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Original article

MicroRNA-93 promotes bladder cancer proliferation and invasion by targeting PEDF

Hua Jiang, (Master of Medicine)*, Qiang Bu, (MD), Minghui Zeng, (Bachelor of Medicine), Dongdong Xia, (Master of Medicine), Aibin Wu, (Bachelor of Medicine)

Department of Urology, People's Hospital of Danyang, Jiangsu, China

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Abstract

Objective: MicroRNA-93 (miR-93) is upregulated in the urine of patients with bladder cancer (BC). Here, we investigated the role of miR-93 in BC progression and explored the underlying mechanism.

Methods: miR-93 expression in BC tissues and cells was detected by real time-polymerase chain reaction. The effects of miR-93 and pigment epithelium-derived factor (PEDF) on cell proliferation and invasion were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and Transwell assays. The binding of miR-93 to the 3′-untranslated region of PEDF was identified by the luciferase reporter assay.

Results: miR-93 expression was higher in BC tissues than in normal controls, and its expression was associated with tumor stage and node stage. Inhibition of miR-93 suppressed the proliferation and invasion of BC cells. PEDF was identified as a target of miR-93 and shown to mediate the effect of miR-93 on cell proliferation and invasion.

Conclusions: The present data suggested that miR-93 promoted BC cell proliferation and invasion by targeting PEDF, providing new biomarkers and targets for BC diagnosis and treatment. © 2018 Published by Elsevier Inc.

Keywords: Bladder cancer; Microrna-93; Pigment epithelium-derived factor; Proliferation; Invasion

1. Introduction

Bladder cancer (BC) is one of the most common urological malignancies in China [1]. Its incidence and mortality rates have increased gradually in recent years. BC is classified into 2 distinct categories: nonmuscle and muscle invasive bladder cancer [2]. Patients with nonmuscle invasive bladder cancer tend to have a high rate of recurrence, whereas patients with localized muscle invasive bladder cancer tend to have a high rate of metastasis and worse prognosis [3,4]. A better understanding of the mechanism, underlying BC development may help improve the treatment of patients with BC.

Increasing evidence suggests that microRNAs (miRNAs) play critical roles in cancer cell behavior [5]. In BC, several miRNAs function as oncogenes or tumor suppressors by regulating gene expression through translational repression [6,7]. miR-93 is a member of the miR-106b-25 cluster that plays important roles in many physiological processes, and its deregulation is associated with many diseases [8–11]. miR-93 is upregulated in multiple types of cancer, including breast cancer [12], osteosarcoma [13], and gastric cancer [14], and functions as an onco-miRNA. It is involved in tumor proliferation, apoptosis, angiogenesis, and metastasis [15]. A recent study showed that the levels of miR-93 in the urine were significantly higher in patients with BC than in healthy controls [16]. However, the role of miR-93 in BC remains largely unclear.

Bioinformatics analysis identified a binding site for miR-93 in pigment epithelium-derived factor (PEDF). PEDF, encoded by SERPINF1, is a member of the serpin

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*Corresponding author. Tel.: 0511-87120120.

E-mail address: dyjianghua@126.com (H. Jiang).

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superfamily with no demonstrable protease inhibitory activity [17]. Numerous studies in various models demonstrated the anti-angiogenic effects of PEDF on tumors [18]. PEDF can also act directly on tumors to induce differentiation to a less malignant phenotype, promote apoptotic tumor cell death, inhibit the proliferation of tumor cells, and block tumor migration, invasion, and metastasis [18].

In the present study, we investigated the role of miR-93 in BC progression by measuring the expression of miR-93 in BC tissues and evaluating the clinical significance of miR-93 in patients with BC. The effects of miR-93 on BC cell proliferation and invasion, and the potential involvement of PEDF were also assessed.

2. Materials and methods

2.1. Clinical specimens

Thirty paired BC and adjacent normal tissue specimens were collected from BC patients who had solely BC and did not receive neoadjuvant chemotherapy or radiotherapy at Danyang People's Hospital between 2011 and 2017. Patients underwent transurethral resection, and tumor tissues were confirmed by pathological examinations as non-muscle invasive bladder transitional cell carcinoma and graded with the WHO/ISUP (2004) [19] and TNM systems [20]. The present study was approved by the local ethics committee, and all patients provided written informed consent.

2.2. Cell culture

The BC cells lines (TCCSUP, 5637, UM-UC-3, and T24) used in this study were obtained from American Type Culture Collection (Manassas, VA). TCCSUP, 5637, UM-UC-3, and T24 were cultured in EMEM (Gibco, Rockville, MD), RPMI 1640 (Gibco), MEM (Gibco), and RPMI 1640, respectively, supplemented with 10% fetal calf serum (Life Technologies, Burlington, Canada), penicillin-G (100 U/ml, Life Technologies), and streptomycin (100 mg/ml, Life Technologies) at 37°C with 5% volume/volume (v/v) CO_2.

2.3. RNA extraction and real-time PCR

Total RNA, including miRNA, was extracted using the mirVana miRNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. After cDNA synthesis, using a Reverse Transcription Kit (Applied Biosystems, Foster City, MA), real-time polymerase chain reaction (PCR) was performed using Power SYBR Green Master Mix (Applied Biosystems). The primers used were as follows: 5′-CCCAAGCTGAAGCTGAGTTAT-3′ and 5′-GTTTGCCTGTGATCTTGCTAAAG-3′ for PEDF, and 5′-CACTCTTCCAGCCTTCCTTC-3′ and 5′-GTACAGGTCTTTGCGGATGT-3′ for β-actin. U6 small nuclear RNA or β-actin mRNA was used as an internal control. Data were analyzed using the 2-△△Ct method.

2.4. PEDF overexpression and knockdown

For PEDF overexpression, cDNA clones were purchased from OriGene Technologies (Rockville, MD), and recombinant pCMV6-Entry (pCMV6-PEDF) vectors were constructed by Hanbio Co. Ltd (Shanghai, China). For PEDF knockdown, a lentiviral shRNA system (Lv-PEDF shRNA) from Santa Cruz Biotechnology (Dallas, TX) with supporting siRNA Transfection Reagent (Santa Cruz) was used.

2.5. Cell proliferation assays

Cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions. Briefly, cells were plated onto 96-well plates (5×10^3 cells per well) and transfected with or without miR-93 mimics, miR-93 inhibitor, pCMV6-PEDF, or Lv-PEDF shRNA. Cells were incubated for 0, 1, 2, 3, and 4 days, and then 20 μl of MTT (Sigma-Aldrich, St. Louis, MO) was added to each well and incubated at 37°C for 4 hours. After incubation with dimethylsulfoxide for 10 minutes, the absorbance of cells in each well was observed at 570 nm with an ELISA reader (Bio-Rad Laboratories, Hercules, CA).

2.6. Invasion assay

Cells were transfected with or without miR-93 mimics, miR-93 inhibitor, pCMV6-PEDF, or Lv-PEDF shRNA for 48 hours, and then plated on the upper surface of modified Boyden chambers (8-mm pores) (BD Biosciences, Bedford, MA) precoated with Matrigel in 24-well plates. Serum-free DMEM and DMEM containing 10% fetal calf serum were added to the upper and lower chambers, respectively. After 48 hours of incubation, the cells on the upper microporous membrane were removed by washing in PBS, and cells on the lower surface of the membrane were fixed with ice cold alcohol. The invasive cells were stained with 0.1% crystal violet and counted under a microscope (400×) in 6 different fields per filter. All experiments were performed in triplicate.

2.7. Western blot analysis

At 48 hours after transfection, the cells were lysed using radio immunoprecipitation assay buffer (Beyotime, Haimen, China). After quantification, protein samples were loaded and separated on 10% sodium dodecyl sulfate-polyacrylamide gels. The protein was then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and incubated with mouse monoclonal PEDF antibody or mouse monoclonal β-actin antibody (both from Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C, followed by

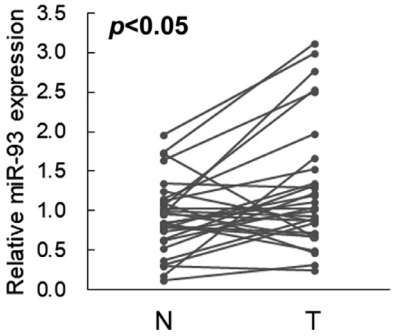

Fig. 1. miR-93 is overexpressed in BC tissues. The relative expression of miR-93 was examined in 30 paired clinical human BC tissues (T) and adjacent normal tissues (N) using real-time PCR. *$P < 0.05$ compared with normal tissue.

incubation with HRP-labeled secondary antibody (Santa Cruz Biotechnology) for 1 hour at room temperature. Finally, protein bands were visualized using the ECL substrate (Millipore, Billerica, MA).

2.8. Luciferase reporter assay

Wild-type sequences of the 3′-untranslated region (3′-UTR) of PEDF were amplified from TCCSUP cells and inserted into the pMIR-REPORT plasmid (Ambion, Waltham, MA). Site-directed mutagenesis of miR-93 binding sites was performed using a QuikChange site-directed mutagenesis kit (Stratagene, Santa Clara, CA). TCCSUP cells in 24-well plates (1×10^5 cells/well) were cotransfected with miR-93 or miR-93 inhibitor, as well as pMIR-REPORT-PEDF wild type 3′-UTR plasmid or pMIR-REPORT-PEDF mutated 3′-UTR plasmid using Lipofectamine 2000 (Invitrogen, Waltham, MA). After 48 hours, cells were lysed, and luciferase activity was measured and normalized to that of the control vector containing Renilla luciferase, according to the manufacturer's protocol.

Table 1
Relationship between miR-93 expression and clinicopathologic factors

Parameters	N	Relative miR-93 expression	*P* value
Age			0.848
<63	16	0.937 ± 0.353	
≥63	14	0.904 ± 0.565	
Gender			
Male	19	0.968 ± 0.41	0.675
Female	11	0.831 ± 0.482	
Tumor size			
<3 cm	21	0.841 ± 0.397	0.343
≥3 cm	9	1.044 ± 0.537	
Pathologic tumor stage			
pTa–pT1	12	0.68 ± 0.339	0.015*
pT2–pT3	18	1.039 ± 0.457	
Pathologic N stage			
NX-N0	15	0.747 ± 0.319	0.033*
N1-N2	15	1.096 ± 0.513	

* $P < 0.05$.

2.9. Statistical analysis

Data are presented as the mean ± standard error. Statistical comparisons between 2 groups were analyzed using two-tailed Student's *t* tests. Variance analysis between multiple groups was performed using one-way ANOVA followed by a Student-Newman-Keuls test. Pearson's correlation analysis was used to evaluate the correlations between the expression of miR-93 and PEDF. A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. miR-93 is overexpressed in BC

Total RNA was extracted from 30 pairs of BC tissues and adjacent normal tissues, and the expression of miR-93 was detected by real-time PCR. As shown in Fig. 1, the expression level of miR-93 was significantly higher in tumors than in matched nontumorous tissues. The tumor samples were then categorized into 2 groups, namely, low- and a high-expressing groups according to miR-93 expression levels below or above the median value. Analysis of the relationship between miR-93 expression and clinicopathological factors (Table 1) showed that high expression of miR-93 was significantly correlated with tumor stage and node (N) stage ($P < 0.05$; Table 1). There were no significant correlations between miR-93 expression and other clinical features such as age, gender, occurrence, and tumor size.

3.2. miR-93 inhibition suppresses the proliferation and invasion of BC cells

To explore the effect of miR-93 on BC cell proliferation and invasion, the expressions of miR-93 in 4 BC cell lines were detected and 2 cell lines with highest expressions, TCCSUP and UM-UC-3 cells, were transfected with miR-93 mimics or inhibitor (Fig. 2A and B). As shown in Fig. 2C, miR-93 mimics slightly increased cell proliferation, whereas miR-93 inhibitor significantly inhibited cell proliferation. Transwell assays demonstrated that cell invasion activity was slightly increased by miR-93 mimics and suppressed by miR-93 inhibitor compared with that in control cells (Fig. 2D).

3.3. miR-93 directly targets PEDF

To identify the molecular target of miR-93, candidate genes were predicted using miRDB, TargetScan, and microrna.org. PEDF was identified as a likely candidate target of miR-93. The correlation between the expression levels of miR-93 and PEDF was examined in primary human

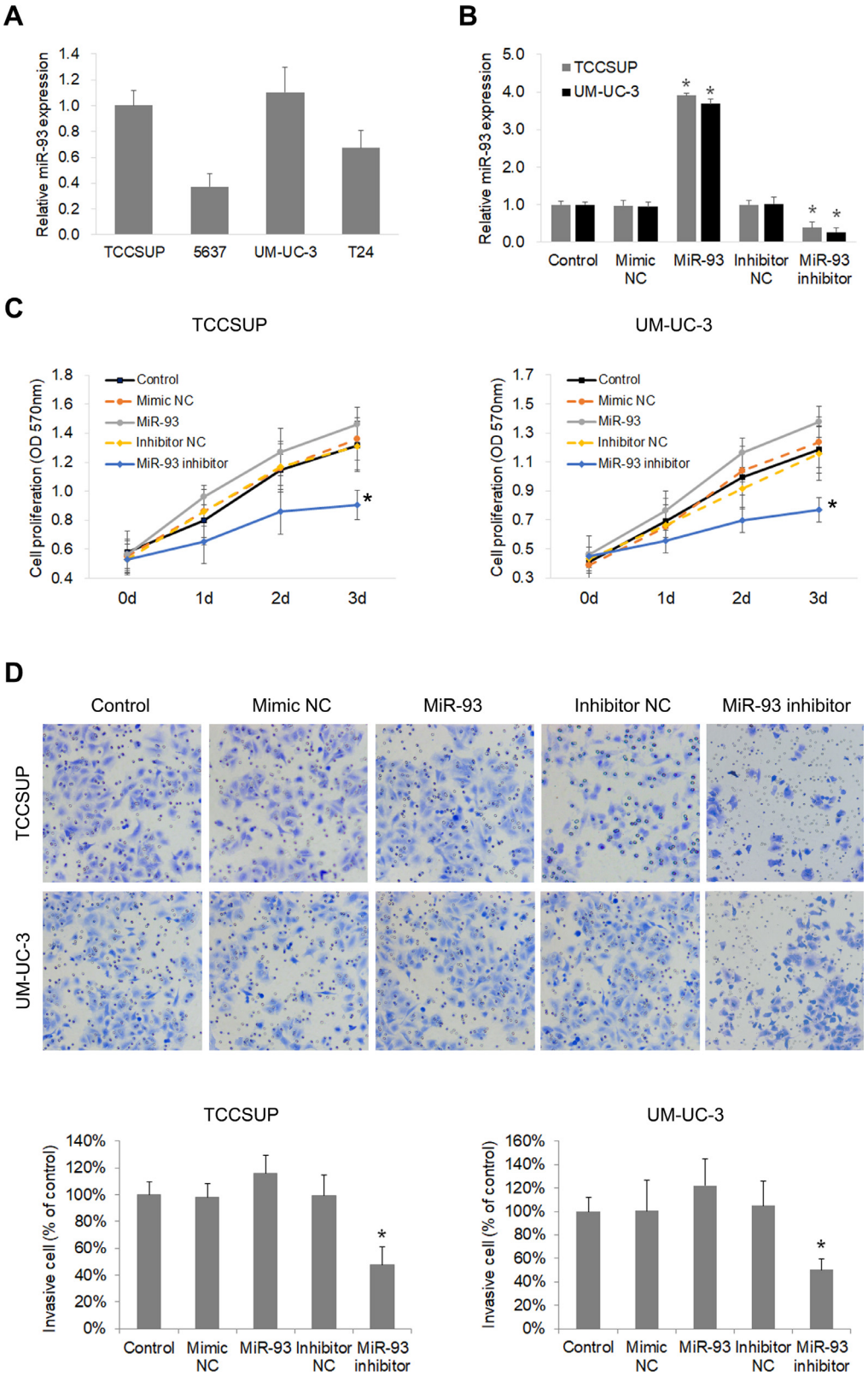

Fig. 2. miR-93 inhibition suppresses the proliferation and invasion of BC cells. (A) miR-93 expressions in four BC cell lines were detected by real-time PCR. TCCSUP and UM-UC-3 cells were transfected with miR-93 mimics, miR-93 inhibitor, or negative control (NC) mimics or inhibitor, respectively. (B) miR-93 expression was detected by real-time PCR. (C) Cell proliferation was measured at the indicated time periods with the MTT assay. (D) Cell invasion was detected with the Transwell assay. $*P < 0.05$ compared with the control.

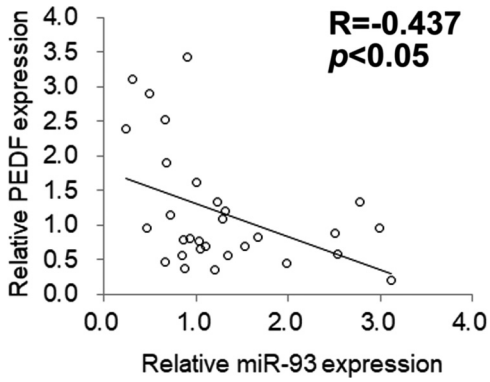

Fig. 3. Correlations between the expression levels of miR-93 and PEDF. The relative expression of PEDF in 30 BC tissues was detected by real-time PCR. Pearson's correlation analysis was used to determine the correlation between the expression levels of miR-93 and PEDF.

BC tissues. Pearson's correlation analysis suggested that the expression of PEDF was significantly inversely correlated with miR-93 expression in BC tissues (Fig. 3).

To determine whether miR-93 directly targeted the 3′-UTR of PEDF, the wild-type and mutant 3′-UTR of PEDF were subcloned into a luciferase reporter vector (Fig. 4A). TCCSUP cells were cotransfected with luciferase vectors and miR-93 mimics or miR-93 inhibitor, and luciferase activity was detected. As shown in Fig. 4B, miR-93 mimics significantly suppressed the luminescence intensity of the reporter containing the wild-type PEDF 3′-UTR, whereas miR-93 inhibitor increased the luciferase activity. Luciferase activity in cells transfected with the mutant PEDF 3′-UTR was not affected by miR-93 mimics or miR-93 inhibitor. These results suggested that PEDF was a direct target of miR-93, and the binding was specific. Moreover, miR-93 inhibition upregulated PEDF mRNA and protein expression in TCCSUP and UM-UC-3 cells (Fig. 4C and D).

3.4. PEDF mediates the effect of miR-93 on cell proliferation and invasion

To determine whether PEDF mediated the effect of miR-93 on BC cell proliferation and invasion, PEDF was overexpressed using the pCMV6-Entry based expression vector and knocked down using lentivirus with PEDF shRNA (Lv-PEDF shRNA). As shown in Fig. 5A and B, PEDF-overexpression vector transfection significantly upregulated the mRNA and protein expression levels of PEDF. Meanwhile, the miR-93 inhibitor-induced increases in PEDF mRNA and protein expression levels were significantly rescued by transfection with Lv-PEDF shRNA. PEDF-overexpression significantly suppressed the proliferation and invasion of TCCSUP cells (Fig. 5C and D). The miR-93 inhibitor-mediated reduction in cell proliferation and invasiveness was rescued by transfection with Lv-PEDF shRNA (Fig. 5C and D). These data indicated that the effects of miR-93 on suppressing cell proliferation and invasion were mediated by PEDF in BC cells.

4. Discussion

Emerging evidence suggests that miRNAs are aberrantly expressed and play important roles in cancer initiation,

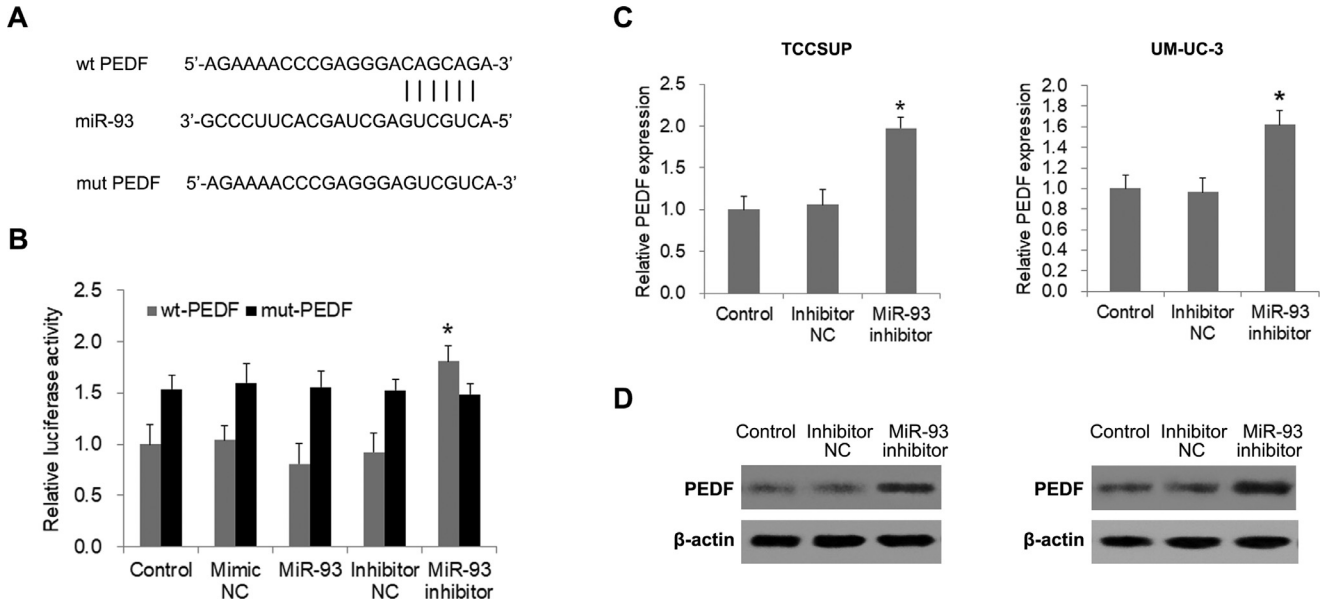

Fig. 4. miR-93 targets PEDF. (A) Predicted miR-93 binding sites within the PEDF 3′-UTR (wt-PEDF) and the mutated (mut) miR-93 binding sites are shown. (B) TCCSUP cells were cotransfected with pMIR-Report vectors and miR-93 mimics or miR-93 inhibitor. Relative luciferase activities were measured and normalized to Renilla luciferase activity. TCCSUP and UM-UC-3 cells were transfected with miR-93 inhibitor for 48 hours. PEDF expression was detected by real-time PCR (C) and western blotting (D). *$P < 0.05$ compared with the control.

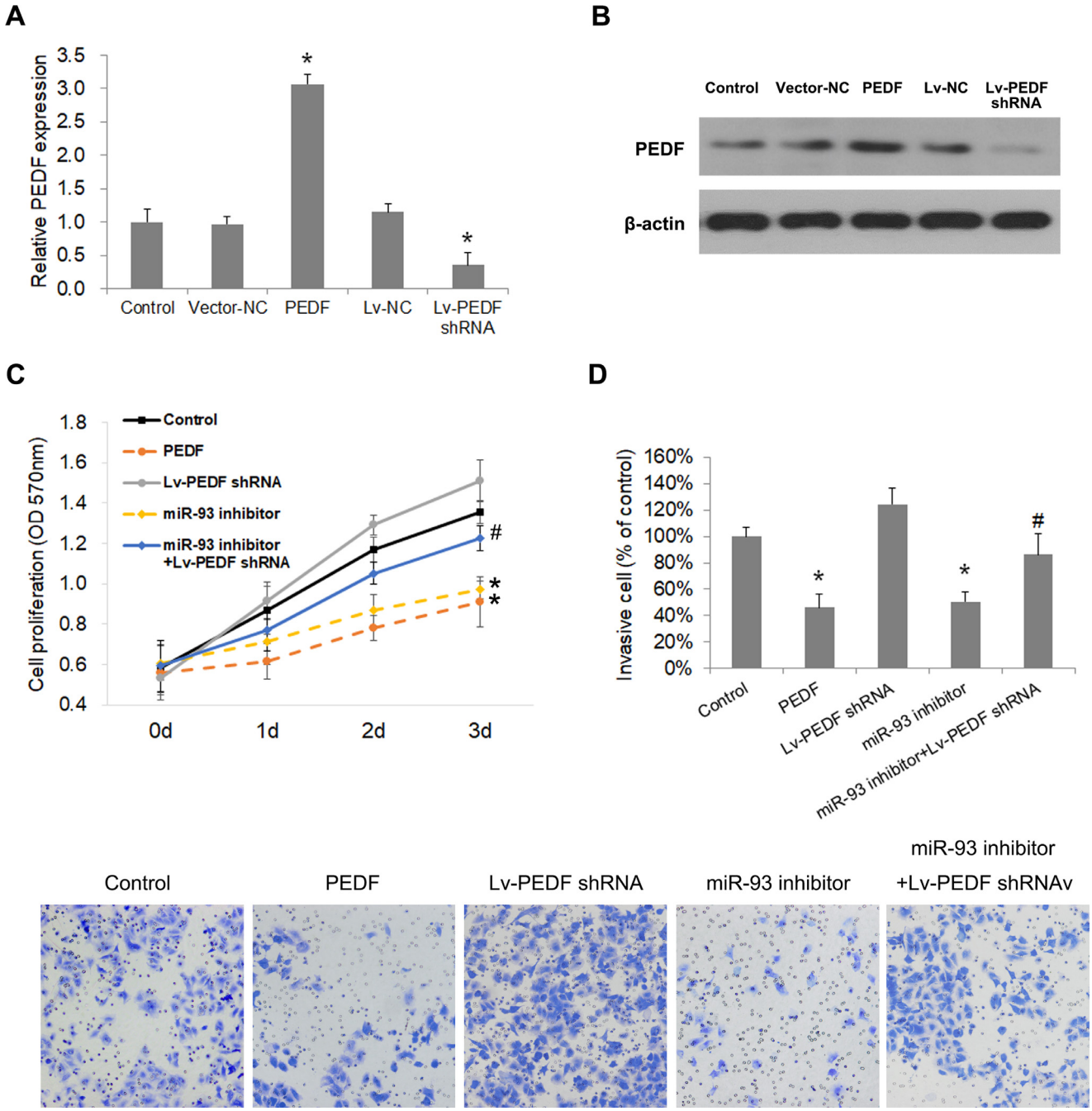

Fig. 5. PEDF mediates the effect of miR-93 on cell proliferation and invasion. TCCSUP cells were transfected with PEDF-overexpression vector (PEDF), Lv-PEDF shRNA, or miR-93 inhibitor. (A) PEDF mRNA expression was detected by real-time PCR. (B) PEDF protein expression was detected by western blotting. (C) Cell proliferation was measured at the indicated time periods using the MTT assay. (D) Cell invasion was detected with the Transwell assay. $^{*}P < 0.05$ compared with the control; $^{\#}P < 0.05$ compared with the miR-93 inhibitor group.

development, and metastasis [21]. Here, we showed that miR-93 was upregulated in BC tissues compared with normal samples, and its expression was associated with tumor stage and lymph node metastasis (N stage). Our results indicated that miR-93 functions as an onco-miRNA involved in BC cell proliferation and invasion by targeting PEDF.

The application of microRNAs (miRNAs) as potential biomarkers and therapy targets has been widely investigated in many kinds of cancers including BC [22,23]. miR-93 is dysregulated in several types of cancer, and high expression of miR-93 is correlated with poor prognosis [24–26]. Consistently, in the present study, miR-93 expression was significantly higher in BC tissues than in normal tissues, and high expression levels of miR-93 correlated with higher tumor stage and increased lymph node metastasis in patients with BC. These results indicated that aberrant expression of miR-93 may be crucial for BC progression. Espinosa-Parrilla et al. found there are single-nucleotide

polymorphisms in miR-93 cluster which may be the mechanism of its aberrant expression [27,28]. We next investigated the function and potential mechanisms underlying the effect of miR-93 on regulating the biological behavior of BC cells. Inhibition of miR-93 significantly suppressed cell proliferation and invasion, whereas miR-93 mimics had a minor effect. These results suggested that the endogenous expression of miR-93 is high in BC cells and may be necessary for BC progression. miR-93 is involved in tumor proliferation and invasion by directly downregulating many genes [13,24,29,30]. In the present study, we identified PEDF as a new target of miR-93 in BC. PEDF plays a role as a tumor suppressor and has anti-angiogenic and antimetastatic activities [18]. Lower PEDF levels were observed to associate with poorer overall survival in colorectal cancer patients [31,32]. E ctopic expression of PEDF during tumor progression results in the inhibition of tumor growth and prolonged survival in various animal models [18]. The interaction of PEDF with several cell surface receptors triggers downstream signaling pathways that mediate the effect of PEDF on regulating tumor cell behavior [18]. PEDF is downregulated in BC and related to tumor angiogenesis [33,34]. Here, we showed that PEDF expression was directly regulated by miR-93 in BC cells. Moreover, PEDF overexpression significantly suppressed the proliferation and invasion of BC cells, whereas PEDF knockdown abrogated the effects of miR-93 inhibition, suggesting that PEDF mediated, at least in part, the anticancer effect of miR-93 in BC cells. In addition to its effect on proliferation and invasion, miR-93 promoted angiogenesis through different molecular pathways [35]. Considering that PEDF is a potent inhibitor of angiogenesis [18], whether it also mediates the function of miR-93 in angiogenesis merits further investigation. Besides, the correlation of miR-93/ PEDF pathway and prognosis of BC patients is lacked in this study, but is also worth to analyze with longer follow-up.

In summary, the present study showed that miR-93 was upregulated in BC and associated with tumor stage and lymph node metastasis. The effect of miR-93 on BC cell proliferation and invasion was mediated by targeting PEDF. Our results provided new potential biomarkers and targets for BC diagnosis and treatment.

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