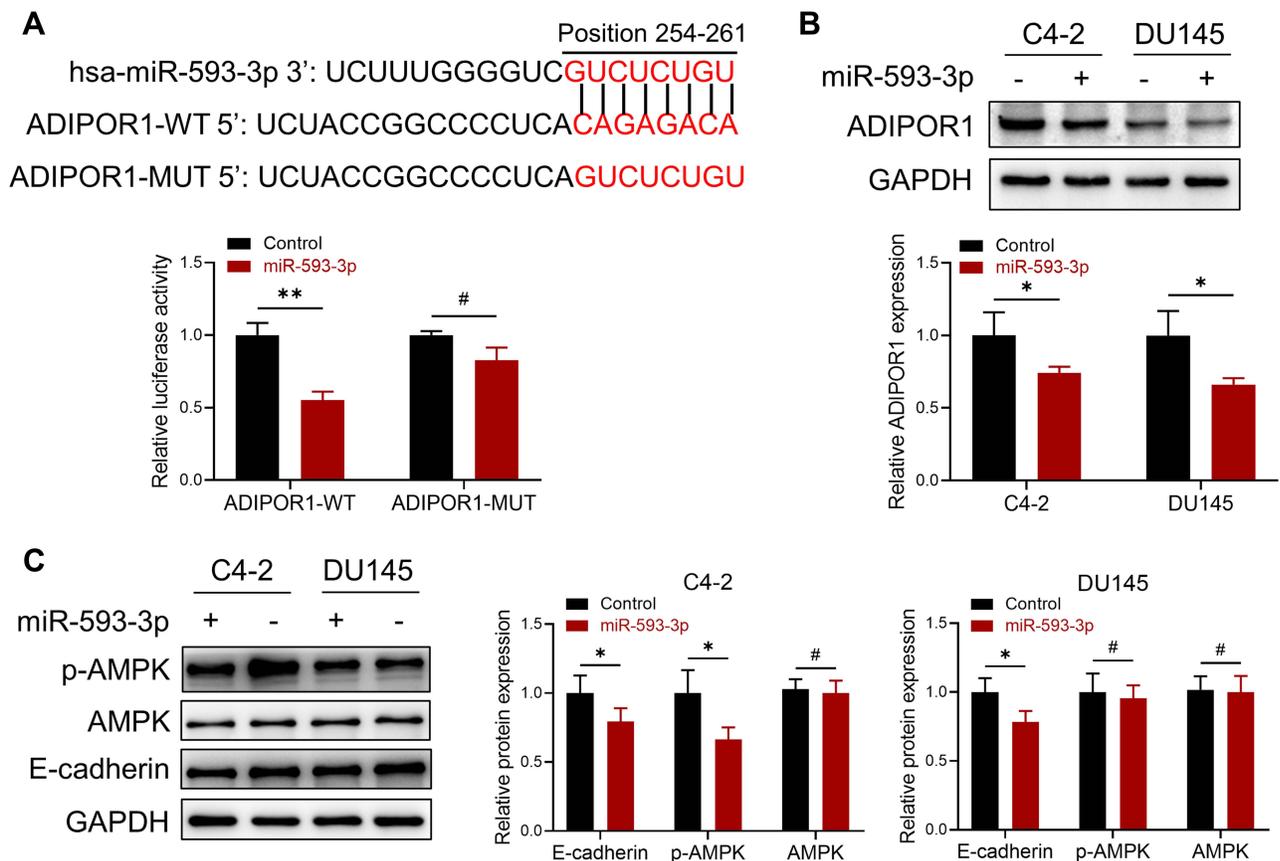


Figure 4 miR-593-3p targets ADIPOR1, affects AMPK signaling pathway and EMT process. **(A)** Wild-type (ADIPOR1-WT) and mutant (ADIPOR1-MUT) luciferase reporter vector were constructed. The luciferase reporter vectors were co-transfected with miR-593-3p mimics or negative control into PCa cells. Luciferase activities were then examined. **(B)** Western blot analysis of ADIPOR1 protein levels after miR-593-3p overexpression. **(C)** Protein levels associated with the AMPK signaling pathway and EMT. (Data are represented as the mean \pm SD; * $P < 0.05$, ** $P < 0.01$, #no significance).

p-AMPK in DU145 cells was not significant. The heterogeneity of tumor cells may account for the phenomenon. More studies need to focus on these metabolic pathways to elucidate the underlying mechanisms and identify therapeutic targets.

This study revealed that miR-593-3p can enhance the mRNA expression of VEGF family in C4-2 cells. VEGF-A can increase the permeability of and promote the formation of new blood vessels.³⁵ A recent study confirms that VEGF-A promotes Snail1 nuclear localization to drive the EMT by combining with the receptor in PCa.³⁶ VEGF-D plays a role in the formation of new blood vessels and lymphatic vessels in cancer tissue.³⁷ In this study, it was observed that miR-593-3p overexpression enhanced the mRNA levels expression of VEGF-A and VEGF-D, which may indicate the crucial role of miR-593-3p in PCa vascularization.

After having established the role of miR-593-3p in PCa progression and metastasis, we sought to therapeutically target miR-593-3p by using LNA-modified

antisense oligonucleotides (ASOs). ASOs are synthetic short nucleic acid fragments (fragments of antisense chain) that can bind to specific DNA and RNA through base complementary pairing and prevent their transcription and translation. ASOs have higher specificity than conventional drug therapy.³⁸ Further studies will involve designing a set of ASOs as previously reported³⁹ and extensively assessing the anti-PCa therapeutic potentials in pre-clinical models.

Conclusion

In summary, this study provided evidence that miR-593-3p overexpression promotes PCa cell proliferation, colony formation, spheroid formation, migration, and invasion. ADIPOR1 is a potential target of miR-593-3p. Furthermore, miR-593-3p also affects downstream AMPK signaling pathway and EMT process. These results aid the understanding of the pathogenesis of PCa and the search for potential therapeutic targets.

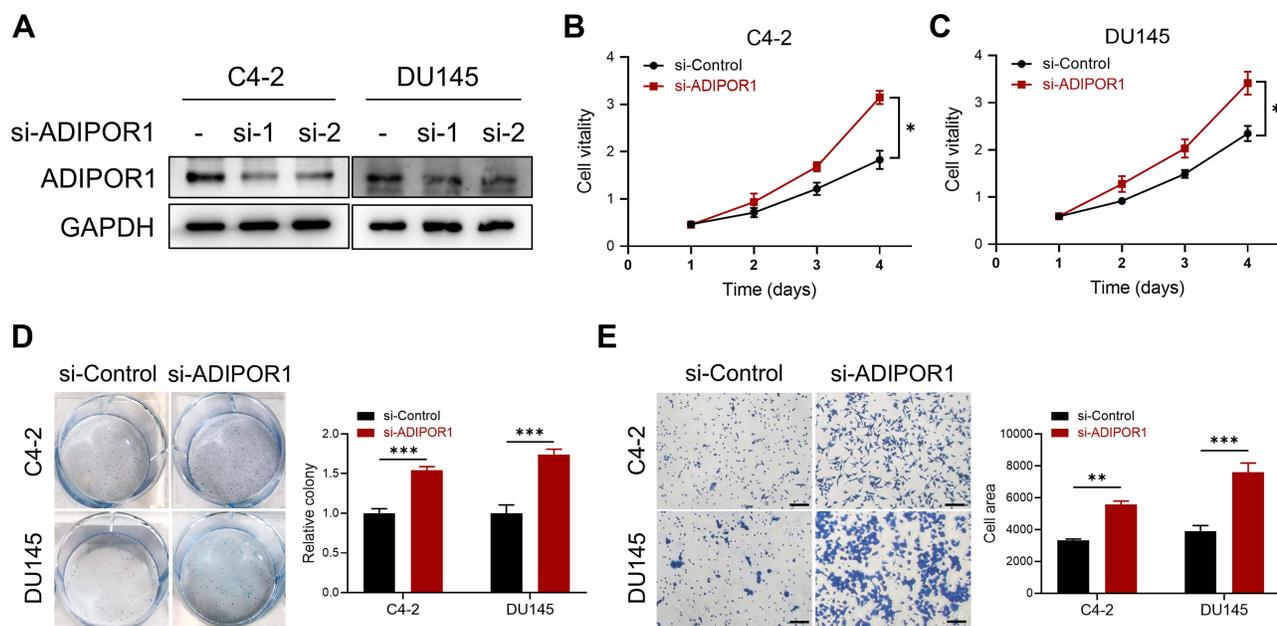


Figure 5 Depletion of ADIPOR1 Promotes PCa Cell Growth and Invasion. **(A)** si-ADIPOR1 was transfected into C4-2 and DU145 cells to induce ADIPOR1 knockdown. The transfection efficiency was detected by Western blot. **(B and C)** The effect of ADIPOR1 depletion on PCa cell proliferation. **(D)** The effect of ADIPOR1 depletion on PCa cell colony formation. **(E)** The effect of ADIPOR1 depletion on PCa cell invasion. Scale bar=100 μ m. (Data are represented as the mean \pm SD; * P < 0.05, ** P < 0.01, *** P < 0.001).

Acknowledgments

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Disclosure

The authors declare no conflicts of interest in this work.

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