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From Cell Biology to Tissue Engineering

The suppressive effects of miR-1180-5p on the proliferation and tumorigenicity of bladder cancer cells

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Summry. In our previous research, we have reported that a candidate microRNA (miR-1180-5p) has the capacity to induce overexpression of tumor suppressor gene *p21* and inhibit the growth of human bladder cancer (BCa) cell lines *in vitro*. However, the exact mechanism as to how miR-1180-5p suppresses BCa cell proliferation remains unknown, and the inhibitory effect of miR-1180-5p *in vivo* also need to be investigated. In the present study, we found that the expression level of miR-1180-5p was lower in BCa cells than in normal human urothelial cells. Furthermore, we found that overexpression of *p21*, activated by miR-1180-5p, interfered with cell cycle progress by inhibiting the cell cycle related proteins (CDK4, CDK6, Cyclin D1 and Cyclin A2), and thereby suppressed BCa cell proliferation. In addition, miR-1180-5p also suppressed the tumor growth *in vivo* significantly. Taken together, our study provides evidence that up-regulation of *p21* is mainly responsible for the suppressive effect of miR-1180-5p on BCa cells and miR-1180-5p can significantly inhibit tumorigenicity *in vivo*.

Key words: miR-1180-5p, Proliferation, Bladder cancer, Tumorigenicity

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Introduction

Bladder cancer (BCa) represents the third most common cancer diagnosed in men of United States, with an estimated annual incidence of 56,320 new cases and 11,510 deaths of men in 2015, which is three times more common in men than in women (Siegel et al., 2015). A high rate of recurrence and progression often results in poor prognosis of patients undergoing conventional treatments such as surgical resection, chemotherapy and radiotherapy (Kaufman et al., 2009). Lack of effective treatment strategies creates an urgent need for molecular targeted therapy.

Endogenous micro RNAs (miRNAs) are a large class of small non-coding RNA which can not only silence specific gene expression at the post-transcriptional level by degrading target mRNAs or suppress their translation, but also can activate indicated gene expression at transcriptional level by targeting their promoter (Bartel, 2004; Guo et al., 2014). We have previously reported that a candidate miRNA (miR-1180-5p) can activate p21 expression by targeting its promoter in human BCa cell lines T24 and EJ (Wang et al., 2014b). Regrettably, the difference between the expression level of miR-1180-5p in BCa cells and that in normal human urothelial cells was not examed. Then, the research referring to miR-1180-5p's inhibitory effect on BCa cell proliferation was superficial and the relevant mechanisms are largely unclear, especially the role of p21 expression on BCa cell growth. Moreover, the question whether miR-1180-5p can activate *p21* expression and suppress BCa cell growth *in vivo* also

needs to be explored.

In the present research, we demonstrated that miR-1180-5p was lowly-expressed in BCa cells compared with normal human urothelial cells. We analyzed the alterations of cell cycle related proteins and evaluated BCa cell proliferation after *p21* was silenced. Besides, we injected nude mice subcutaneously with EJ cells infected with indicate recombinant lentivirus to observe the effect of miR-1180-5p on tumorigenicity. Our results not only confirmed that overexpression of *p21* is responsible for the inhibitory effect of miR-1180-5p on BCa cell proliferation, but also verified that miR-1180-5p can activate *p21* expression and significantly suppress tumorigenicity *in vivo*.

Materials and methods

Recombinant Lentivirus and small non-coding RNA

Lenti-miR-1180-5p, Lenti-NC, miR-1180-5p mimics and siP21 were all synthesized by GenePharma (Shanghai, China). Lenti-NC (5'-TTCTCCGAACG-TGTCACGT-3') was used as a nonspecific control (Liu et al., 2015; Zhou et al., 2015). siP21 is a small interfering RNA used to silence p21 expression (Li et al., 2014). All the sequences of RNAs are listed in Table 1.

Cell culture, infection with Lentivirus and transfection with dsRNA

Human BCa cell lines T24 and EJ (ATCC) were cultured in RPMI 1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Gibco, USA) at 37°C with 5% CO_2. Normal human urothelial cell SV-HUC-1 (ATCC) was cultured in F12K medium (Sigma, USA) supplemented with 10% fetal bovine serum (Gibco, USA) under the same conditions.

The day before infection, $3\sim5\times10^3$ cells were seeded in a 96-well plate with 100 μL culture medium, to ensure cells were at a density of 40-60% in each well during infection. T24 cells were infected with Lenti-miR-1180-5p or Lenti-NC at 20 Multiply of Infection (MOI) and EJ cells were infected at 40 MOI according to the manufacturer's protocol with modification. Culture medium was substituted 24 h later. Fluorescence expression was observed at 72~96 h after infection. Then cells were harvested and reseeded into Petri dishes for further experimental treatment.

Table 1. Sequences for RNAs used in present study.

	Sequences (5'-3')
miR-1180-5p	GGACCCACCCGGCCGGGAAUA
siP21 Sense	CUUCGACUUUGUCACCGAG[dT][dT]
siP21 Antisense	CUCGGUGACAAAGUCGAAG[dT][dT]

Transfection with indicated miRNA or siP21 at a final concentration of 50 nM was performed as previously described (Wang et al., 2016).

Cell proliferation assay

1000 cells per plate were grown on 96-well plates. Cell proliferation ability was analyzed daily for 5 days using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit (MTS Assay kit, Promega, USA). At each time point, the culture medium of each plate was replaced with 100 μL fresh medium premixed with 10 μL of CellTiter 96® AQ_{ueous} One Solution, and then incubated at 37°C for 2 h. The absorbance at 490 nm of each plate was measured by a microplate reader (Bio-Rad, USA).

Colony formation assay

Cells were seeded into 6-well plates with 1000 cells/plate. After being incubated for 10 days, the colonies were fixed with methanol for 15 min at room temperature, and then stained with 0.5% crystal violet (Sigma, USA) for 30 min at the same temperature. The crystal violet was washing gently with running water, aired and then counted. Colony formation rate = number of colonies/number of seeded cells × 100%.

Cell cycle analysis

Cells were harvested with trypsin, and then were handled as follows: fixed in 70% ethanol at 4°C for one night, washed twice with PBS, resuspended with RNase A (Sigma, USA) at 37°C for 30 min, and then added with propidium iodide (PI) (0.05 mg/mL, Keygen Biotech, China) at 4°C for 30 min without light. The processed cells were accessed using a FACSort flow cytometer (BD Biosciences, USA). The data were analyzed using CellQuest software (BD Biosciences, USA).

RNA extraction, real-time quantitative PCR (qPCR) and miRNA analysis

Total cellular RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. Reverse transcription of RNA (500 ng) was carried out using a reverse transcription kit (TaKaRa, Dalian, China) according to the manufacturer's instructions with modifications. qPCR was performed with SYBR Premix Ex Taq II (TaKaRa) on a Mx3000P system (Stratagene, USA), and the detailed procedure can be seen as previously described (Wang et al., 2016). Mature miRNAs were reverse transcribed and then quantitated. U6 snRNA was used as internal control miRNAs. All primers were synthesized by Invitrogen (Shanghai, China), and the sequences are listed in Table 2.

Western blotting

Western blotting analysis was performed as previously described (Wang et al., 2016). The primary antibodies: p21 (1:1000, Cell Signaling Technology, USA), CDK4 (1:500, Affinity, USA), CDK6 (1:500, Affinity, USA), Cyclin D1 (1:500, Affinity, USA), and α-Tubulin (1:500, Boster, China).

Nude mice and tumor implantation

12 BALB/c-nu mice (4 weeks old, 15.4-17.1g) were provided by Hua Fukang Biological Technology Co., Ltd (Beijing). EJ cells (About 5×10^6, 200 μL) infected with Lenti-miR-1180-5p or Lenti-NC were injected subcutaneously into the right back of each mouse (n=6 / group). Tumor length and width were measured using calipers every 4 days for 28 days. Tumor volume was calculated as follows: $V=length\times width^2\times0.5$.

Immunohistochemistry

Immunohistochemical staining was performed as previously described (Reis et al., 2012), using p21 (Cell Signaling Technology, USA), CDK4 (Cell Signaling Technology, USA), CDK6 (Affinity, USA), Cyclin D1 (Cell Signaling Technology, USA) and Cyclin A2 (Affinity, USA) antibody.

Statistical analysis

All numeral data were presented as means ± standard deviation (means ± SD). Statistical analyze was carried out using SPSS 13.0 software (Chicago, IL, USA). Differences were accessed using Student's t-test. $P<0.05$ was considered to be statistically significant.

Table 2. Primers used in this study.

	Sequences (5'-3')
miR-1180-5p (RT)	GTCGTATCCAGTGCAGGGTCCGAG-GTATTCGCACTGGATACGACTATTCC
U6 (RT)	CGAATTTGCGTGTCATCCT
miR-1180 (forward)	GGACCCAACCGGCCG
miR-1180 (reverse)	CAGTGCAGGGTCCGAGGTAT
U6 (forward)	CTCGCTTCGGCACATA
U6 (reverse)	CGAATTTGCGTGTCATCCT
p21 (forward)	GCCCAGTGGACAGCGAGCAG
p21 (reverse)	GCCGGCGTTTGGAGTGGTAGA
GAPDH (forward)	TCCCATCACCATCTTCCA
GAPDH (reverse)	CATCACGCCACAGTTTCC
CDK4 (forward)	ATGGCTACCTCTCGATATGAGC
CDK4 (reverse)	CATTGGGGACTCTCACACTCT
CDK6 (forward)	TCTTCATTCACACCGAGTAGTGC
CDK6 (reverse)	TGAGGTTAGAGCCATCTGGAAA
Cyclin D1 (forward)	GCTGCGAAGTGGAAACCATC
Cyclin D1 (reverse)	CCTCCTTCTGCACACATTTGAA
Cyclin A2 (forward)	AGGAGAGTCGTGACTGTACCT
Cyclin A2 (reverse)	GAAGGTCCATGAGACAAGGC

RT, reverse transcription; GAPDH, glyceraldehide-3-phosphate dehydrogenase.

Results

miR-1180-5p attenuates BCa cell proliferation, inhibits colony formation and induces cells cycle arrest

We previously have reported that miR-1180-5p was lower expressed in BCa tissues than adjacent non-tumor tissues (Wang et al., 2014a). To further identify the expressing levels of miR-1180-5p in cell lines, we performed real-time PCR and found that miR-1180-5p was lowly-expressed in T24 and EJ cells compared with SV-HUC-1 cell (Fig. 1A). Based on these results, miR-1180-5p may act as a tumor suppressor in BCa.

To explore the effect of miR-1180-5p on BCa cell growth, we infected BCa cells with Lenti-miR-1180-5p or Lenti-NC. Fluorescence was observed using fluorescence microscopy 72 h after infection with indicate lentivirus (Fig. 1B). MTS assay demonstrated that the proliferation ability of both T24 and EJ infected Lenti-miR-1180-5p was notably weakened compared with Lenti-NC infected group (Fig. 1C). Moreover, colony formation assay showed that the ability to form colonies was significantly suppressed as the size of colonies was smaller and the number was fewer in Lenti-miR-1180-5p infected group (Fig. 1D,E). To analyze the mechanism leading to alterations of BCa cell proliferation induced by Lenti-miR-1180-5p, we carried out flow cytometry (FCM) to detect DNA content at each phase of the cell cycle. FCM revealed that compared with Lenti-NC infected group, Lenti-miR-1180-5p infected BCa cells represented a notable increase in the G0/G1 percentage and a significant decrease in the S percentage (Fig. 1F,G). These data demonstrated that miR-1180-5p induced G0/G1 arrest by interfering cell cycle progression, and thus inhibited the proliferation of BCa cells.

miR-1180-5p induces p21 expression and inhibits cells cycle regulators such as CDK4, CDK6, Cyclin D1 and Cyclin A2

We speculated that, as we have reported previously, the underlying molecular mechanism that miR-1180-5p suppresses the proliferation of BCa cells, was related with the overexpression of *p21* activated by miR-1180-5p. Compared with Lenti-NC group, *p21* expression was up-regulated in Lenti-miR-1180-5p infected BCa cells as shown in Fig. 2A,B.

As the G0/1-S transition of cell cycle progress was interfered in Lenti-miR-1180-5p group, we conjectured that cell cycle-related proteins (such as CDK4, CDK6, Cyclin D1 and Cyclin A2) might be suppressed. qPCR analysis showed the expression of CDK4, CDK6, Cyclin D1 and Cyclin A2 mRNA was notably down-regulated

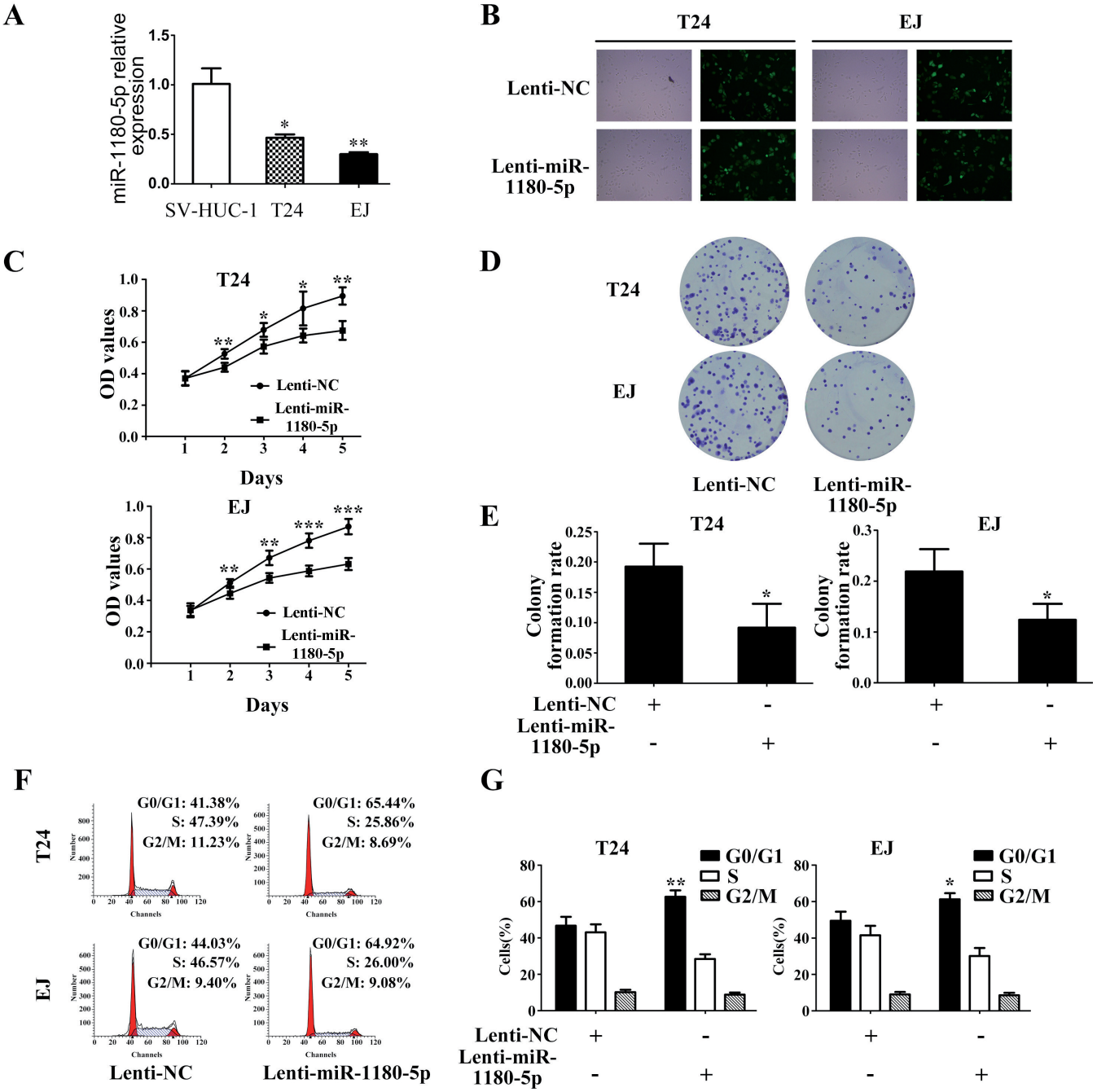

Fig. 1. miR-1180-5p mediated by lentivirus vector inhibits BCa cell proliferation, suppresses colony formation and induces cell cycle arrest. **A.** The expression of miR-1180-5p among three cell lines was detected. *P<0.05, **P<0.01 compared with SV-HUC-1 cell. **B.** Representative micrographs of BCa cells at 72 h after infection with Lenti-miR-1180-5p or Lenti-NC. **C.** Cell growth curves of infected cells were measured by MTS assay. Take OD values as the detection index. *P<0.05, **P<0.01 and ***P<0.001 compared with the Lenti-NC group at the same time point. **D.** Representative micrographs of cell colonies formed in each group. **E.** Relative quantification of cell colonies formed in each group. *P<0.05 compared with the Lenti-NC group. **F.** Representative micrographs of the percentage of cells in distinct cell cycle phases detected by FCM. **G.** Quantification of cell cycle distribution in each group. *P<0.05 and **P<0.01 compared with the Lenti-NC group.

(Fig. 2C). The results were further confirmed by Western blotting (Fig. 2D).

miR-1180-5p suppresses BCa cells tumorigenicity in subcutaneously transplantation

We performed tumorigenicity assay to evaluate the inhibitory effect of miR-1180-5p on BCa cells proliferation *in vivo*. Apparently, subcutaneous tumorigenesis capacity of Lenti-miR-1180-5p infected BCa cells was suppressed as the tumors were markedly smaller in volume and lighter in weight compared with those in Lenti-NC group (Fig. 3A-C). As shown in Fig. 3D, the expression of CDK4, CDK6, Cyclin D1 and Cyclin A2 mRNA was down-regulated, whereas the expression of p21 was up-regulated in subcutaneous transplantation tumor. It was further confirmed by IHC staining (Fig. 3E). These results together demonstrated

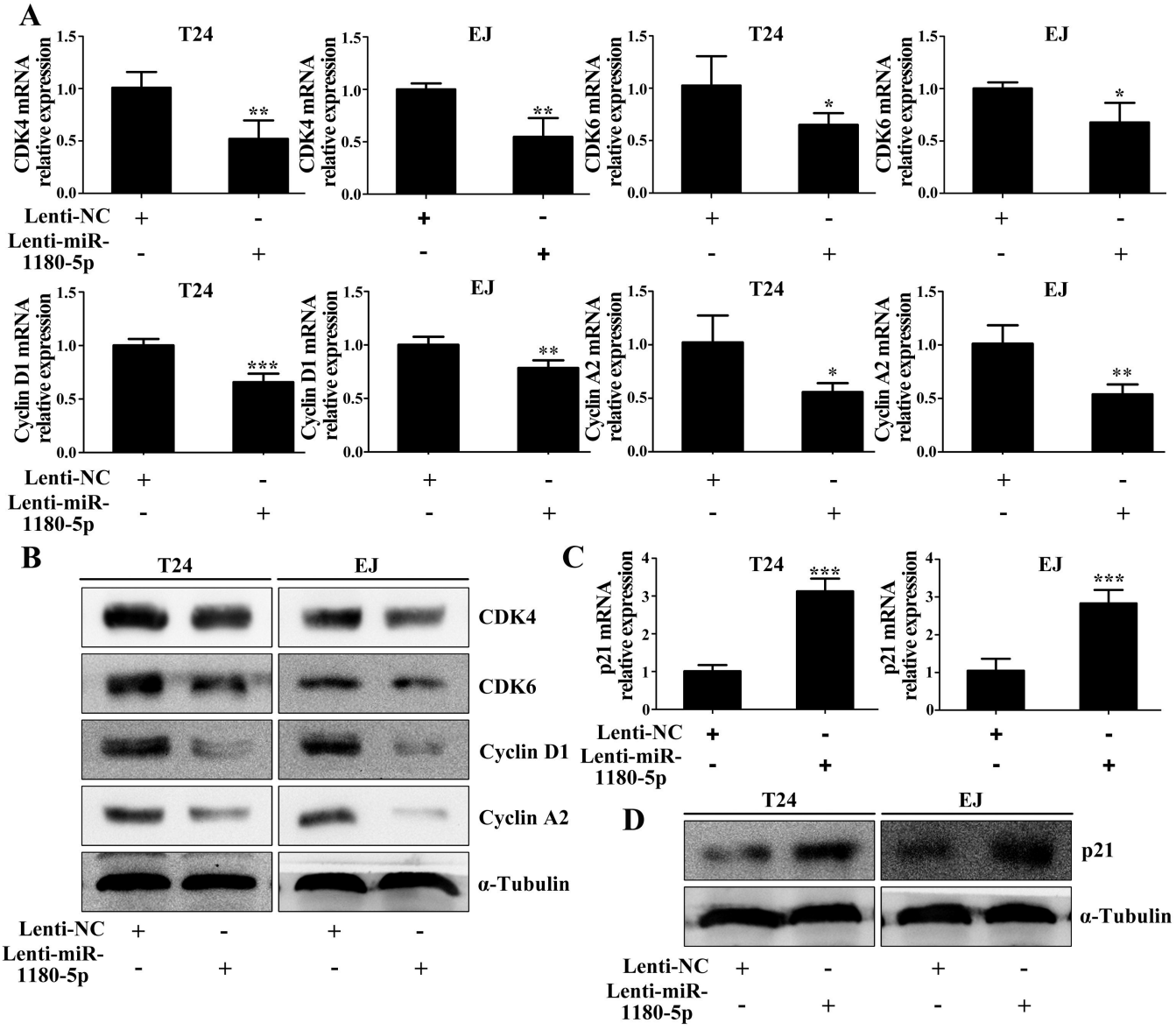

Fig. 2. miR-1180-5p mediated by lentivirus vector induces the down-regulation of CDK4, CDK6, Cyclin D1 and Cyclin A2 and activates the expression of p21. **A.** QPCR analyzed the relative expression of CDK4, CDK6, Cyclin D1 and Cyclin A2 mRNA. Take GAPDH as a loading control. *P<0.05, **P<0.01 and ***P<0.001 compared with the Lenti-NC group. **B.** Expression of CDK4, CDK6, Cyclin D1 and Cyclin A2 proteins was detected by Western blotting. Take α-Tubulin as a loading control. **C.** QPCR analyzed the relative expression of p21 mRNA. Take GAPDH as a loading control. ***P<0.001 compared with the Lenti-NC group. **D.** Expression of p21 protein was detected by Western blotting. Take α-Tubulin as a loading control.

that miR-1180-5p can significantly suppress the tumorigenicity of BCa cells.

miR-1180-5p inhibits the expression of CDK4, CDK6, Cyclin D1 and Cyclin A2 mainly by up-regulating p21 expression

To investigate the effect of *p21* on the expression of cell cycle regulator proteins, we transfected both cell lines with miR-1180-5p mimics or co-transfected miR-1180-5p mimics and siP21. As seen from Fig. 4A,B, p21 mRNA and protein expression were obviously interfered in co-transfected miR-1180-5p mimics and siP21 at 72 h. Then we analyzed the expression of CDK4, CDK6, Cyclin D1 and Cyclin A2. Western blotting showed that compared with miR-1180-5p group, the expression of CDK4, CDK6, Cyclin D1 and Cyclin A2 was up-regulated (Fig. 4C). The observations were further confirmed by qPCR (Fig. 4D). In conclusion, our data suggested that the down-regulation of CDK4, CDK6 and Cyclin D1 and Cyclin A2 is induced by overexpression of *p21*.

miR-1180-5p interferes with BCa cell proliferation mainly by activating p21 expression

At 72 h post co-transfection of miR-1180-5p and siP21, FCM revealed that cell G0/G1 arrest was interfered and the G0/1-S transition of cell cycle progress was restored to a certain extent (Fig. 5A,B).

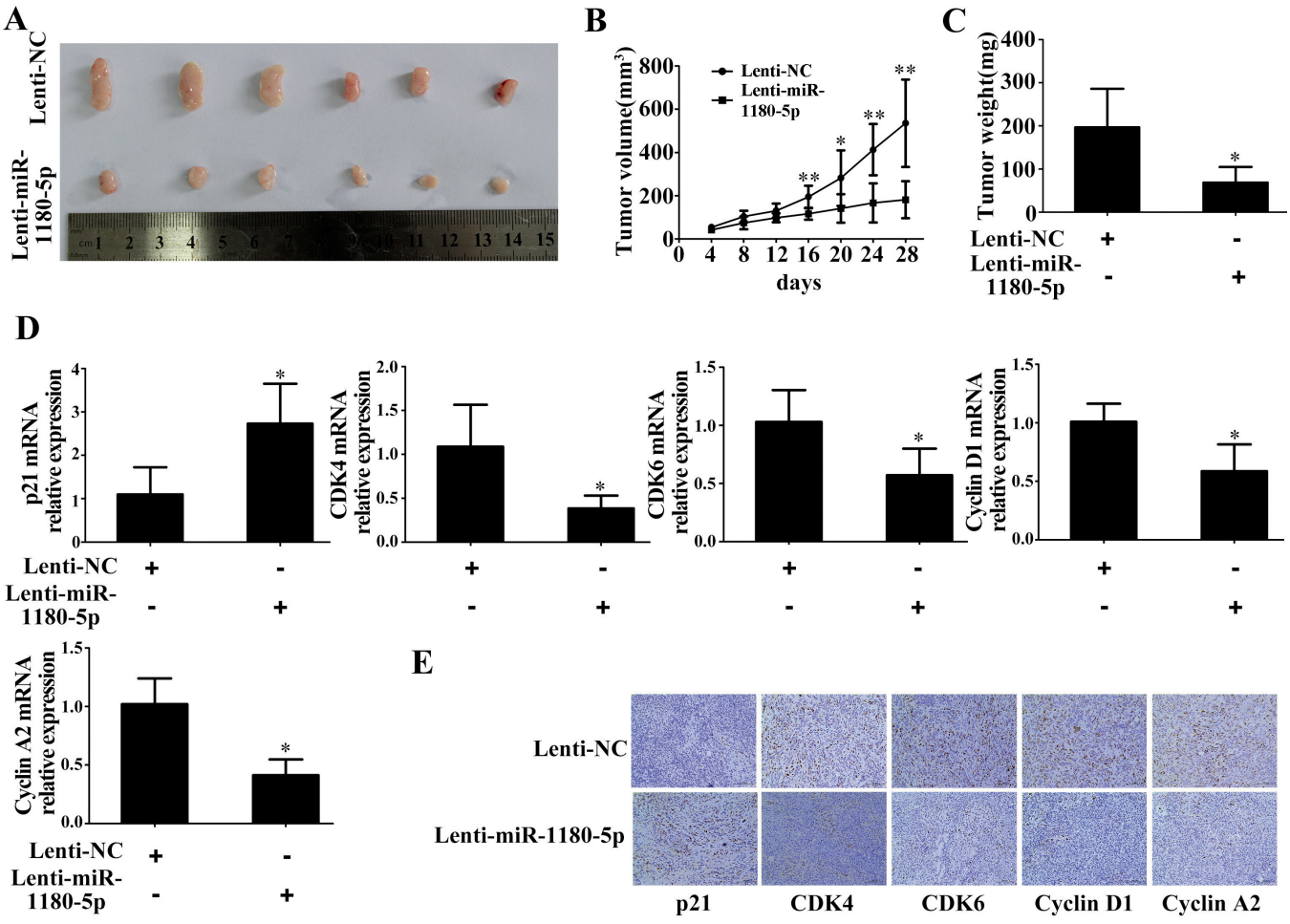

Fig. 3. miR-1180-5p mediated by lentivirus vector inhibits the tumorigenicity of BCa cells *in vivo*. **A.** Photograph of tumors formed 28 days later after injected EJ infected with candidate lentivirus subcutaneously. **B.** Mean tumor growth curves at each group. *P<0.05, **P<0.01 compared with the Lenti-NC group. **C.** Comparison of tumor weights in two groups. *P<0.05 compared with the Lenti-NC group. **D.** Expression of p21, CDK4, CDK6, Cyclin D1 and Cyclin A2 mRNA in subcutaneous transplantation tumor. **E.** Representative micrographs of IHC staining for CDK4, CDK6, Cyclin D1, Cyclin A2 and p21 proteins in slices of tumors formed in each group. E, × 200

MTS assay also showed that, compared with miR-1180-5p group, the proliferation ability of BCa cells was remarkably promoted post *p21* expression was silenced (Fig. 5C). Furthermore, the colony formation capacity was up-regulated (Fig. 5D,E). Taken together, our results indicated that overexpression of *p21* activated by miR-1180-5p was in charge of BCa cell proliferation suppression.

Discussion

In the present study, we demonstrated that miR-1180-5p was lowly-expressed in BCa cells compared with normal human urothelial cell. It indicated that the expression of miR-1180-5p was related with the growth of BCa. Then we investigated the mechanism concerning the inhibitory effect of miR-1180-5p on BCa cell

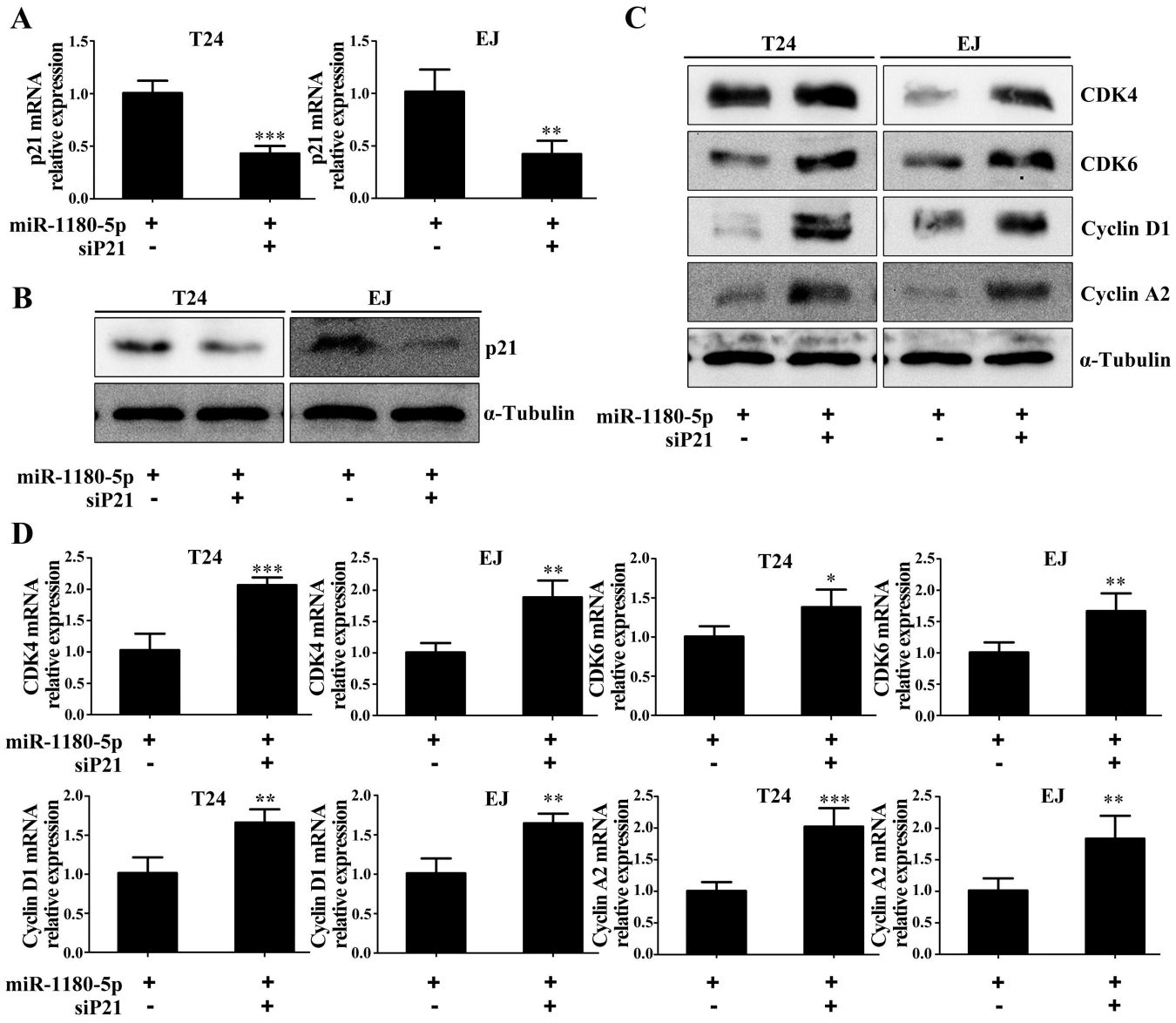

Fig. 4. miR-1180-5p fails to suppress the expression of CDK4, CDK6 and Cyclin D1 after p21 expression is silenced. T24 and EJ cells were transfected with the indicated small non-coding RNAs for 72 h. **A.** Relative expression of p21 mRNA was analyzed by qPCR. GAPDH serves as a loading control. **P<0.01 and ***P<0.001 compared with the miR-1180-5p group. **B and C.** Expression of p21, CDK4, CDK6, Cyclin D1 and Cyclin A2 proteins was detected by Western blotting. Take α-Tubulin as a loading control. **D.** QPCR analyzed the relative expression of CDK4, CDK6, Cyclin D1 and Cyclin A2 mRNA. Take GAPDH as a loading control. *P<0.05, **P<0.01 and ***P<0.001 compared with the miR-1180-5p group.

proliferation and explored whether miR-1180-5p can suppress BCa cell growth *in vivo*. We confirm that the suppressive effect of miR-1180-5p is directly caused by the overexpression of *p21* and several cell cycle related proteins are involved. Moreover, to our best knowledge, this is the first study focused on the suppressive effect of p21 expression activated by miR-1180-5p in BCa *in vivo*.

As MTS assay and Colony formation assay showed, miR-1180-5p can suppress BCa cell proliferation significantly. Uncontrolled proliferation capacity is one distinctive characteristic of cancer cells, which is mainly induced by cell cycle disorder (Sperka et al., 2012; Liao et al., 2015). Cell cycle disorder is a common phenomenon in cancer (Dickson, 2014). This prompted us to inspect that miR-1180-5p may suppress cancer proliferation by inducing cell cycle arrest. Then we performed FCM assay which showed that miR-1180-5p could significantly inhibit G0/1 to S transition.

As we know, the cell cycle is partly regulated by CDKs (cyclin-dependent kinases) and cyclins (Konecny, 2016). CDK4 and CDK6 are members of the serine/threonine protein kinase family and have a significant effect on tumor cell proliferation by controlling G1 phase progression and the G1-S transition of cell cycle (Graf and Koehler, 2010; Sherr et al., 2015). Much research has demonstrated that dysregulated activation and expression of CDKs is related with cancers, and targeting CDKs in cancer cells has become a promising therapeutic strategy (Liao et al., 2015). On the other hand, CDKs have no enzymatic activity without activating subunits such as Cyclin

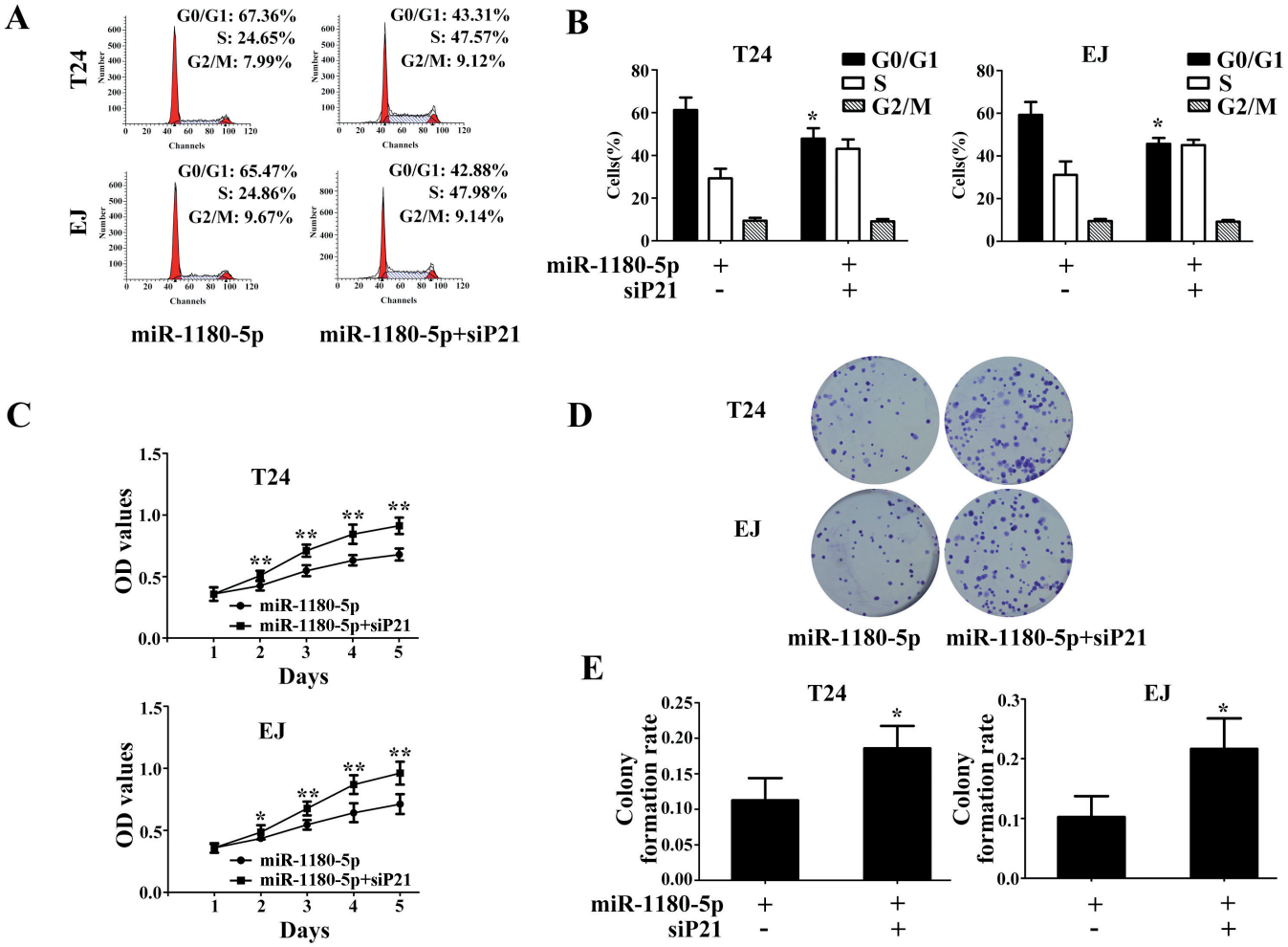

Fig. 5. miR-1180-5p fails to inhibit BCa cell proliferation without overexpression of p21. **A.** FCM detected the percentage of cells in distinct cell cycle phases. **B.** Quantification of cell cycle distribution in each group. *P<0.05 compared with the miR-1180-5p group. **C.** Indicated cell growth was accessed by MTS assay. *P<0.05, **P<0.01 compared with the miR-1180-5p group. **D.** Representative micrographs of cell colonies formed in each group. **E.** Relative quantification of cell colonies formed in each group. *P<0.05 compared with the miR-1180-5p group.

D1and Cyclin A2 (Krystof and Uldrijan, 2010). CDK4/6 or CDK2 assemble with Cyclin D1 or Cyclin A2 respectively to form enzymatically active complexes like Cyclin D1-CDK4/6 and Cyclin A2-CDK2. Cyclin A2 is an important regulator in S phase of cell cycle and so is usually related with cell proliferation (Obaya et al., 2002). Besides, Cyclin A2 is often found to be overexpressed in human cancers (Bendris et al., 2012). Both *in vitro* and *in vivo,* we found that miR-1180-5p transfection leads to suppression of CDK4, CDK6, Cyclin D1 and Cyclin A2. As a broad-acting CDK inhibitor, p21 can inhibit the level of CDK4 and CDK6 (Stivala et al., 2012). With a cyclin binding motif, p21 can also competitively bind with Cyclin and then reduce the formation of Cyclin-CDK complexes (Chen et al., 1996; Parry et al., 1999). Our results demonstrate that the overexpression of p21 is mainly responsible for suppression of CDK4, CDK6, Cyclin D1 and Cyclin A2, as completely silencing p21 further up-regulated CDK4, CDK6, Cyclin D1 and Cyclin A2. It is confirmed that miR-1180-5p can notably activate the expression of *p21*, suppress cell cycle regulator proteins, induce cell cycle arrest and thereby suppress BCa cell proliferation.

RNA activation (RNAa) is a phenomenon in which specific gene expression can be activated by small double-stranded RNAs (dsRNAs) through targeting complementary sequences on the gene promoter (Li et al., 2006). The activating effect emerges 24-48 h later, peaks 72 h later, and lasts for about 14 days post transfection. Unfortunately, the exact mechanism still remains uncertain (Huang et al., 2010). More and more research has revealed that not only can miRNAs interfere with gene expression by targeting 3'UTR regions, but can also activate gene expression by targeting promoter elements (Place et al., 2008; Huang et al., 2012; Turner et al., 2014). Most miRNAs either have a tumor-suppressive or -promoting effect (Xiao et al., 2015). We have reported that miR-1180-5p was down-regulated in BCa tissues of ten patients compared with adjacent non-tumor bladder mucosal tissues (Wang et al., 2014b). Besides, miR-1180-5p is also shown to be significantly associated with survival in papillary renal cell carcinoma patients (Ge et al., 2015). We hereby speculate that miR-1180 has a tumor-suppressive effect in some cancers.

Taken together, we have demonstrated that miR-1180-5p can suppress BCa cell growth both *in vitro* and *in vivo*. The suppressive effect of miR-1180-5p on BCa cell proliferation mainly depends on the overexpression of p21, and several cell cycle related proteins are also involved. It is well-known that *p21* is frequently lowly-expressed in BCa cells and low *p21* expression predicated poor prognosis and even tumor recurrence in BCa (Behnsawy et al., 2011; Tang et al., 2015). Therefore, miR-1180-5p may be a promising strategy for BCa treatment. However, further studies are needed to explore the exact mechanism of miR-1180-5p's activating effect on *p21*.

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Conflicts of interest. All authors declare no conflicts of interest regarding the publication of the paper.

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