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microRNA-183 plays as oncogenes by increasing cell proliferation, migration and invasion via targeting protein phosphatase 2A in renal cancer cells

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ABSTRACT

The aim of this study was to investigate the function of miR-183 in renal cancer cells and the mechanisms miR-183 regulates this process. In this study, level of miR-183 in clinical renal cancer specimens was detected by quantitative real-time PCR. miR-183 was up- and down-regulated in two renal cancer cell lines ACHN and A498, respectively, and cell proliferation, Caspase 3/7 activity, colony formation, *in vitro* migration and invasion were measured; and then the mechanisms of miR-183 regulating was analyzed. We found that miR-183 was up-regulated in renal cancer tissues; inhibition of endogenous miR-183 suppressed *in vitro* cell proliferation, colony formation, migration, and invasion and stimulated Caspase 3/7 activity; up-regulated miR-183 increased cell growth and metastasis and suppressed Caspase 3/7 activity. We also found that miR-183 directly targeted tumor suppressor, specifically the 3′UTR of three subunits of protein phosphatase 2A (PP2A-Cα, PP2A-Cβ, and PP2A-B56-γ) transcripts, inhibiting their expression and regulated the downstream regulators p21, p27, MMP2/3/7 and TIMP1/2/3/4. These results revealed the oncogenes role of miR-183 in renal cancer cells via direct targeting protein phosphatase 2A.

1. Introduction

Renal cancer is the most common urinary cancer all over the world, with 63,920 (39,140 are male and 24,780 are female) estimated new cases and 13,860 (8900 are male and 4960 are female) estimated deaths in 2014 [1]. The distribution of renal cancer varies all over the world, and the incidence is increasing [1–3]. The exact causing of renal cancer remain unclear, but researchers suggested there is a connection between the development of renal cancer and several environmental risk factors [4,5]. In nowadays, therapeutic options for metastatic renal cancer are limited in their efficacy and specificity [6]. Thus, there is an increasing requirement to develop therapeutic alternatives.

MicroRNAs (miRNAs) are a class of non-coding endogenous single-strand RNAs (20–23 nt) that mediate post-transcriptional inhibition of target gene expression by inducing transcript degradation, translational repression, and gene silencing through binding to the 3′-untranslated region (3′UTR) of target mRNA, and play critical roles in cells [7,8].

Researchers have indicated that miRNAs are involved in cell growth, differentiation, and death; they regulate the initiation, development, and progression of human cancers, including tumor growth, apoptosis, invasion, and metastasis [9]. In malignancy, miRNAs can function as oncogenes or tumor suppressors depending on the function of the target genes [10]. MiR-183 is a member of miR-183-96-182 cluster, and researchers have reported that the miR-183-96-182 cluster plays as oncogenes by regulating growth and progression of human cancers [11]. Researchers always pay their attentions to investigating the function of miR-183-96-182 cluster, but a few to the individual miR-183. In previous studies, researchers have indicated that either individually or as a cluster, the expression levels of miR-183, miR-96, and miR-183 is up-regulated in several cancers, including breast cancer, lung cancer,

Abbreviations: 3′UTR, 3′-untranslated region; ANTs, adjacent nontumorous tissues; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, metal matrix proteinase; MTS, CellTiter 96 AQueous One Solution Cell Proliferation assay; PP2A, protein phosphatase 2A; PVDF, polyvinylidene fluoride; qRT-PCR, quantitative real-time PCR; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TIMP, tissue inhibitor of metalloproteinase.

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medulloblastoma, prostate cancer, urothelial carcinoma and etc [11–15]. These suggest that miR-183 may function as oncogenes in renal cancer. Otherwise, protein phosphatase 2A (PP2A) is an important member of serine threonine phosphatase and plays a critical effect in cellular processes via dephosphorylate many critical cellular molecules like Akt, p53, c-Myc and beta-catenin [16]. PP2A is composed of catalytic, scaffold and regulatory subunits, structurally, and observed in various human tumors, suggesting that PP2A play as a tumor suppressor [16].

In this study, we investigated the effect of miR-183 expression and suppression in renal cancer cell lines ACHN and A498 and explored its possible mechanisms of this action.

2. Materials and methods

2.1. Tissue specimens

Matched fresh renal cancer specimens and adjacent nontumorous tissues (ANTs) were obtained from 16 patients at the Affiliated Hospital of Guangdong Medical College, Zhanjiang, China from 2013 to 2014. All the specimens were immediately frozen in liquid nitrogen and then stored at -80 °C for RNA isolation. For the use of these clinical specimens for research purposes, prior written informed consents to participate were obtained and the study was approved by the Medical Ethics Committee of Affiliated Hospital of Guangdong Medical College (Certificate No. PJ2012130).

2.2. Cell lines and cell culture

Human renal cancer cell lines (ACHN and A498) were purchased from Guangzhou Jennio Biological Technology (Guangzhou, China). ACHN cells were cultured in High glucose DMEM medium (GIBCO) supplemented with 10% FBS (GIBCO). A498 cells were cultured in RPMI-1640 medium (GIBCO) supplemented with 10% FBS. Both cell lines were cultured at 37 °C in a humidified 5% CO_2 atmosphere.

2.3. miRNA transfection

The DNA fragment encoding miR-183/control miRNA and miR-183 inhibitor/control siRNAs were purchased from GeneCopoeia, Inc. (Guangzhou, Guangdong, China) and inserted into a lentiviral ORF/shRNA/miRNA expression plasmid. Transfection was performed according to manufacturer protocols. Briefly, ACHN and A498 cells were cultured in medium supplemented with 10% FBS for 24 h before miRNAs transfection, and then with the virus vectors were added into the medium for transfection, respectively. Cells were then cultured in medium containing 1 μg/mL puromycin at 48 h post-transfection for selecting the positive cells. For maintaining the stable expression and inhibition, the positive cells were cultured in the medium containing 0.5 μg/mL puromycin after stable expression and inhibition were achieved. The related miRNA sequences were in Supplementary Information 1.

2.4. Luciferase reporter assay

To determine the influence of miR-183 on the 3′UTR of PP2A-Cα, PP2A-Cβ and PP2A-B56-γ mRNAs, we used the Dual-Luciferase Reporter 1000 Assay System (Promega). Briefly, 293 cells were cultured in 24-well cell culture cluster (Corning) until the cells reached 70% confluence; the cell were co-transfected with has-miR-183/control miRNA and the 3′UTR of PP2A-Cα, PP2A-Cβ and PP2A-B56-γ. After 48 h transfection, cells were harvested and firefly and Renilla luciferase activities were assayed; results were normalized to Renilla luciferase.

2.5. Quantitative real-time PCR (qRT-PCR)

Quantitative real-time RT-PCR was performed on a Thermal Cycler Dice Real Time System (TP800, TaKaRa; Dalian, Liaoning, China) with the PrimeScript miRNA qPCR Starter Kit Ver.2.0 (TaKaRa; Dalian, Liaoning, China) and SYBR Premix Ex Taq II (TaKaRa; Dalian, Liaoning, China). For the miRNA expression assay, we employed a two-step qRT-PCR with specific primers for miR-183 and RNU6B (internal control) according to manufacturer protocols. The cycling conditions were 95 °C for 10 s, followed by 40 cycles of amplification at 95 °C for 5 s and 55 °C for 20 s. For relative mRNA expression analysis, we employed a two-step qRT-PCR with specific primers for GAPDH (internal control), MMP2, MMP3, MMP7, TIMP1, TIMP2, TIMP3, and TIMP4 with the following cycling conditions: 95 °C for 30 s, followed by 40 cycles of amplification at 95 °C for 5 s and 56 °C for 30 s. Data are representative of three independent assays, and expression was expressed as $2^{-\Delta\Delta CT}$. Primers are listed in Table 1.

2.6. Western blot analysis

ACHN and A498 cells were lysed with RIPA buffer (Beyotime Institute of Biotechnology; Shanghai, China) and total proteins were extracted at 4 °C. Proteins were separated by SDS–PAGE and then transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were incubated with anti-PP2A-B56-γ mouse monoclonal antibody (61 kD, 1:400, sc-374379, Santa Cruz Biotechnology), anti-PP2A C subunit rabbit polyclonal antibody (38kD, 1:1000, #2038, 36, Cell Signaling Technology), anti-PP2A-Cα rabbit polyclonal antibody (36 kD, 1:400, sc-130237, Santa Cruz Biotechnology), anti-PP2A-Cβ rabbit polyclonal antibody (36 kD, 1:1000, AB61085a, Life Science), anti-p21 rabbit antibody monoclonal (21 kD, 1:1000, #2947, Cell Signaling Technology), anti-p27 rabbit monoclonal antibody (27 kD, 1:1000, #3686, Cell Signaling Technology), or anti-β-tubulin mouse monoclonal antibody (55 kD, 1:50000, #70004, Pure Earth Biotechnology; loading control), and then probed with a secondary antibody (1:10,000, Pure Earth Biotechnology Co. Ltd.).

2.7. Cell proliferation and Caspase-Glo 3/7 assays

Cell proliferation was measured by using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation (MTS) assay according to manufacturer protocols. Briefly, cells were seeded in 96-well cell culture cluster (Corning) at a density of 3000 cells per well in 100 μL culture medium and cultured for up to 8 d. The medium

Table 1
Sequences for target gene primers.

Gene	Primer sequence 5′–3′		Tm (°C)
miR-183	F:	CTATGGCACTGGTAGAATTCACT	58.39
	R:	TCGTATCCAGTGCAGGGTC	59.72
GAPDH	F:	TGCACCACCAACTGCTTAG	57.56
	R:	AGTAGAGGCAGGGATGATGTTC	60.07
MMP2	F:	CCACAGGAGGAGAAGGCTGT	61.90
	R:	CTCCAGTTAAAGGCGGCATC	59.85
MMP3	F:	CTCTTCCTTCAGGCGTGGAT	59.85
	R:	CACGGTTGGAGGGAAACCTA	59.85
MMP7	F:	TGACATCATGATTGGCTTTGC	56.06
	R:	AGCATCTCCTCCGAGACCTG	61.90
TIMP1	F:	CAATTCCGACCTCGTCATCAG	59.97
	R:	CTTGGAACCCTTTATACATCTTGG	58.55
TIMP2	F:	CAGATGTAGTGATCAGGGCCAA	60.07
	R:	TTCTCAGGCCCTTTGAACATC	58.01
TIMP3	F:	CAACTCCGACATCGTGATCC	59.85
	R:	GTGAAGCCTCGGTACATCTTCA	60.07
TIMP4	F:	CACTCGGCACTTGTGATTCG	59.85
	R:	TCTCAAACCCTTTGAACATCTTT	54.82

was removed and replaced with the same volume of medium containing CellTiter 96 AQ_{ueous} One Solution reagent and then incubated at 4 °C for 2 h. Absorbance was detected at 490 nm in a 96-well plate reader. For Caspase-Glo 3/7 assay, equal volume of Caspase-Glo 3/7 reagent was added into each well of the 96-well plate at 5 d and 7 d and incubated for 30 min at room temperature keeping in dark place. The luminescence of each well was measured by the luminometer manufacturer.

2.8. Colony formation assay

A colony formation assay was performed according to a slightly modified method [17], briefly, cells were seeded into 60 mm plastic dishes (Nest Biotechnology, Hong Kong, China) at 1000 cells per well, and cultured at 37 °C in a humidified 5% CO_2 atmosphere (ACHN cells were cultured for 3 weeks and A498 cells were cultured for 2 weeks). Colonies were counted after staining with Coomassie Brilliant Blue (CBB).

2.9. In vitro wound healing (migration) assay

ACHN and A498 cells were seeded in 12-well cell cultured plate (3×10^5 cells per well; Nest Biotechnology). Would healing assays were performed with a sterile pipette tip to make a scratch through the confluent monolayer when cells reached 100% confluence. Medium was exchanged and cell migration was observed after incubation up to 24 h.

2.10. In vitro transwell (invasion) assay

In vitro transwell (invasion) assay was performed by a modified method [17], briefly, 3×10^5 cells in 150 μL serum-free medium supplemented with 1% FBS were placed in the upper chamber of the insert (membrane pore size, 8 μm; Corning) with Matrigel (BD Biosciences, MA), and 500 μL medium supplemented with 10% FBS was added to the lower chamber of the 24-well plastic plate. After 24 h culture at 37 °C, cells remaining in the upper chamber or on the upper membrane were removed. Cells adhering to the lower membrane were counted after staining with Crystal Violet Staining Solution (Beyotime Institute of Biotechnology; Shanghai, China) for 10 min.

2.11. Statistical analysis

Cell proliferation assays and Caspase-Glo 3/7 assays were performed in five independent experiments and other analyses were repeated in three independent experiments. Results are presented as mean ± SD. All data were analyzed using SPSS 18.0 by one-way ANOVA, and differences between treatments were assessed using a Fisher's Least Significant Difference test [LSD (L)]. Significant differences were inferred for $P < 0.05$ and extremely significant difference $P < 0.01$ and $P < 0.001$.

3. Results

3.1. miR-183 was up-regulated in renal cancer tissues and cell lines

To determine the potential significance of miR-183 in renal cancer, we firstly measured the levels of miR-183 in renal cancer specimens and cell lines by qRT-PCR. As shown in Fig. 1A, higher levels of miR-183 were detected in renal cancer tissues as compared with that in paired adjacent nontumorous tissues (ANTs; $P < 0.001$); in parallel, miR-183 was expressed at higher levels in two renal cancer cell lines than that in normal renal cell line HK-2 (Fig. 1B, $P < 0.001$). These data suggested that miR-183 expression was significantly stimulated in renal cancer.

3.2. Decreased endogenous miR-183 expression suppressed cell proliferation, migration, and invasion in renal cell lines

To determine the biological functions of miR-183, expression of miR-183 was stably inhibited in ACHN ($P < 0.001$; Fig. 1C) and A498 ($P < 0.05$; Fig. 1C) cells. We performed a cell proliferation assay (MTS) to examine the effect of miR-183 on renal cancer cell growth. As shown in Fig. 2, decreased endogenous miR-183 expression suppressed proliferation in ACHN and A498 cells ($P < 0.01$; Fig. 2A) and increased Caspase 3/7 activity ($P < 0.01$ at 5 d and $P < 0.001$ at 7 d; Fig. 2B). Down-regulated endogenous miR-183 expression reduced colony formation in ACHN ($P < 0.05$; Fig. 2C) and A498 ($P < 0.01$; Fig. 2C) cells, consistent with the results of the MTS assay. We also performed wound healing and transwell assays to investigate the biological function of miR-183 in renal cancer cells metastasis. Decreased endogenous miR-183 expression significantly decreased wound healing in ACHN ($P < 0.05$; Fig. 2E) and A498 ($P < 0.01$; Fig. 2E); transwell assays with Matrigel demonstrated that down-regulation of endogenous miR-183 reduced the invasive capacity of both cell lines ($P < 0.001$; Fig. 2D). These results suggested that inhibition of endogenous miR-200c inhibited renal cancer cell growth and metastasis *in vitro*.

3.3. Ectopic miR-183 expression promoted cell proliferation, migration, and invasion in renal cell lines

Expression of miR-183 was stably up-regulated in ACHN ($P < 0.001$; Fig. 1D) and A498 ($P < 0.001$; Fig. 1D) cells to reveal the biological significance of miR-183. In these cells, we observed increased proliferation ($P < 0.01$; Fig. 3A) and colony formation ($P < 0.01$; Fig. 1C), and decreased Caspase 3/7 activity ($P < 0.05$; Fig. 1B) in both cell lines. Wound healing and transwell assays revealed that ectopic miR-183 stimulated migration ($P < 0.05$; Fig. 1E) and invasion ($P < 0.001$; Fig. 1D) in both cell lines. These results showed that miR-183 promoted cell growth and metastasis in renal cancer cells.

3.4. miR-183 regulated cell proliferation and metastasis by directly targeting PP2A-Cα, PP2A-Cβ, and PP2A-B56-γ

To dissect the molecular mechanism of miR-183, we searched for candidate targets by using TargetScan, a bioinformatics tool for miRNA target screening, and identified PP2A-Cα, PP2A-Cβ, and PP2A-B56-γ as putative targets of miR-183 (Fig. 4A). We then performed a miR-183-based luciferase assay in 293 cells and found that miR-183 directly bind to 3′UTRs of PP2A-Cα, PP2A-Cβ, and PP2A-B56-γ, as shown by a significant decrease in luciferase expression ($P < 0.001$; Fig. 4B). Western blotting and qRT-PCR revealed down-regulation of miR-183 increased expression of PP2A-Cα, PP2A-Cβ, and PP2A-B56-γ, p21, p27, and TIMP1/2/3/4, and decreased expression of MMP2/3/7 (Fig. 4C and D). Inhibition of PP2A-Cα, PP2A-Cβ, and PP2A-B56-γ, p21, p27, and TIMP1/2/3/4, and induction of MMP2/3/7 expressions were detected in the presence of miR-183 over-expression (Fig. 4C and D). These data demonstrated that miR-183 regulated renal cancer cell growth and metastasis by directly targeting the 3′UTR of PP2A-Cα, PP2A-Cβ, and PP2A-B56-γ, altering their expression and that of downstream genes in in cell proliferation and metastasis.

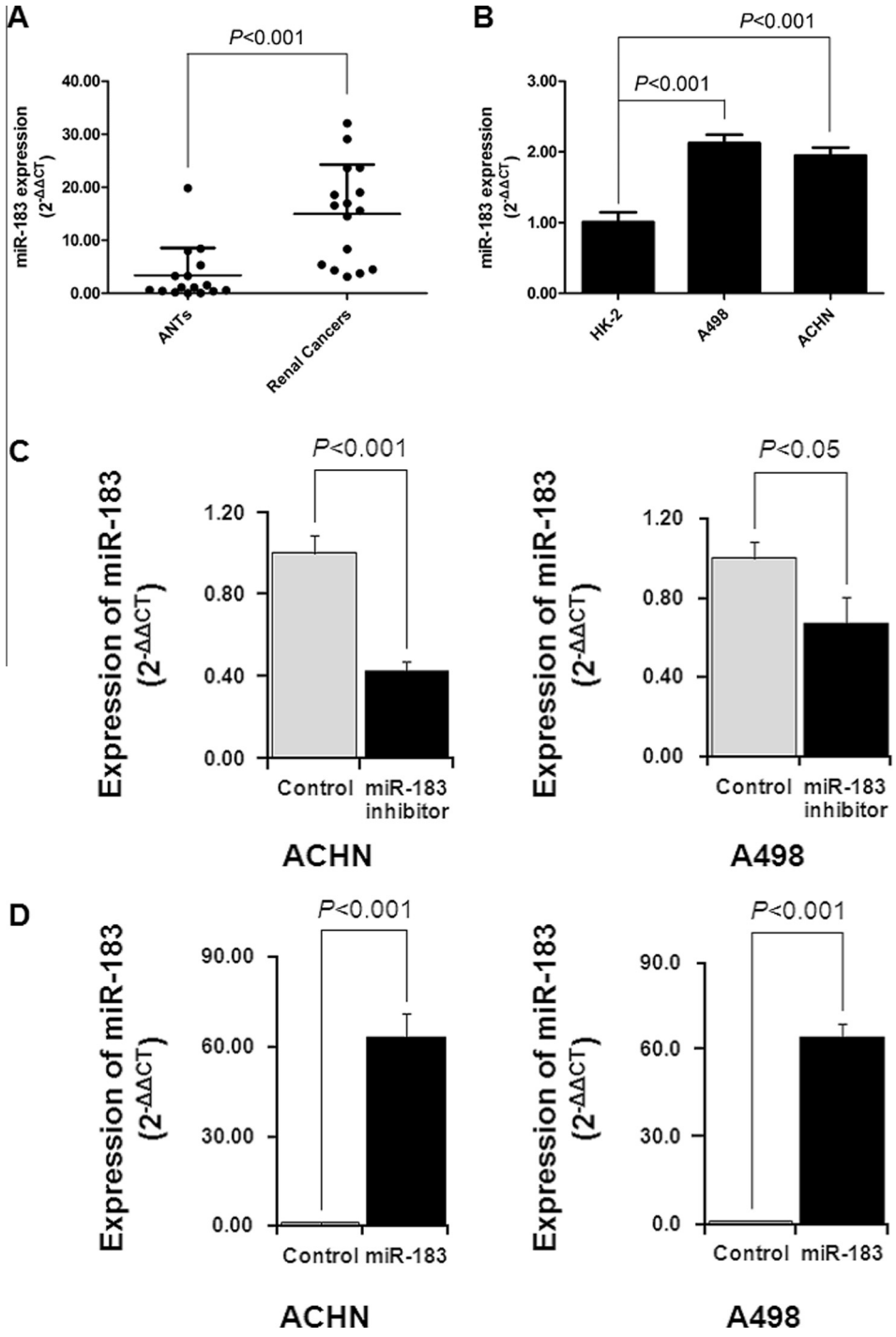

Fig. 1. miR-183 levels in renal cancer tissues and cell lines. (A) miR-183 levels in 16 pairs of renal cancer tissues and their corresponding adjacent nontumorous tissues (ANTs); (B) miR-183 levels in normal renal cell line HK-2 and two renal cancer cell lines ACHN and A498. (C) Endogenous miR-183 was down-regulated by stable transfection with a specific inhibitor; (D) miR-183 was induced in ACHN and A498 cells by a viral vector.

4. Discussion

Previous studies have demonstrated that microRNAs played critical roles as oncogenes or tumor suppressors in human cancers [18–21]. Elevated levels of miR-183 were observed in a wide range of tumors and cell lines derived from corresponding tumors [22], suggested that up-regulation of miR-183 may be a critical event in the tumorigenesis/transformation process. We investigated miR-183 regulation of cell proliferation, migration, and invasion in renal cancer cells. Our results demonstrated that suppression of endogenous miR-183 significantly inhibited cell growth, migration, and invasion, and ectopic miR-183 restored cell proliferation and metastasis in renal cancer cells; miR-183 promoted renal cancer cell growth and metastasis by directly targeting tumor suppressor protein phosphatase 2A and modulating its downstream targets.miR-183 was a member of miR-183-96-182 cluster, and altered expression level of miR-183 was observed in a wide range of tumors and cell lines derived from the corresponding tumors

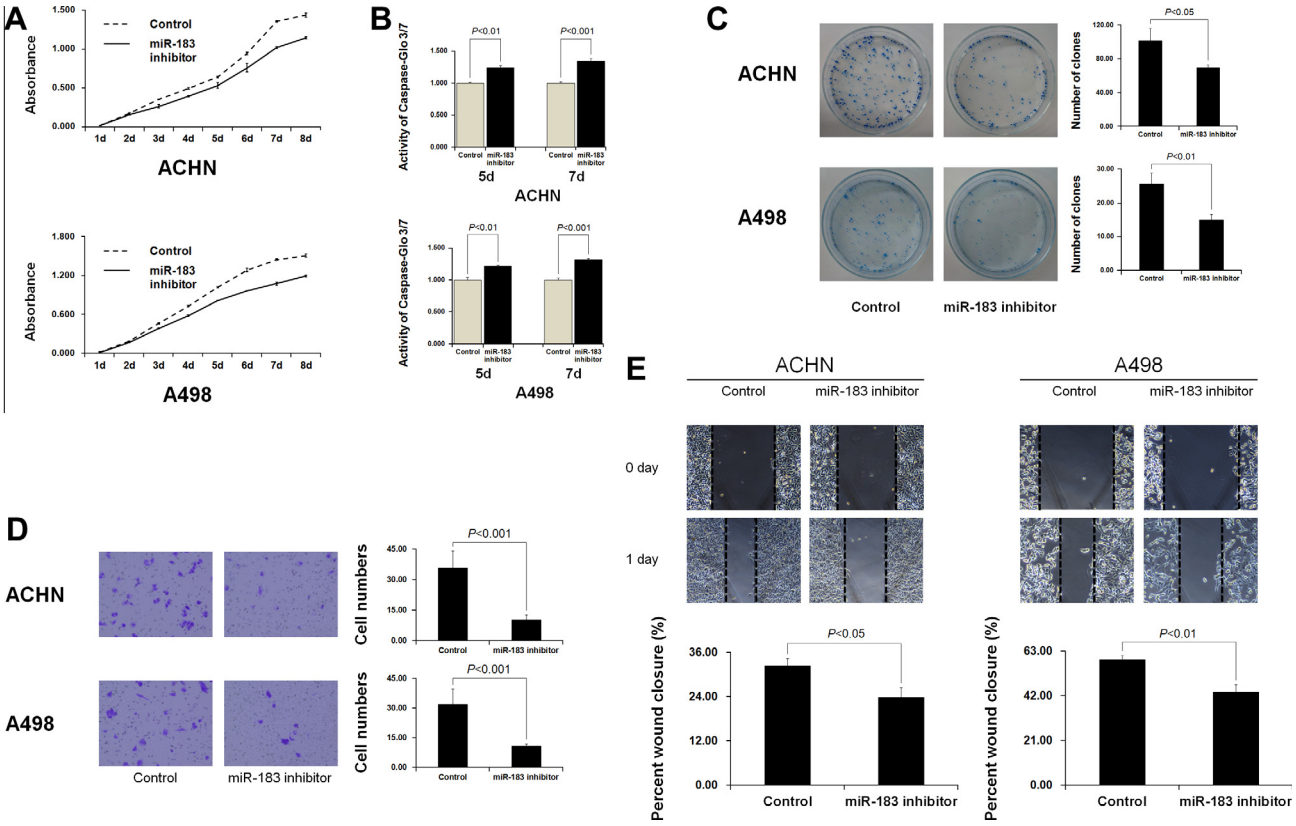

Fig. 2. Inhibitor of endogenous miR-183 suppressed renal cancer cell growth and metastasis *in vitro*. (A) Proliferation of renal cancer cells was measured by MTS assay; (B) Caspase 3/7 activity was examined by Caspase-Glo 3/7 assay; (C) Colony formation was assessed after miRNA transfection; (D) Transwell invasion assays; (E) *In vitro* wound healing of renal cancer cells after miRNA transfection.

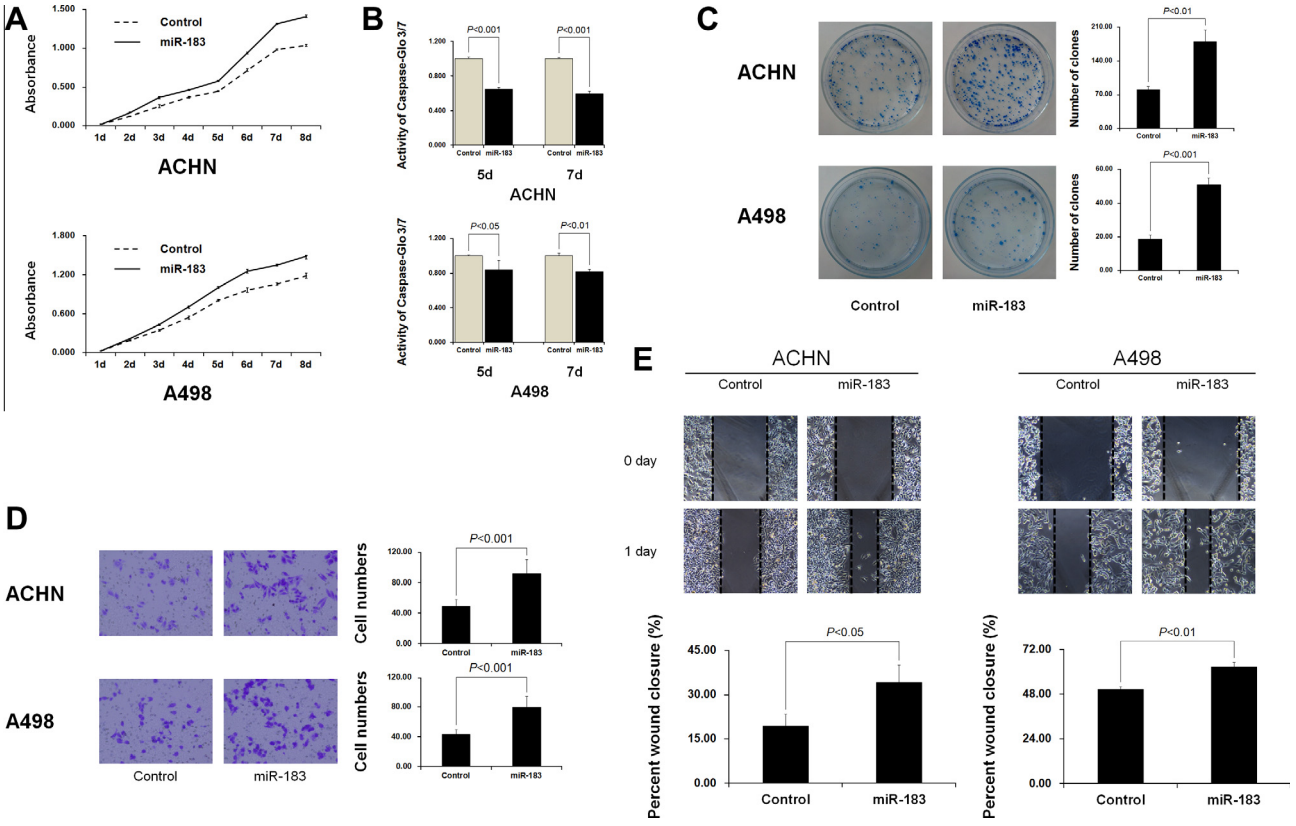

Fig. 3. Ectopic miR-183 promoted renal cancer cell growth and metastasis *in vitro*. (A) Proliferation of ACHN and A498 cells was analyzed by MTS assay; (B) Caspase-Glo 3/7 assay; (C) Colony formation after miRNA transfection; (D) Transwell invasion assays; (E) *In vitro* wound healing after miRNA transfection.

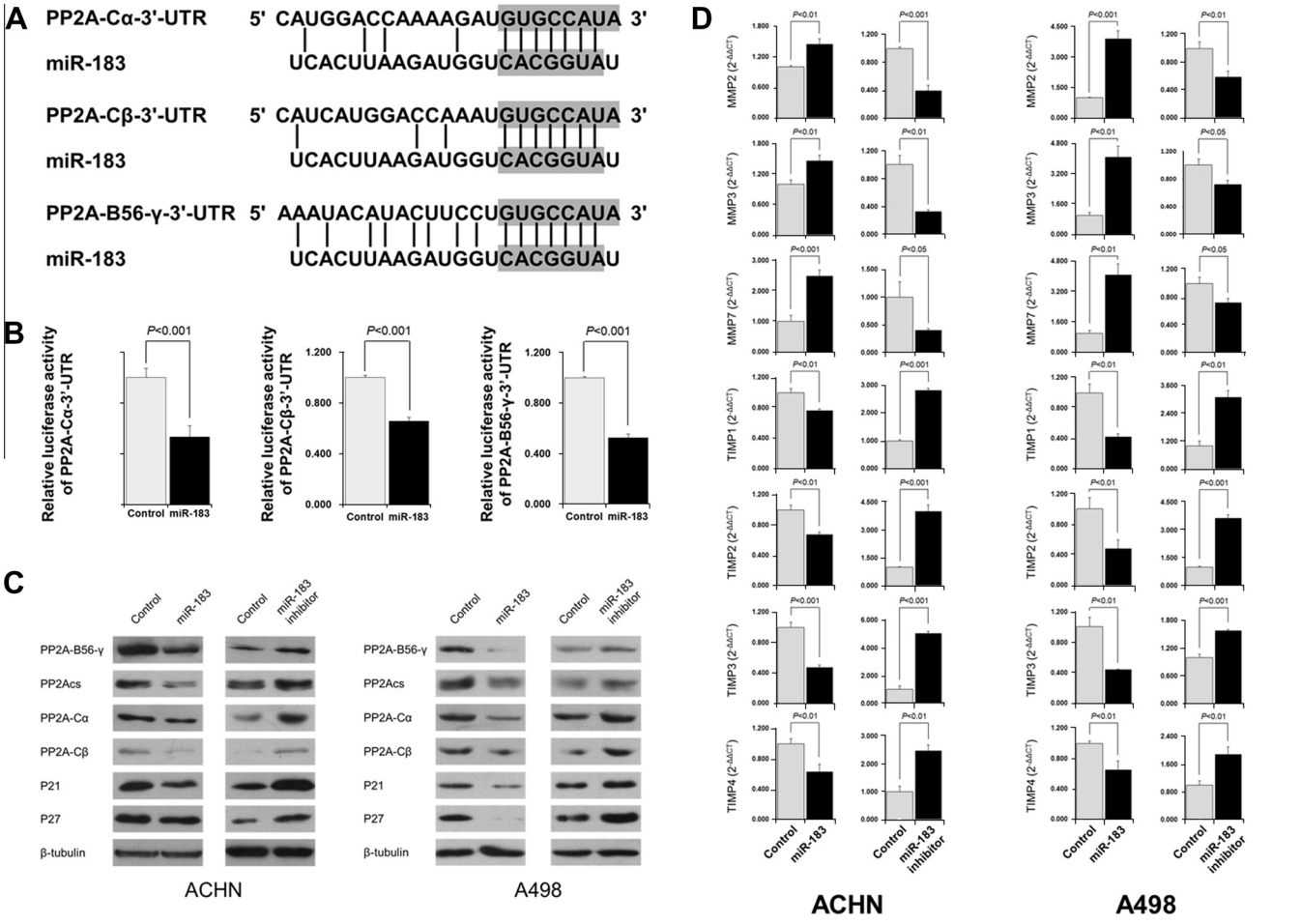

Fig. 4. miR-183 played oncogenes roles by directly binding to tumor suppressor PP2A-Cα, PP2A-Cβ, and PP2A-B56-γ. (A) miR-183 and its putative binding sequences in the 3′UTR of PP2A-Cα, PP2A-Cβ, and PP2A-B56-γ; (B) miR-183 suppressed luciferase activity; (C) regulation of miR-183 on relative protein expression; (D) influence of miR-183 on relative transcript levels: MMP2, MMP3, MMP7, TIMP1, TIMP2, TIMP3, and TIMP4.

[11,22,23]. Researchers have recognized and demonstrated that miR-183-96-182 cluster acted by stimulating tumorigenesis in cancers via regulating multiple biological processes [11,14]. Aaron et al. knocked down expression of miR-183 in the synovial sarcoma and colon cancer cell lines to determine the influence of miR-183, and found decreases in tumor cell migration [11]. Liu et al. measured the effects of miR-83–96-182-cluster on bladder cancer cells by transfecting a miR-83–96-182-cluster-mower into bladder cancer cells, and decreased cell growth, increased cell apoptosis, and inhibited migration were observed [24]. These revealed that miR-183/miR-183-96-182-cluster functions as a potential oncogene in cancers. In the present study, we firstly inhibited endogenous miR-183 expression to investigate the function of endogenous miR-183 in renal cancer cells, and cell growth inhibition, decreased invasion and migration, and increased Caspase 3/7 activity was observed. These results were similar to the results in the studies above [22,24], and indicated that endogenous miR-183 expression could maintain the basal capacity of cell growth, invasion and migration in renal cancer cells. In otherwise, over-expression of miR-183 was performed and increased cell proliferation, invasion and migration, and reduced Caspase 3/7 activity was observed as expected. These results consisted with the results above and suggested that ectopic single miR-183 promoted cell growth, invasion and migration, and suppressed cell apoptosis in renal cancer cells.

It is well known that the basic function of miRNAs is to regulate the target genes by directly cleave the target mRNA and/or by suppressing protein synthesis, according to the degree of complementarity with the 3′UTR of mRNA of target genes [25]. Protein phosphatase 2A (PP2A) is an essential kind of serine/threonine phosphatase conserved in all eukaryotes and acts as a tumor suppressor in human cancers through dephosphorylating many critical cellular molecules like Akt, p53, c-Myc, and β-catenin in various biological processes [16,26]. p21 and p27 are potent members of cyclin-dependent kinase inhibitors, and function as cell cycle inhibitory proteins by inducing attenuating of G1, G2/M phase arrest and reduction in apoptosis [27–29]. Otherwise, tumor-associated matrix metalloproteinases (MMPs) are recognized as tumor promoters and increase tumor metastasis [30,31], and their inhibitors tissue inhibitors of metalloproteinases (TIMPs) are able to inhibit MMPs activity by controlling MMP-mediated extracellular matrix (ECM) breakdown [31]. In human cancers, a disturbed balance of MMPs and TIMPs is found by researchers [32]. In this study, we have found that individual miR-183 could directly target mRNA 3′UTRs of three subunits of protein phosphatase-2A including PP2A-Cα, PP2A-Cβ, and PP2A-B56-γ, suppress these protein expressions; and down-regulation of PP2A result in reduce expression levels of p21 and p27, suppressed expressions of TIMP1/2/3/4, and increased levels of MMP2/3/7 expressions. These results confirmed to the researches above.

In summary, we investigated the function of miR-183 in renal cancer cells, and demonstrated that miR-183 was up-regulated in renal cancer tissues and possessed the potency to increase renal cancer cells growth and metastasis by regulating protein phospha-

tase 2A. Therefore, miR-183 could function as oncogenes in renal cancers. The identification of miR-183 in renal cancer would provide a better understanding of the molecular mechanisms underlying renal cancer development. These findings would provide us important basic information and a wider perspective on renal cancer intervention/ prevention and treatment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.067.

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