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Original Article

Targeted p21 activation by a new double stranded RNA suppresses human prostate cancer cells growth and metastasis

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Abstract: We have previously demonstrated that miR-1236-3p and its sequence homology dsRNA, dsRNA-245 (which is completely complementary to the p21 promoter) had potential ability to upregulate p21 expression by targeting specific promoter sequence and inhibited bladder cancer (BCa). However, we still know little about the effect of miR-1236-3p on prostate cancer and which dsRNA has an inhibitory effect on prostate cancer (PCa)? Here, we confirmed that miR-1236-3p was decreased in PCa cells and tissues. MiR-1236-3p inhibited PCa cells growth and metastasis by activating p21. Furthermore, we demonstrated that dsP21-245 could inhibit PCa cells growth and metastasis by activating p21 expression. Microarray experiments displayed that miR-1236-3p could affect AKT signaling pathway. We demonstrated that miR-1236-3p significantly suppressed the AKT pathway by inhibiting TLR2 expression while activating p21 expression in PCa cells; this influence was independent of p21 activation. In summary, our results provided evidence that both endogenous and exogenous small RNAs might function to induce p21 expression by interacting with the same promoter region, therefore impeding PCa development. Additionally, our results indicated that miRNA activation could activate the expression of some unknown genes as well as cell signaling pathways. This indicated the need for the further study of clinical applications of RNA activation.

Keywords: miR-1236-3p, RNA activation, p21, prostate cancer, AKT pathway

Introduction

PCa represents the second most common cause of cancer-related death in males in the USA, with a reported 26730 deaths in 2017 and an estimated annual incidence of 161360 new cases [1]. Similarly, the incidence and mortality rates of PCa have increased in China over the past a few decades [2]. Androgen deprivation therapy (ADT) is the main treatment of advanced PCa. Unfortunately, most androgen-dependent PCa patients progressed to castration-resistant state after a median time of 18-24 months [3]. Thus, there is an urgent need for further study of the carcinogenesis and development of PCa. Regulation of specific anti-tumor genes was verified to contribute to PCa initiation and development, the current study data have led the scholars to explore novel therapies based on targeted gene therapy for cancer treatment [4].

RNA interference (RNAi) is a silencing mechanism of evolutionary conserved gene in which small RNAs, such as exogenous double stranded RNAs (dsRNAs) or endogenous miRNAs, target specific mRNA sequences to inhibit mRNA translation or degrade them [5]. In contrast, RNA activation (RNAa) is a currently discovered phenomenon that dsRNAs or miRNAs can activate target gene expression by binding complementary sequences of the promoter [6]. As tumorigenesis may result from functional silence of anti-tumor genes, inhibited expression of the suppressor genes by RNAa would offer potential therapies for cancers.

Studies reported that several miRNAs or dsRNAs could influence the proliferation and metastasis of PCa cells. In a previous study, we demonstrated that E-cadherin could be activation through mature miR-373 or the corresponding dsEcad-640 which is perfectly complemen-

tary to the specific sequences of promoter [7, 8]. Moreover, dsP53-285 could up-regulate p53 expression and the overexpression of dsP53-285 potently inhibited the proliferation of PCa and BCa cells [9]. Studies also proved that p21 had the potential ability to inhibit tumor growth and metastasis by regulating epithelial mesenchymal transition (EMT) process [10]. P21 gene was proved to be induced by dsP21-322 and played an anti-tumor role in various of human cancers [11-13]. Besides, we found that a miRNA played different roles in different tumors. MiR-1236 can activate the expression of p21 in bladder cancer and lung cancer cells, but has no regulatory effect on p21 gene in liver cancer and pancreatic cancer cells [14]. In addition, we found that miR-1236 up-regulated the oncogene Skp2 expression while activating p21 gene in BCa cells, and that expression of Skp2 attenuated the anti-tumor effect of miR-1236. There was no effect on the expression of Skp2 while the corresponding dsRNA (dsP21-245) activated p21 [15]. More and more studies have shown that miRNA played an important role in the development of human tumors. However, the mechanism of action on tumor cells remains unclear. In the present study, we transfected miR-1236-3p and four dsRNAs (dsP21-242, dsP21-243, dsP21-244, and dsP21-245) corresponding to the miR-1236-3p target sequence into PCa cells and examined the p21 expression. Our results showed that the dsP21-245 could active p21 gene expression and also significantly inhibit PCa cells proliferation and metastasis.

TLR2 induces an inflammatory cascade predominately in response to products of bacterial lipoprotein. Besides, TLR1 or TLR6 which is heterodimerization of TLR2 expands the spectrum of ligand recognition to enable the innate immune system to sense a greater variety of lipoproteins amongst various pathogens and leads to the activation of nuclear factor kappa B (NF-κB), phosphoinositide-3-kinase (PI3K)/Akt, signal transducer and activator of transcription 3 (STAT3) pathways and extracellular-regulated kinase (ERK) [16]. There are many studies have confirmed that PI3K/Akt signaling pathway plays an important role in the development of PCa [17]. In present study, we discovered through genetic genomics that miR-1236-3p could inhibit the AKT pathway by inhibiting TLR2 expression. To determine whether and by what mechanism miR-1236-3p can induce AKT expression in human PCa cells, we transfected four dsRNAs (dsP21-242, dsP21-243, dsP21-244, and dsP21-245) corresponding to the miR-1236-3p target sequence to PCa cells and found that only dsP21-245 could activate p21 expression. However, dsP21-245 had no effect on AKT pathway while activating p21 in PCa cells.

Materials and methods

MiRNA and dsRNA

MiRNA mimics and dsRNAs were synthesized by RiboBio Co., Ltd. (Guangzhou, China). A small interfering RNA (sip21) was utilized to silence p21 expression, and a control miRNA, which lacks of significant homology to all known human sequences, was used as a negative control [18, 19]. The sequences of siP21: sense 5'-CUUCGACUUUGUCACCGAG-3', antisense 5'-CUCGGUGACAAAGUCGAAG-3'.

Cell culture, miRNA and dsRNA transfection

The human PCa cell lines DU145, PC3 and normal prostate epithelial cells RWPE-1 (ATCC) were cultured in RPMI 1640 medium (HyClone, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA). The day before transfection, cells were plated at a density of 30-50%. Both dsRNAs and miRNAs were transfected into PCa cells at a final concentration of 50 nM using Lipofectamine RNAiMax (Invitrogen, USA) according to the manufacturer's instruction.

RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

TRIzol reagent (Invitrogen, USA) was used to extract total RNA according to the manufacturer's instruction. Then, 500 ng of RNA was reverse transcribed into cDNA using a Takara reverse transcription kit (Takara, China) according to the manufacturer's protocol. The resulting cDNA was amplified by SYBR Premix Ex Taq II (Takara, China) on an Mx3000P instrument (Stratagene, USA). All the primers included in this study were offered by Invitrogen (Shanghai, China) and the primer sequences used were listed in **Table 1**. Genes expression levels were assessed by relative quantification using GAPDH as the endogenous reference gene.

Table 1. Primers used in this study

Name	Sequences (5'-3')	Assay used for
p21 (F)	GCCCAGTGGACAGCGAGCAG	PCR
p21 (R)	GCCGGCGTTTGGAGTGGTAGA	PCR
E-cadherin (F)	ACCAGAATAAAGACCAAGTGACCA	PCR
E-cadherin (R)	AGCAAGAGCAGCAGAATCAGAAT	PCR
GAPDH (F)	TCCCATCACCATCTTCCA	PCR
GAPDH (R)	CATCACGCCACAGTTTCC	PCR
Cyclin D1 (F)	GCTGCGAAGTGGAAACCATC	PCR
Cyclin D1 (R)	CCTCCTTCTGCACACATTTGAA	PCR
CDK4 (F)	ATGGCTACCTCTCGATATGAGC	PCR
CDK4 (R)	CATTGGGGACTCTCACACTCT	PCR
Vimentin (F)	GACGCCATCAACACCGAGTT	PCR
Vimentin (R)	CTTTGTCGTTGGTTAGCTGGT	PCR
TLR2 (F)	GCCTCTCCAAGGAAGAATCC	PCR
TLR2 (R)	TCCTGTTGTTGGACAGGTCA	PCR

Protein extraction and western blotting analysis

Total proteins were extracted using RIPA lysis buffer supplemented with a protease inhibitor cocktail (Roche, Switzerland) according to the manufacturer's protocol. Then, 50 μg of each protein sample was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% bovine serum albumin (BSA) (Sigma-Aldrich, USA) and then incubated with primary antibodies, including those against P21 (1/500) (BD Biosciences), E-cadherin (1/500) (BD Biosciences), Vimentin (1/1000) (Affinity, USA), CDK4 (1/1000) (Boster, China), Cyclin D1 (1/500) (Boster, China) and GAPDH (1/500) (Boster, China), overnight at 4°C. After three washes, the membranes were incubated with second antibodies and visualized using an enhanced chemiluminescence (ECL) assay kit (Millipore, USA).

Cell growth assay

Cell viability was detected daily for five days using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) method (Promega, USA) according to the manufacturer's protocol. MTS was added to cells, and then the plate was incubated for 2 h at 37°C at each time point. Absorbance was measured on a microplate reader (Bio-Rad, USA) at 490 nm.

Clonogenic survival assay

Transfected PCa cells were reseeded in 6-well plates at approximately 1000 cells per well, and the medium was replaced every 3 days. The colonies were then fixed 10 days later and stained with 0.5% crystal violet (Sigma, USA) for 30 min at room temperature. The colony formation rate was calculated using the following equation: colony formation rate = number of colonies/number of seeded cells × 100%.

Cell cycle analysis by flow cytometry

Cells were collected 72 h after transfection and fixed with 70% ethanol at 4°C overnight. Then, the fixed cells were washed and incubated with RNase A (0.1 mg/mL) for 30 min at room temperature. Cellular DNA was stained with propidium iodide (PI) (0.05 mg/mL) and analyzed on a FACSort flow cytometer (BD Biosciences, USA). All experiments were repeated 3 times, and a total of 10,000 events were analyzed for each sample. The data were processed using CELL Quest software (BD Biosciences, USA).

5-ethynyl-2'-deoxyuridine proliferation assay

Transfected BCa cells were plated into 96-well plates and the Cell-Light EdU DNA cell kit (Ribobio, China) was used to detect cell proliferation. The cells were incubated with 50 mM 5-ethynyl-2'-deoxyuridine (EdU) for 3 hours at 37°C. Then, 4% paraformaldehyde was used to fix the cells. The cells were treated with 0.5% Triton X-100 for 15 min, 1 mg/ml DAPI (Sigma, USA) was used to stained the cell nuclei for 30 minutes. The EdU-labeled cells were observed by fluorescence microscopy.

Migration and invasion assay

A 24-well Boyden chamber with an 8 μm pore size polycarbonate membrane (Corning, USA) was used to detect the cell motility. For the invasion assay, Matrigel (BD Biosciences, USA) pre-coated into the membranes to form a matrix barrier. Cells were plated in the upper chamber with serum-free medium. Medium containing 10% FBS, which served as a chemoattractant, was added to the lower chamber. The membranes were fixed and stained with 0.5% crystal violet (Sigma, USA). Cotton swabs

were used to remove the non-motile cells from the top of the membranes, randomly extract 5 visual fields with 200× magnification for counted.

About 5×10^5 PCa cells were plated in a six-well plate following transfection. The confluent cell monolayers were scratched. Then, PBS was used to wash the non-adherent cells and serum-free medium was added to the wells. The migrated distances were observed at 0 and 24 h post-scratch.

Chromatin immunoprecipitation assay (ChIP assay)

Cells transfected with biotin-labeled dsRNA were used for ChIP assay using a ChIP assay kit (Millipore) at 72 h according to the manufacturer's protocol. Collection of 3×10^6 cells were used for a single immunoprecipitation. Degradation of RNA was avoided by RNase inhibitor (Thermo, USA) with a final concentration of 50 units/ml. One percent formaldehyde was used to cross-link chromatin for 10 minutes at 37°C. Then, SDS lysis buffer was used to wash and re-suspend the fixed cells. Biotin (Santa Cruz Biotechnology, USA) antibody and normal rabbit IgG (Millipore, USA) (negative control) were used for immunoprecipitation after the chromatin was pre-cleared with protein A agarose/Salmon sperm DNA overnight at 4°C. Then, the antibody/antigen/DNA complexes were collected and reversed. The column-purified (Omega bio-tek, USA) DNA was used as a template for real-time PCR.

Statistical analysis

Statistical analyses were performed in the SPSS17.0 software (SPSS Inc., Chicago, IL, USA). All data are shown as the mean ± standard deviation (SD) for the three independent experiments. Differences between groups were analyzed with Student's t-tests. Statistical significance between three or more groups was analyzed by one-way ANOVA. $P<0.05$ was considered significant.

Results

MiR-1236 and p21 expression are reduced in both PCa tissues and PCa cell lines

To investigate the potential roles of miR-1236 and p21 in PCa, we first examined their expression levels in prostate tumor/normal tissues and cells. MiR-1236 and p21 mRNA levels were significantly down-regulated in 3 PCa cell lines (LNCaP, PC3, and DU145) compared with normal prostate epithelial cells RWPE-1 (**Figure 1A** and **1B**). We then estimated the expression levels of miR-1236 and p21 mRNA levels in 17 pairs of PCa and corresponding adjacent normal tissues. The average expression levels of miR-1236 and p21 mRNA levels were lower in the PCa tissues than in the adjacent normal tissues (**Figure 1C** and **1D**). Spearman's correlation test was used to evaluate the relationship between miR-1236 and p21 expression levels. The outcomes showed that miR-1236 expression was positively correlated with p21 expression (**Figure 1E** and **1F**, Normal, $r=0.9341$, $P=0.0128$; Tumor, $r=1.165$, $P=0.0115$).

Activation of p21 by miR-1236 and its corresponding dsRNAs

We found that miR-1236 was robustly able to induce p21 expression by targeting the promoter sequences (**Figure 2A** and **2B**) in PCa cells. To evaluate whether sequence homologous dsRNAs fully complementary to the putative miR-1236 target site could induce p21 expression in PCa cells, we designed four different dsRNAs through an Excel macro template and obtained detailed scores according to the design rules (**Figure 2C**) [20]. Each duplex was named according to its target within the p21 promoter.

Next, we transiently transfected the four dsRNAs (dsP21-242, dsP21-243, dsP21-244 and dsP21-245) and miR-1236 mimics into PC3 and DU145 cells for 72 h. As seen in **Figure 2D**, the p21 mRNA expression was significantly enhanced by miR-1236 and dsP21-245 in PC3 and DU145 cells. Additionally, the expression of dsP21-245 caused higher p21 mRNA expression levels than the expression of miR-1236 in PC3 and DU145 cells. However, other dsRNAs failed to significantly induce p21 mRNA expression in the two cell lines. The gene activation effect was further confirmed by immunoblot analysis (**Figure 2E**).

MiR-1236 overexpression can inhibit PCa cells proliferation and metastasis

We then investigated whether miR-1236-activated p21 expression could inhibit PCa cell proliferation and metastasis. In consistent with our speculation, after transfection with miR-

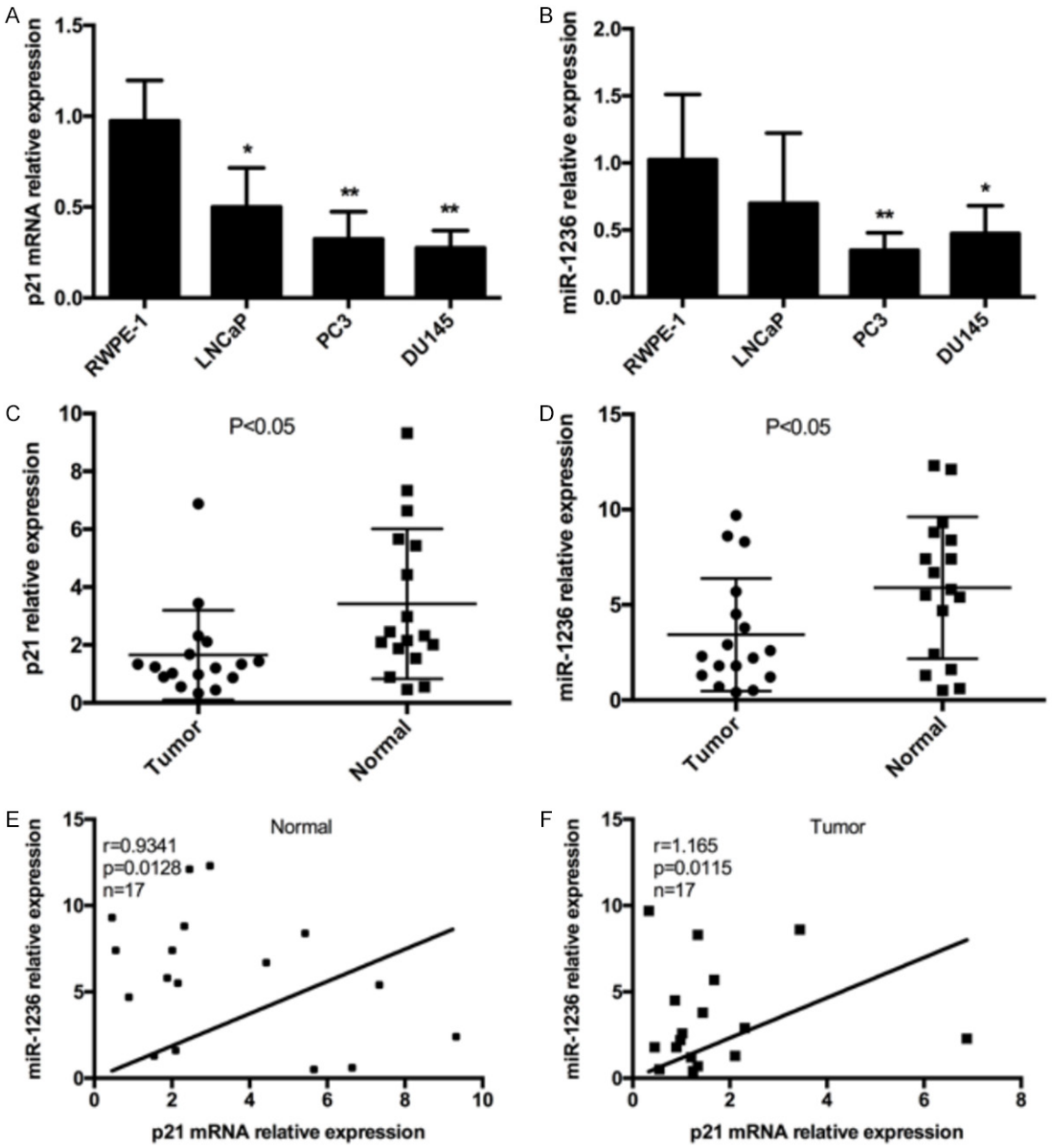

Figure 1. The expression pattern of p21 and miR-1236 are detected in prostate cancer cells and tissues. (A and B) Expression of miR-1236 and p21 mRNA levels in prostate cell lines were assessed by real-time PCR. *P<0.05, **P<0.01 compared to RWPE-1 group. (C and D) The expression of miR-1236 and p21 mRNA levels in prostate cancer tissues were assessed by real-time PCR. GAPDH and U6 served as corresponding loading controls. *P<0.05 compared to Normal group. Both (E) and (F) showed positive correlation between miR-1236 and p21 expression levels in prostate cancer and adjacent normal tissues.

1236 mimics, a significant increase in miR-1236 expression was detected using qRT-PCR. An MTS assay showed that the proliferation rate of DU145 and PC3 cells was significantly repressed after overexpression of miR-1236 (**Figure 3A** and **3B**). Moreover, the ability of colony formation was notably weakened (**Figure 3C-E**). To further detect the alterations of cell metastasis caused by miR-1236, a wound healing assay was conducted to analyze overexpression of miR-1236 that inhibited cell invasion in both PC3 and DU145 cells (**Figure 3F**).

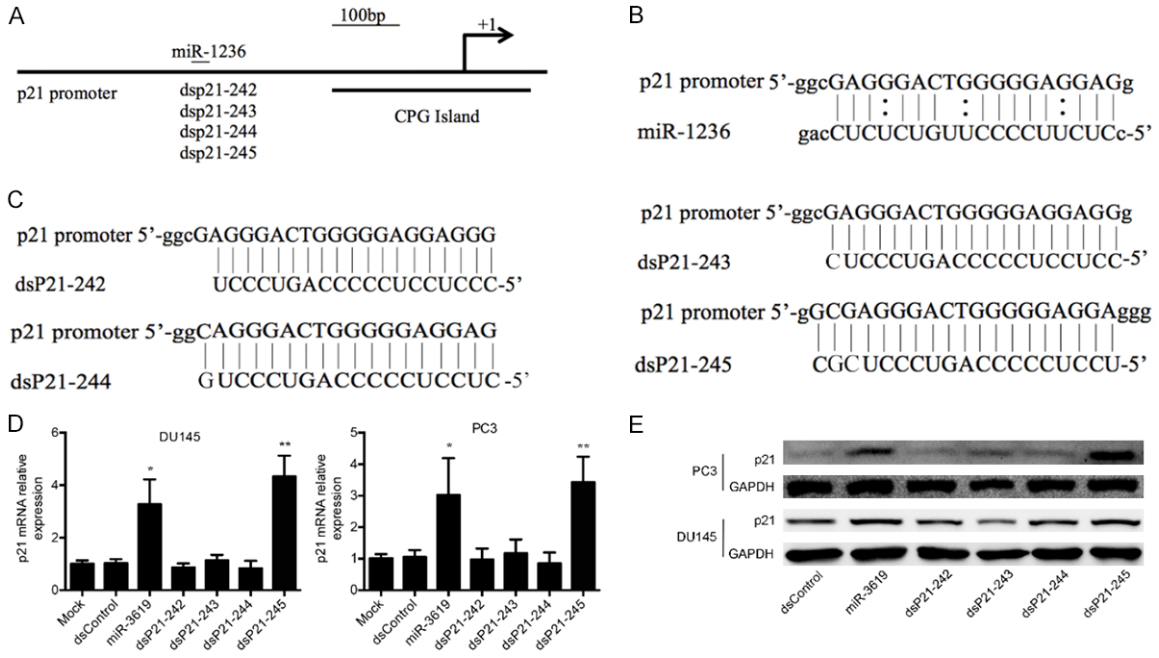

Figure 2. Expression of p21 was induced by miR-1236 and corresponding dsRNAs through promoter targeting prostate cancer cells. A. Schematic representation of the p21 promoter and corresponding dsRNA target sites. B. The miR-1236 seed sequence is complementary to the p21 promoter. C. The four dsRNAs are fully complementary to the putative miR-1236 target site. D. p21 mRNA levels were assessed by qRT-PCR. GAPDH was used as an internal control. *P<0.05, **P<0.01 compared to untreated or control miRNA group. E. Expression of p21 protein was examined by Western blot analysis. GAPDH level was used as an internal control.

DsP21-245 inhibits PCa cells proliferation through activating p21 expression

As shown in **Figure 4A**, compared to the dsControl group, both tested cells exhibited progressive retarded growth after 48 h as measured by CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay. And knockout of p21 evidently weakened the anti-proliferative effect regulated by dsP21-245 in both cell lines. The DU145 and PC3 cells both showed less colony formation after transfection of dsP21-245 (**Figure 4B**). And the colony formation ability of the PCa cells was restored after co-transfection of siP21 compared with dsControl group (**Figure 4B**). Furthermore, DU145 and PC3 cells accumulated in G1/G0 phase and displayed reduced S phase and G2/M phase cells after dsP21-245 transfection. However, G1/G0 phase cells as well as S phase and G2/M phase cells were restored after co-transfection of siP21 compared with dsControl group (**Figure 4C**). An EdU assay was conducted to further detect the alterations in cell proliferation, and the outcomes were consistent with the above results (**Figure 4D** and **4E**).

DsP21-245 restrains PCa cells migration and invasion through activating p21 expression

We next analyzed the effect of dsP21-245 on PCa cell motility in vitro. We conducted migration and Matrigel invasion chamber assays to evaluate the migration and invasion abilities of the transfected cells, respectively. The results showed that dsP21-245 inhibited cell migration and invasion. Similarly, after knockout of the p21 gene in PC3 and DU145 cells, the invasion and metastasis inhibitory effects of dsP21-245 were significantly weakened (**Figure 5A** and **5B**).

DsP21-245 interacts with the p21 gene promoter directly

Studies have verified that the binding of miRNAs to target gene promoters is a possible mechanism for RNA activation [21-23]. ChIP assay was conducted to identify whether dsP21-245 activated p21 by targeting a specific site in its promoter. 72 hours after transfection with biotinylated dsP21-245 or the dsControl, the target promoter DNA was pulled down

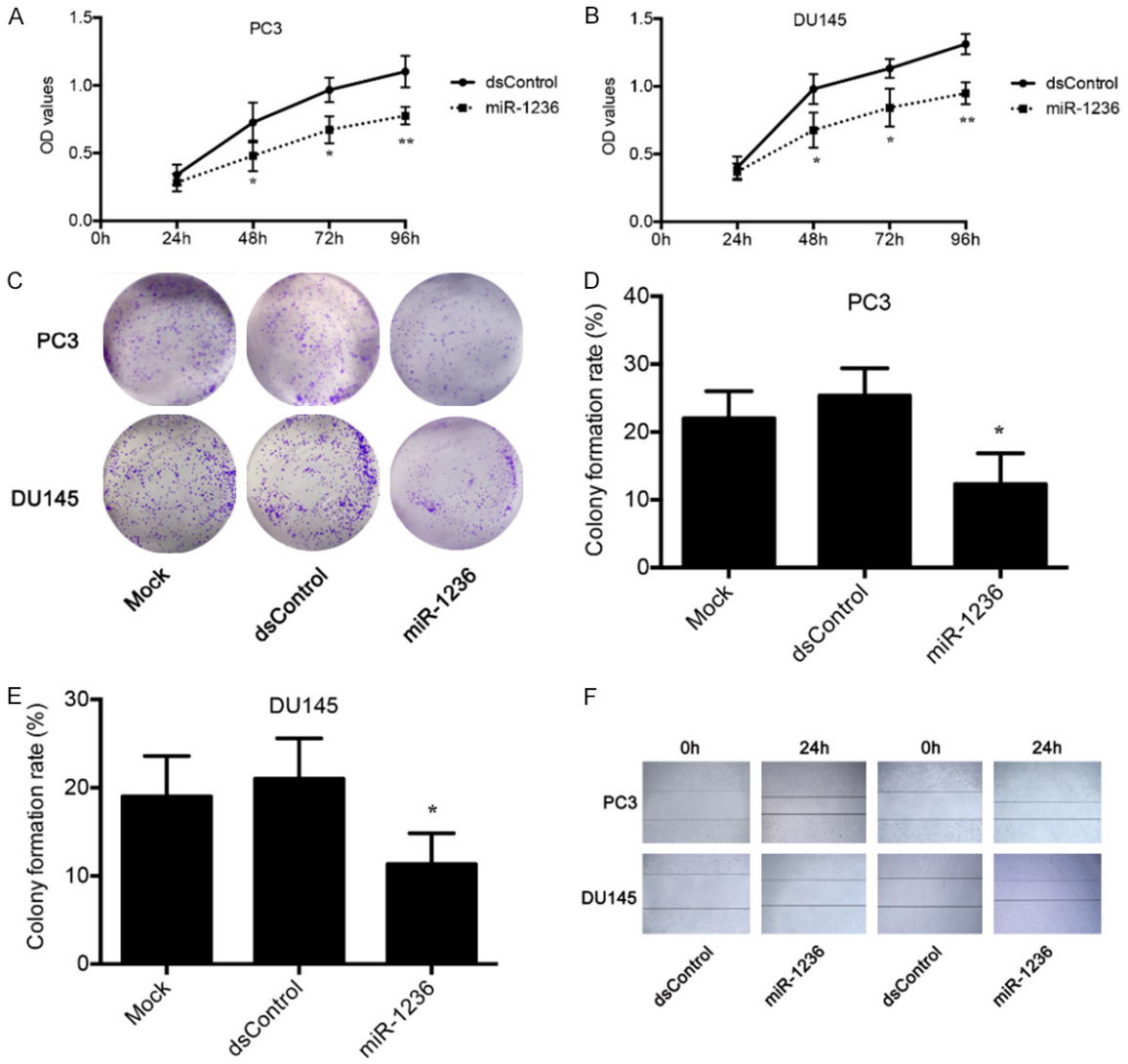

Figure 3. miR-1236 inhibits prostate cancer cells proliferation, clonogenesis and metastasis. DU145 and PC3 cells were transfected with 50 nM of the indicated RNAs for 72 h. A, B. Viable cells were measured from 24 h to 96 h following transfection using the Cell Titer 96 AQ_{ueous} One Solution Cell Proliferation Assay kit. Results were plotted as OD values. *P<0.05, **P<0.01 compared to dsControl group. C. Representative photographs of colony formation assay. D, E. Quantification of the cell colonies formation. *P<0.05 compared to Mock and dsControl groups. F. Representative wound-healing images at 0 and 24 h.

using a corresponding biotin antibody and amplified by qRT-PCR. The primer set amplifying the p21 promoter from -1288 bp to -1069 bp relative to the TSS (transcription start site) served as a negative control (**Figure 6A**). As seen in **Figure 6B** and **6C**, both the 5'-end and 3'-end of the biotin-labeled dsP21-245 pulled down promoter proximal DNA (from -326 to -107) more obviously than the dsControl in DU145 and PC3 cells. In contrast, there was no remarkable change in the covalently linked of the dsP21-245 and the dsControl RNAs to the negative control region. These results indicated that dsP21-245 activated p21 gene expression by directly combining with the specific promoter sequence.

DsP21-245 modulates PCa cells cell cycle and EMT associated genes mainly by enhancing p21 gene

We further determined the effects of dsP21-245 transfection on the expression of downstream genes associated with cell cycle and mitotic checkpoint in PCa cells. We first detected the expression of p21 mRNA after transfec-

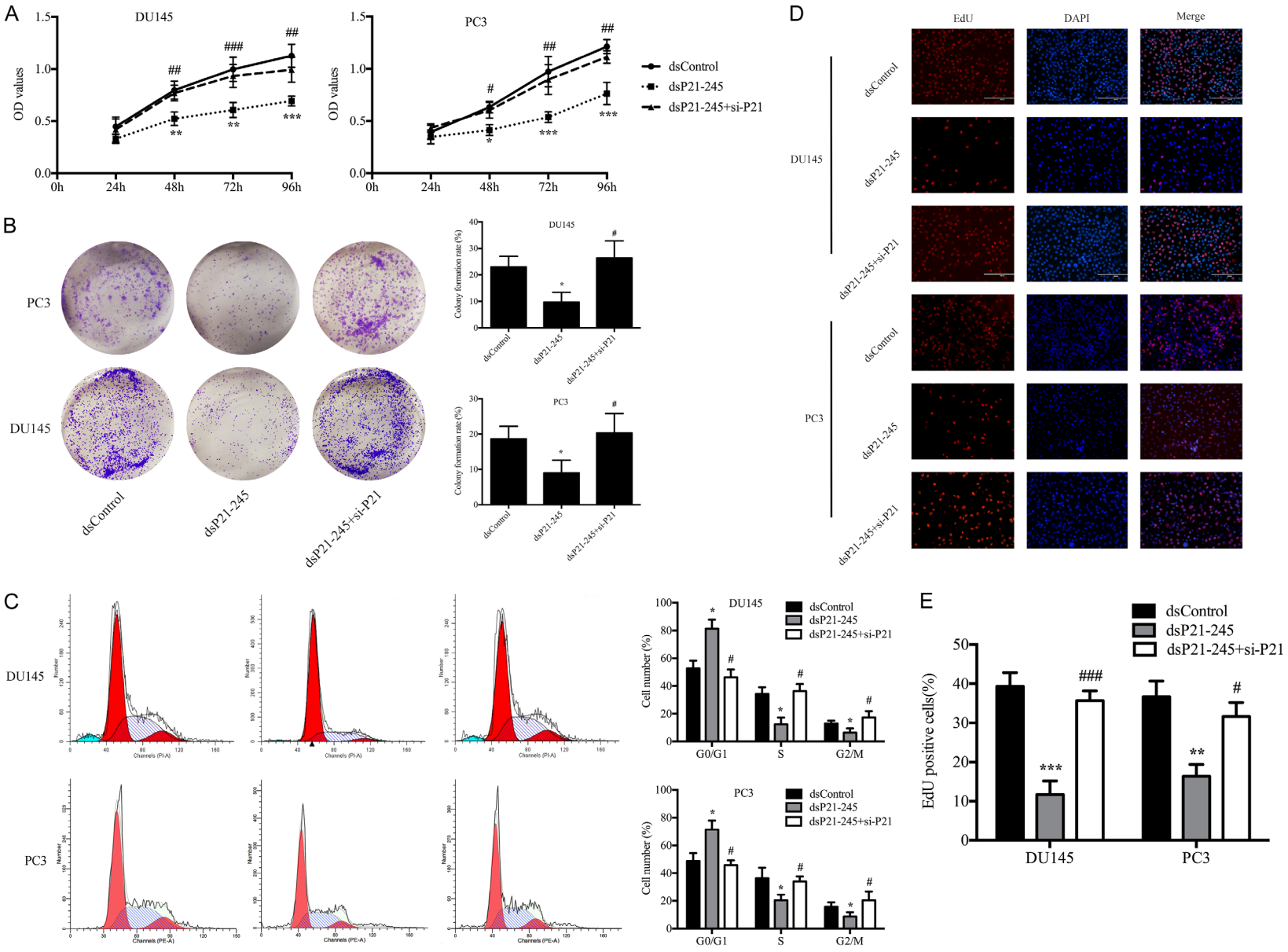

Figure 4. Dsp21-245 inhibits prostate cancer cells growth mainly via up-regulating p21 expression. DU145 and PC3 cells were transfected with 50 nM of the indicated RNAs for 72 h. A. Viable cells were measured from 24 h to 96 h following transfection using the Cell Titer 96 AQ_{ueous} One Solution Cell Proliferation Assay kit.

Results were plotted as OD values. B. Representative photographs of colony formation assay and quantification of cell colony formation. C. Representative images of cell cycle analysis in DU145 and PC3 cells as well as quantification of cell cycle distribution. D. Representative micrographs of EdU-positive cells (red). The nucleus was stained with DAPI (blue). E. Quantification of EdU-positive cells. *P<0.05, **P<0.01 and ***P<0.001 compared to dsControl group. #P<0.05, ##P<0.01 and ###P<0.001 compared to dsP21-245 group.

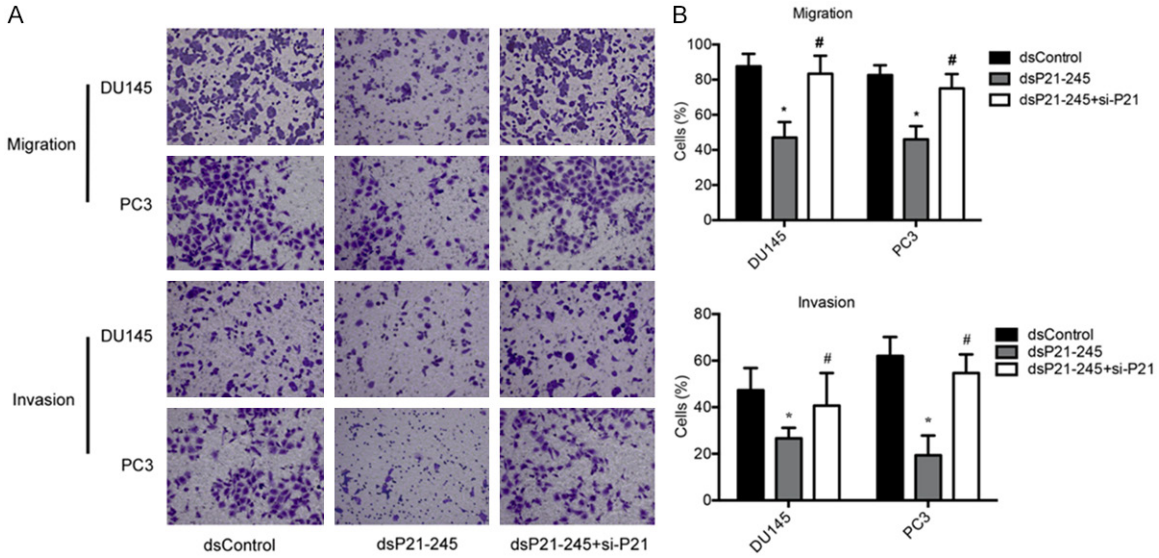

Figure 5. Dsp21-245 inhibits prostate cancer cells migration and invasion mainly through activating p21 expression. DU145 and PC3 cells were transfected with 50 nM indicated RNAs for 72 h. A. Representative photographs of the transwell assay (200×). B. The number of migrated and invaded cells was quantified. *P<0.05 compared to dsControl group. #P<0.05 compared to dsP21-245 group.

tion of dsP21-245, dsP21-322 was used to activate p21 expression (positive control). After co-transfection with sip21, the activated effect of dsP21-245 was disappearance (**Figure 7A** and **7B**). As shown in **Figure 7C** and **7D**, transfection of dsP21-245 caused a significant decrease of mRNA levels of Cyclin D1 and CDK4 in both tested cell lines. DsP21-245 failed to down-regulated Cyclin D1 and CDK4 mRNA levels after siP21 co-transfection. The suppressing effects on protein levels of these genes were further identified by western blotting analysis (**Figure 7G**). Moreover, some essential genes related to the EMT process were also detected post dsP21-245 transfection. As seen from **Figure 7E** and **7F**, compared to dsControl group, dsP21-245 significantly up-regulated the mRNA expression of epithelial marker, E-cadherin, and down-regulated mesenchymal marker, Vimentin in both cell lines, respectively. We next assessed the expression levels of the specific genes mediated EMT process after siP21 transfection. Compared to dsP21-245 group, the mRNA of epithelial marker E-cadherin was down-regulated, whereas mesenchymal marker Vimentin was up-regulated after co-treatment of siP21 for 72 h in both PC3 and DU145 cells. Immunoblot analysis also revealed the same trend with qRT-PCR trend (**Figure 7G**). Taken together, our data strongly imply that dsP21-245 modulates PCa cells cell cycle and EMT associated genes largely depended on modulating p21 expression.

miR-1236 inhibits the Akt signaling pathway in PCa cells

Genetic genomics results showed that miR-1236-3p could inhibit the AKT pathway by inhibiting TLR2 expression. In the present study, we verified the influence of miR-1236-3p on the protein levels of AKT and p-AKT. Our results showed that high levels of miR-1236-3p decreased the expression of TLR2. Besides, we found that miR-1236 induced TLR2 expression independent of p21 activation. However, dsP21-245 had no effect on the expression of TLR2 (**Figure 8A**). Similarly, over-expression of miR-1236-3p decreased the phosphorylation

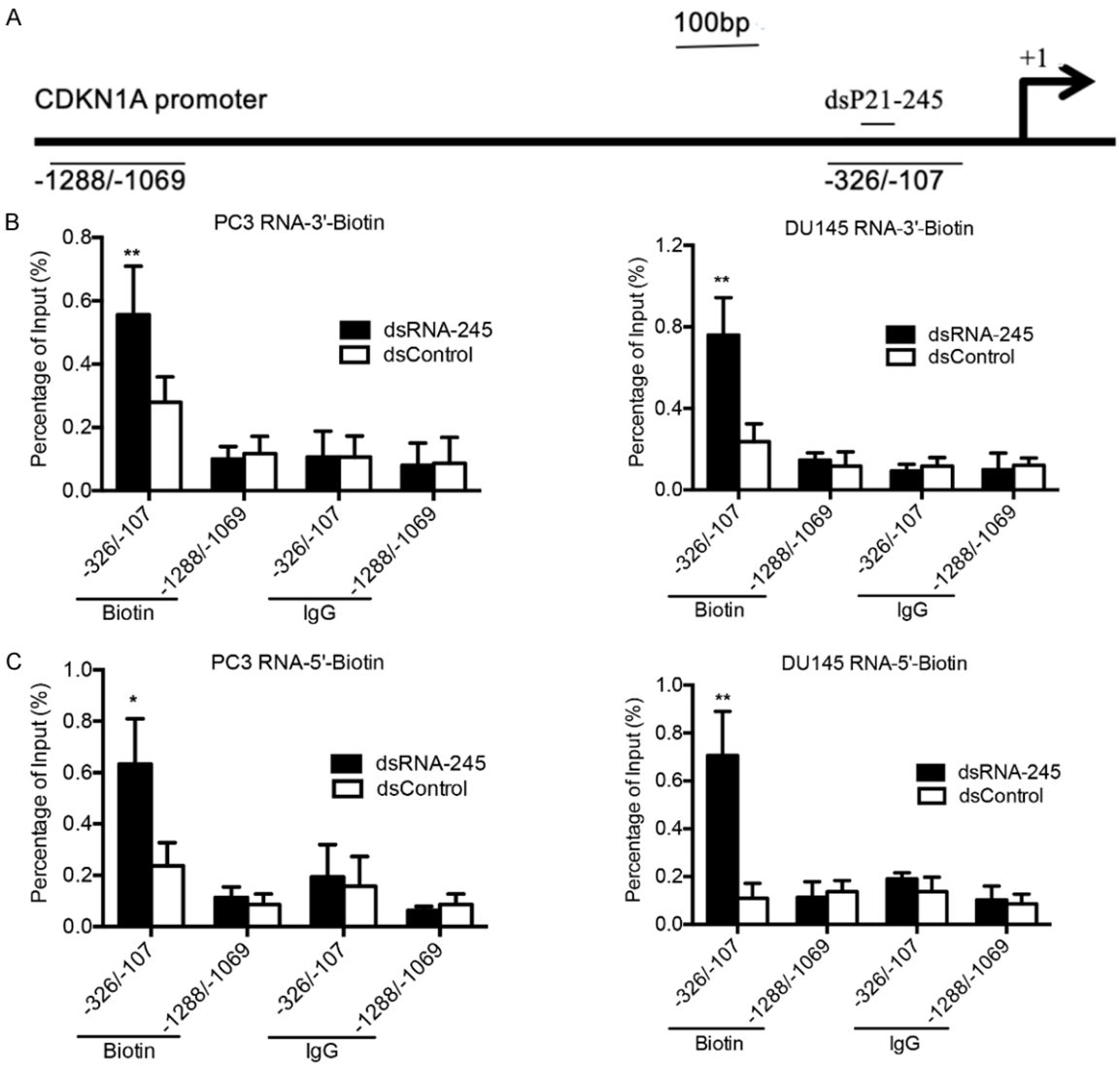

Figure 6. DsP21-245 directly interacts with the p21 promoter. The specific cells were transfected with 50 nM biotinylated dsP21-245 or dsControl for 72 h. A. Schematic illustration of the primers capable of amplifying the p21 promoter at different regions. Locations are shown relative to the TSS. B, C. ChIP assay showed that biotin-labeled dsP21-245 pulled down promoter proximal DNA (-326/-107) more effectively than the dsControl RNA (-1288/-1069). In contrast, there were no differences in the binding of dsP21-245 and dsControl RNAs to DNA upstream of the p21 promoter in IgG group that served as a negative control in PC3 and DU145 cells. *P<0.05, **P<0.01 compared to the corresponding dsControl group.

of AKT (P-AKT) and dsP21-245 had no influence on the P-AKT level. Whereas, miR-1236-3p failed to down-regulating phosphorylation of AKT levels after siTLR2 co-transfection in DU145 and PC3 cells (**Figure 8B** and **8C**). Collectively, our results revealed that miR-1236-3p suppressed the Akt signaling pathway partly through its target TLR2.

Discussion

One distinctive feature of cancer cells is their sustained proliferative activity. Thus, blockade of cell cycle is regarded as effective strategy for cancer therapy [24]. Cell cycle is mainly controlled by Cyclin-CDK [25]. Inhibition of Cyclin D1-CDK4/6 complexes activity elicits reduction in G0/G1-to-S phase progression [26]. It is now well known that p21 is a powerful CDK inhibitor involved in many antigrowth pathways including promoting cell cycle arrest. The p21 protein has a cyclin binding motif and inhibits Cyclin-CDK complexes by direct interaction [27]. Earlier studies indicated that the expression of p21 is decreased in PCa cells [28]. Up-regulation of

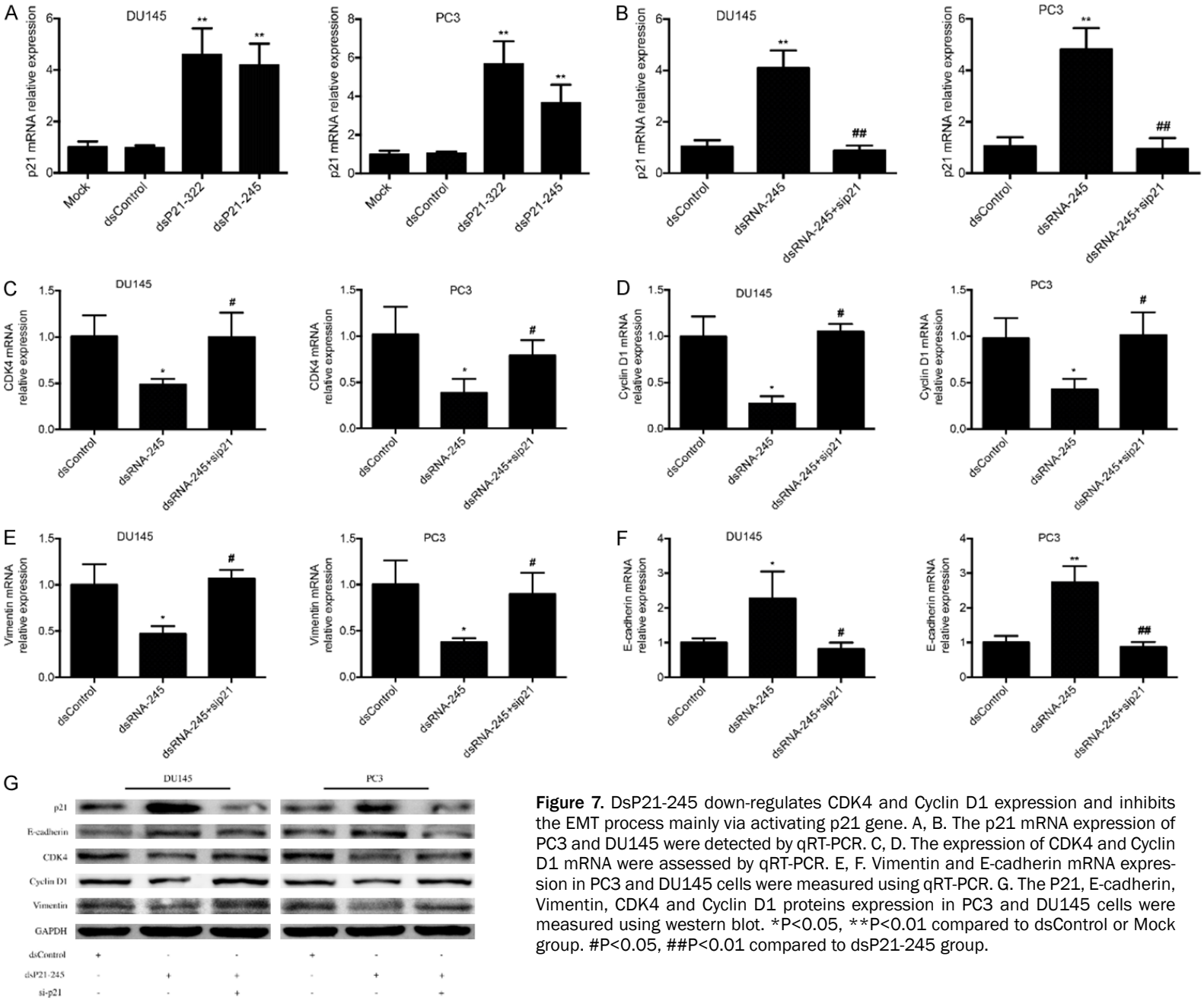

Figure 7. DsP21-245 down-regulates CDK4 and Cyclin D1 expression and inhibits the EMT process mainly via activating p21 gene. A, B. The p21 mRNA expression of PC3 and DU145 were detected by qRT-PCR. C, D. The expression of CDK4 and Cyclin D1 mRNA were assessed by qRT-PCR. E, F. Vimentin and E-cadherin mRNA expression in PC3 and DU145 cells were measured using qRT-PCR. G. The P21, E-cadherin, Vimentin, CDK4 and Cyclin D1 proteins expression in PC3 and DU145 cells were measured using western blot. *P<0.05, **P<0.01 compared to dsControl or Mock group. #P<0.05, ##P<0.01 compared to dsP21-245 group.

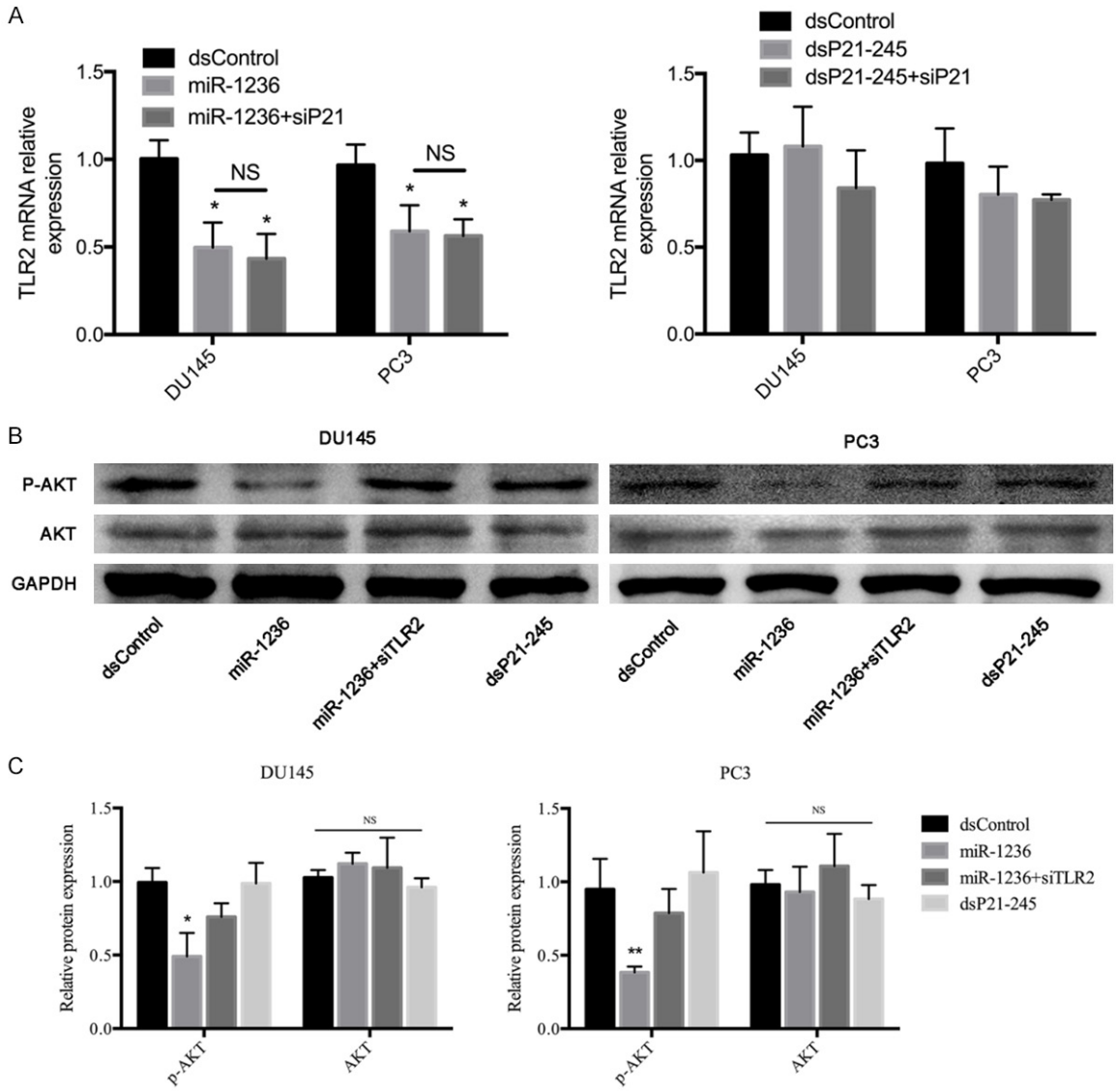

Figure 8. MiR-1236 suppresses the Akt signaling pathway partly through its target TLR2 independent of p21 activation. A. The TLR2 mRNA expression after transfection of miR-1236 and dsP21-245 were measured through qRT-PCR in PC3 and DU145 cells. Knockdown with sip21 had no influence on the downregulation of TLR2 by miR-1236. B. Phosphorylation levels of AKT were measured using western blot in different groups. C. Quantification of protein expression levels. *P<0.05 and **P<0.01 compared to dsControl group.

p21 gene through RNAa would exert potent anti-tumor capacity [29, 30]. Moreover, we have reported that exogenous dsRNA or endogenous miRNA could influence the expression of p21 in variable levels by targeting different regions of promoter [31].

In the present study, we demonstrated that a synthetic dsRNA (dsP21-245), which is completely complementary to putative miR-1236 target site of p21 promoter, exhibits considerable potency to activate p21 expression in PCa cells. Moreover, dsP21-245 possesses a similar capacity with miR-1236 to enhance p21 expression. Several Cyclin-CDK genes (Cyclin D1 and CDK4) associated with cell cycle are down-regulated following dsP21-245 transfection. Hereby, transfection of dsP21-245 causes PCa cell cycle arrest and impedes proliferation. In addition, this dsRNA's anti-tumor function is mainly achieved by regulating p21 expression.

EMT process is a crucial initiator and contributor to tumor metastasis. We confirmed that

dsP21-245 increased the expression of the epithelial biomarker E-cadherin and inhibited the expression of the mesenchymal biomarkers Vimentin in PCa cells which indicated that dsP21-245 suppressed the EMT progression in PCa cells. Furthermore, we found that exogenous dsP21-245 significantly inhibited the metastasis of PCa cells. The inhibition of dsP21-245 was significantly weakened after silencing the p21 expression in PCa cells. Experimentally enforced expression of dsP21-245 in PCa cells resulted in cell cycle arrest and metastasis suppression. Thus, dsP21-245 fulfilled the criteria of a tumor suppressor gene in the context of PCa.

The same miRNAs play different roles in different cell types. We demonstrated that miR-370 and miR-1236 inhibited tumor growth by activating p21 gene expression in bladder cancer T24, EJ cells, and non-small-cell lung carcinoma A549 cells, while in hepatocellular cells both microRNAs cannot effectively induce the expression of p21. In pancreatic cancer PANC-1 cells, only miR-370-5p had the potent abilities to induce p21 expression rather than miR-1236-3p [28]. The specific mechanism of RNA activation may not be a single one-to-one model. In previous study, we identified that miR-3619 suppressed the expression of CDK2 and β-catenin while activing the p21 exprssion [32]. Besides, we confirmed that miR-1236 increased the expression of S-phase kinase-associated protein 2 (Skp2) while activing the expression of p21 and the corresponding of dsRNA had no effect to the expression of Skp2 while inducing p21 expression [15]. In present study, we found that miR-1236 overexpression significantly decreased the Toll-Like Receptor 2 (TLR2) expression while activating p21 expression in PCa cells. Besides, we confirmed that miR-1236-3p could inhibit the AKT pathway by inhibiting TLR2 expression. Moreover, we found that miR-1236-3p inhibited the expression of TLR2 independent of the expression of activated p21. However, the effect of AKT signaling pathway on the role of miR-1236 in inhibiting PCa cells function needs to be further clarified.

Conclusions

Taken together, our results provide evidences that dsP21-245 stimulated p21 expression by directly targeting promoter-derived sequences which is identical to miR-1236 target site. Besides, miR-1236 and dsP21-245 also has the capacity to induce cell cycle arrest and inhibit proliferation and metastasis by activating p21 expression in PCa cells. Furthermore, miR-1236 inhibits PI3K/Akt through targeting TLR2 expression while activating p21 gene, which independent of the expression of activated p21. We speculate that miRNA activation may lead to the aberrant expression of some unknown genes. Therefore, identifying more natural targets induced by miRNAs may provide additional insights into the gene expression profiles associated with certain miRNAs.

Disclosure of conflict of interest

None.

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