

TUG1 promotes prostate cancer progression by acting as a ceRNA of miR-26a

Bin Yang^{1*}, Xiaodi Tang^{2*}, Zhixin Wang³, Daju Sun⁴, Xin Wei³, Youpeng Ding³

1, Department of Breast surgery, China-Japan Union Hospital of Jilin University, Changchun 130033, P.R.China; 2, Department of Radiation Oncology, China-Japan Union Hospital of Jilin University, Changchun 130033, P.R.China; 3, Department of Urology, China-Japan Union Hospital of Jilin University, Changchun 130033, P.R.China; 4, Department of pathology, China-Japan Union Hospital of JiLin University, Changchun 130033, P.R.China.

Correspondence to: Zhixin Wang, Department of Urology, China-Japan Union Hospital of Jilin University, No. 126, Xiantai Street, Changchun 130033, P. R. China

Email: zhangtang1617@163.com

* Equal contributors

PURPOSE:

Previous studies have demonstrated that taurine-upregulated gene 1 (TUG1) was aberrantly expressed and involved in multiple types of cancer, however, the expression profile and potential role of TUG1 in prostate cancer (PCa) remains unclear. The aim of the present study was to evaluate the expression and function of TUG1 in PCa.

METHODS:

In the present study, we analyzed TUG1 expression levels of PCa patients in tumor and adjacent normal tissue by real-time quantitative PCR. Knockdown of TUG1 by RNA interference was performed to explore its roles in cell proliferation, migration, and invasion.

RESULTS:

Here we report, for the first time, that TUG1 promotes tumor cell migration, invasion and proliferation in PCa by working in key aspects of biological behaviors. TUG1 could negatively regulate the expression of miR-26a in PCa cells. The bioinformatics prediction revealed putative miR-26a binding sites within TUG1 transcripts.

CONCLUSION:

In conclusion, our study suggests that lncRNA TUG1 acts as a functional oncogene in PCa development.

Keywords

TUG1; LncRNA; miR-26a; PCa; metastasis; EMT

Introduction

Prostate cancer is the second most frequently diagnosed cancer in men which leads to second or third cancer-related death worldwide [1]. Despite improved therapy concept and method, the recurrence of prostate cancer within five years remains about 25% [2]. Metastasis and invasion are the main causes of these lethal consequences of prostate cancer [3]. Thus, deciphering the mechanism of invasive and metastatic behavior are of great importance for early diagnosis and therapy of prostate cancer.

Long non-coding RNAs (lncRNAs) are important new members of the family of ncRNAs with limited or no protein-coding capacity [4-5]. Cumulative evidence is emerging that lncRNAs impact the biological functions of many different cancer types, including PCa [6-8]. Recent studies have shown that the biological behavior of the tumor could be regulated by specific lncRNA factors [9]. Taurine-upregulated gene 1 (TUG1) is a 7.1-kb lncRNA, which was initially identified as a transcript that upregulated in response to taurine treatment of developing mouse retinal cells [10]. Recently, TUG1 has been found to be elevated in a number of cancers, including head and neck, gallbladder, lung and pancreatic cancer [11-13]. However, limited data are available for the molecular mechanisms of TUG1 in PCa, largely due to a lack of specific

investigation. In order to evaluate whether PCa cells would be sensitive to TUG1 blockade, we showed that TUG1 is over expressed in PCa, that TUG1 inhibition increased cell apoptosis, that TUG1knockdown in PCa cells resulted in a significant decrease in cell growth, migration and invasion.

Materials and methods

Patients and specimens

This study included 86 consecutive men diagnosed with PCa affirmed by clinical resection and pathology during 2009–2014. Cancer tissues and adjacent normal tissues surgically removed from PCa patients were immediately frozen in liquid nitrogen and stored at -80°C . The present research was sanctioned by the Institutional Research Ethics Committee of China-Japan Union Hospital of Jilin University, and informed consent was signed by all the 86 patients.

Cell Lines

RWPE1 and PCa cell lines DU145, PC-3, LNCaP, and 22Rv1 were purchased from American Type Culture Collection (ATCC, Rockville, MD). PCa cell were cultured in RPMI-1640 or minimum essential Eagle's medium, supplemented with 10% fetal bovine serum (FBS) and antibiotics. The human nontumorigenic prostate epithelial cell line RWPE-1 was cultured in keratinocyte serum-free medium supplemented with 5 ng/ml human recombinant epidermal growth factor and 30 mg/ml

bovine pituitary extract (Invitrogen, Carlsbad, CA). Cultures were maintained in a 5% CO₂ humidified atmosphere at 37 °C.

RNA preparation and qRT-PCR.

Total RNA from tissues or whole-cell lysates were isolated using TRIzol (Life Technologies, Carlsbad, CA, USA). For quantitative real-time PCR (qRT-PCR), cDNA was synthesized with the PrimeScript RT Master Mix (Takara, Dalian, China) from 500 ng of RNA. The real-time PCR analyses were performed using SYBR Premix Ex Taq II (Takara).

Small interfering RNA

Two small interfering RNAs (siRNAs) against TUG1 (si-TUG1) at different sites and one negative control (si-NC) with no definite target were employed and synthesized by GenePharma (Shanghai, China). Cells were seeded on six-well plates at a density of 3×10^5 /well overnight, and then transfected with siRNA or the negative control at a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen, USA). Forty eight hours after transfection, the cells were harvested to detect the overexpression or knockout efficiency via quantitative real-time PCR (qRT-PCR). The sequences of the three designed IncRNA TUG1 siRNAs were as follows: si-TUG1 1#, CAGUCCUGGUGAUUUAGACAGUCUU; si-TUG1 2#, CCCAGAAGUUGUAAGUUCACCUUGA.

CCK-8 assay

The proliferation of PCa cells was tested by CCK-8 kit (Doindo, Japan). Approximately transfected 3.5×10^3 cells in 100 ml were incubated in triplicate in 96-well plates. Following 48 h incubation at 37 °C in a humidified atmosphere containing 5% CO₂, the CCK-8 reagent (10 ml) was added to each well and incubated for another 2 h. The optical density at 450 nm was measured using FLx800 Fluorescence Microplate Reader (Biotek).

Flow cytometric analysis

Cells were harvested directly or 48 h after siRNA transient transfection and washed with ice-cold phosphate-buffered saline (PBS). The PI/RNase staining kits (Multisciences, Hangzhou, China) and annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kits (KeyGEN Biotech, Nanjing, China) were used to detect cell cycle and apoptosis in a FACScan instrument (Becton Dickinson, Mountain View, CA, USA), respectively.

Transwell migration/invasion assay

Transwell chamber was used to measure cell migration and invasion abilities. In brief, culture inserts with 8-mm pore size (Transwell; Corning, NY) were placed into 24-well plates. Before the measurement of invasion ability, the plates were pre-coated with matrigel. 2 h before the addition of matrigel, 500 µL of serum-free medium was independently added to the upper and lower chambers, followed by incubation at 37°C for

hydration. Cells were digested by trypsin, and resuspended in serum-free medium. The cell density was adjusted to 1×10^5 /mL. Then, 200 μ L of cell suspension was added into the upper chamber, and 500 μ L of DMEM containing 10% FBS into the lower chamber. After incubation at 37°C with 5% CO₂ for 24 h, the Transwell chamber was removed, cells were washed with 1×PBS, fixed in paraformaldehyde for 20 min, and then stained with 0.1% crystal violet for 20 min. The cotton swab was used to clean the non-migrated cells in the upper chamber, cells migrating through the membrane were counted in 5 randomly selected fields under a microscope (Nikon) at a magnification of $\times 100$.

Western Blot Analysis

Proteins were quantified by Bradford method. Then, total protein extracts was fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (GE Healthcare, Piscataway, NJ, USA). The membranes were blocked with 5% milk at room temperature for 2h. The primary antibodies including anti E-cadherin, anti-N-cadherin, anti-Vimentin (Santa Cruz Bio-technology, Santa Cruz, CA, USA), and anti-GAPDH antibody (Cell Signaling Technology) were added and incubated with blots at 4°C for 12 h. Target protein bands were visualized using the enhanced chemiluminescence method.

Luciferase reporter assay.

Cells were seeded in 96-well plates at a density of 5×10^3 cells per well 24 h before transfection. The cells were co-transfected with a mixture of miR-26a or miR-control and wild-type or mutant TUG1 fragment, using Lipofectamine 2000 (Invitrogen). After 48 h, the luciferase activity was measured with a dual luciferase reporter assay system (Promega, Madison, WI). The relative luciferase activity was normalized against to the Renilla luciferase activity.

Statistical analysis

Continuous variables were expressed as means \pm standard deviation; differences were assessed for significance using Student's t-test or the Mann–Whitney test. Categorical variables were evaluated using chi-square or Fisher exact tests, as appropriate. A *P* value < 0.05 was considered statistically significance. All tests were performed on SPSS Statistics 20.0 software.

Results

Expression of TUG1 and miR-26a in PCa tissue samples

We used qRT-PCR assay to study the TUG1 expression in primary PCa tissues. Expression of TUG1 in PCa tissues is significantly higher than that of in normal paired tissues (Figure 1A). Then, we used the qRT-PCR assay to detect the expressions of miR-26a in the PCa tissues. When compared with normal tissues, the miR-26a expression reduced significantly in the PCa tissues ($P < 0.05$; Figure 1B), and miR-26a

expression was negatively associated with TUG1 expression in PCa tissues (Figure 1C).

TUG1 promoted PCa cells proliferation *in vitro*

TUG1 was highly expressed in diverse PCa cell lines including LNCaP, DU145, PC3, and 22Rv1, as compared to that in an immortalized nontumorigenic human prostate epithelial cell line RWPE1 cells, a finding confirmed by qRT-PCR assays ($P<0.05$; Figure 1D). DU145 and PC3 cell lines have the highest levels of TUG1. To evaluate the effectiveness of TUG1 in PCa, silencing of TUG1 by siRNA was performed in DU145 and PC3 cells. The qPCR assays revealed that TUG1 expression was significantly reduced in DU145 and PC3 cell lines (Figure 2A and B, $P<0.05$). There was a significant decrease in proliferation both in DU145 and PC3 cells after knockdown of TUG1 determined by CCK8 (Figure 2C-D, $P<0.01$).

Knockdown of TUG1 induced PCa cells apoptosis

To further determine whether the effect of TUG1 on PCa cells proliferation reflected cell apoptosis, we performed flow cytometry. The results showed that DU145 and PC3 cells transfected with TUG1 siRNA had higher apoptotic rate in comparison with control cells (Figure 3A-B). Experiments using siRNA confirmed the effects of knockdown TUG1 on cell arrest. Flow cytometric analysis revealed that the knockdown TUG1

resulted in cell arrest in G1 phase of cell cycle both in DU145 and PC3 cell lines (Figure 3C-D).

TUG1 promoted PCa cell invasion via regulating EMT

Next, transwell assays showed the number of DU145 and PC3 cells in lower section were significantly reduced in the TUG1 knockdown groups compared with the control groups, which indicated that upregulation of TUG1 promote cell invasion and metastasis (Figure 4 A-B, $P<0.05$). Because EMT is vital for cell invasion, we next examined whether silencing TUG1 expression inhibited mesenchymal features. As expected, TUG1 knockdown decreased the expression of Vimentin and N-cadherin, and increased E-cadherin expression in DU145 and PC3 cells (Figure 4 C-D, $P<0.05$). Therefore, inhibition of TUG1 in PCa cells changed the cell morphology from a mesenchymal to a more epithelial phenotype.

TUG1 inhibited miR-26a expression in PCa cells

To investigate whether miR-26a was involved in the inhibitory effect of TUG1 on PCa cells, we applied the online software starBase v2.0. The prediction showed that TUG1 contains binding sequences complementary to miR-26a seed regions. For further confirmation, we used the luciferase assay to detect the association between TUG1 and miR-26a. The results showed that overexpression of miR-26a reduced the luciferase activity of the pMIR luciferase reporter containing wild-type TUG1 (WT-TUG1) but not mutant reporter (MUT-TUG1) vector (Figure 5A). Next, we

measured the levels of miR-26a expression in various PCa cell lines. As shown in Figure 5B, the expression of miR-26a was obviously decreased in DU145 and PC3 cell lines, indicating the opposite result to TUG1 expression. In addition, we cloned the TUG1 into pcDNA3.1 vector and co-transfected into DU145 and PC3 cells with or without miR-26a overexpression. The results showed that overexpression of TUG1 inhibited the miR-26a expression (Figure 5C). All these data demonstrated that TUG1 associated with the miR-26a and may function as a ceRNA.

Discussion

Some studies have suggested that increased lncRNAs levels are essential for tumorigenesis in a variety of biological cellular processes and multiple cancers. Numerous new lncRNA including HOTAIR, MALAT-1, and GAS5 [16-17] regulates numerous cellular processes important for tumorigenesis and progression of PCa. TUG1 was originally reported to be upregulated in response to taurine treatment of developing mouse retinal cells. Aberrant expression of TUG1 in various tumors is related to increased cell proliferation and invasion and reduced apoptosis [18]. The clinical impact and molecular mechanisms of TUG1 in PCa patients remain uncertain. To shed additional light on this issue, we conducted an analysis investigating the clinical significance of TUG1 in human normal and cancerous tissues from prostate, and PCa cell lines,

as well as the role of TUG1 in the regulation of tumor cell proliferation and invasion *in vitro*.

Consistent with previous reports, we confirmed that TUG1 was overexpressed in PCa tissues. Silencing of TUG1 was found to significantly impair PCa cell proliferation and invasiveness, suggesting that TUG1 is a critical oncogene. Our results demonstrated that inhibition of TUG1 could potentially have a suppressive effect on the migratory and invasive ability of PCa cells by regulating EMT.

To investigate the mechanism underlying the migration process, we measured the protein level of EMT markers following downregulation of TUG1 expression. EMT is a process defined by cells losing their junctions, repressing E-cadherin expression, and exhibiting increased cell mobility. We further identified that expression of EMT related markers were significantly altered following TUG1 knockdown. Thus, our data suggests that TUG1-positive PCa cells possessed invasive properties by regulating EMT.

LncRNAs could act as miRNA sponges to regulate expression of various miRNAs and their target mRNAs [19-20]. Inspired by the 'ceRNAs' regulatory network, we hypothesized that TUG1 may also serve as a ceRNA. Then we searched for the interactions of TUG1 and potential miRNAs. Through bioinformatics analysis and luciferase assays, we discovered miR-26a could form complementary base on the full-length

TUG1 transcript and induce translational repression using a RLuc-TUG1 reporter gene. Our study showed the miRNA-26a expression reduced in PCa tissues, and qRT-PCR analysis showed that miR-26a expression was inversely correlated with TUG1 expression in PCa. Moreover, ectopic overexpression of TUG1 inhibited the miR-26a expression.

In conclusion, we found that inhibition of TUG1 with specific siRNA results in potent anti-tumor activity in PCa *in vitro*, indicating that TUG1 has the potential to be an oncogene for PCa. Our data highlight the important role of TUG1 in the treatment of PCa.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Research involving patients All procedures performed in studies involving humans were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Zhixin Wang conceived and designed the experiments. Bin Yang and Xiaodi Tang performed the experiments. Daju Sun and Xin Wei performed the proliferative ability analysis. Youpeng Ding performed cell migration assay. Bin Yang and Xiaodi Tang performed cell cycle analysis and statistical analysis. Zhixin Wang oversight of all aspects of the study. All authors read and approved the final manuscript.

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Figure 1 A: qRT-PCR showing expression level of TUG1 in PCa tissues and adjacent noncancerous tissues; B: qRT-PCR showing expression level of miR-26a in PCa tissues and adjacent noncancerous tissues; C: miR-26a expression was negatively associated with TUG1 expression in PCa tissues; D: qRT-PCR showing expression level of TUG1 in PCa cell lines; All tests were at least performed three times. Data were expressed as mean \pm SD. *P < 0.05, **P < 0.01

Figure 2 A and B: We employed siRNA to enhance efficiency of TUG1 knockdown in PCa cell lines; C: CCK8 assay showing knockdown of TUG1 inhibited cell proliferation of DU145 cells; D: CCK8 assay showing knockdown of TUG1 inhibited cell proliferation of PC3 cells; All tests were at least performed three times. Data were expressed as

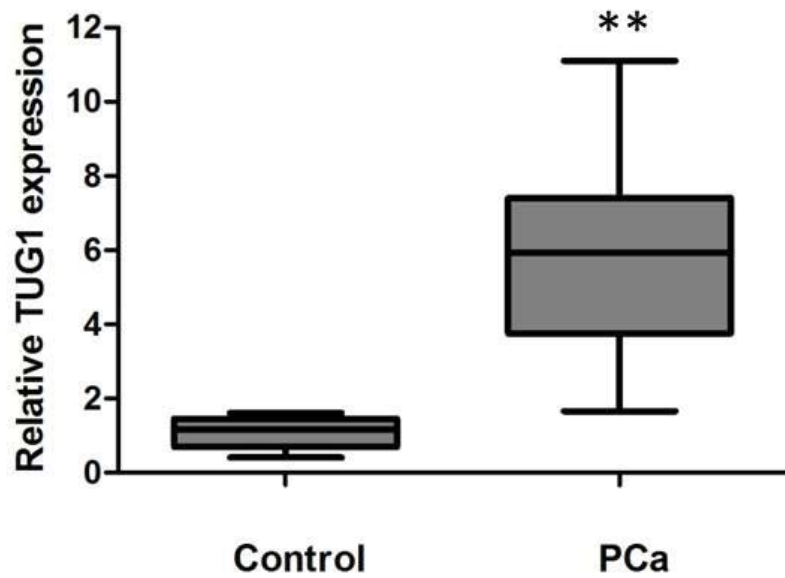
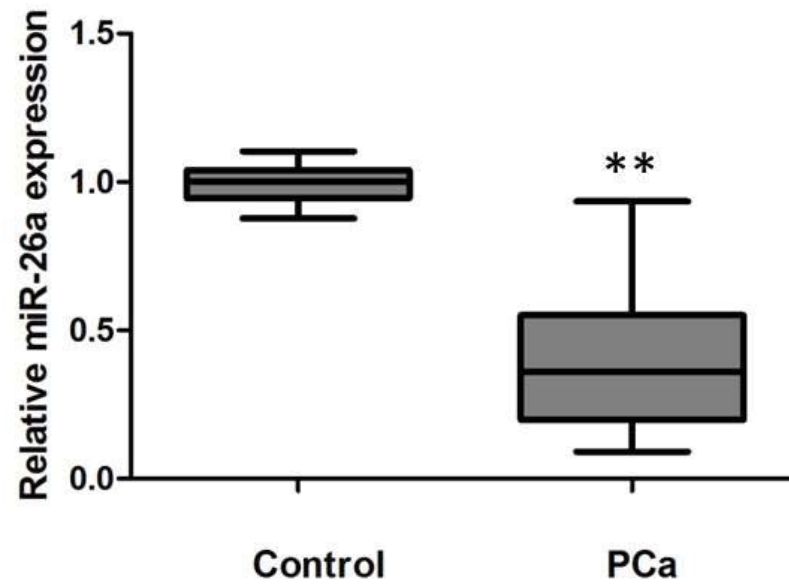
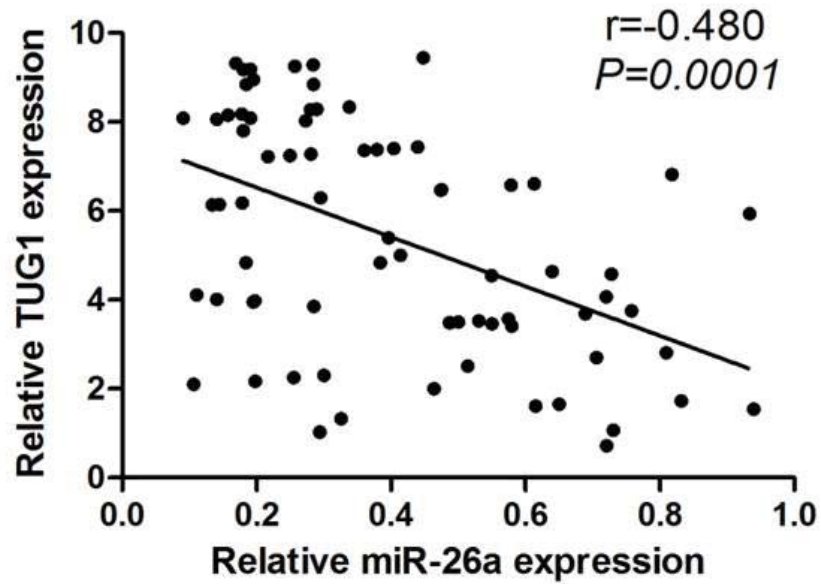
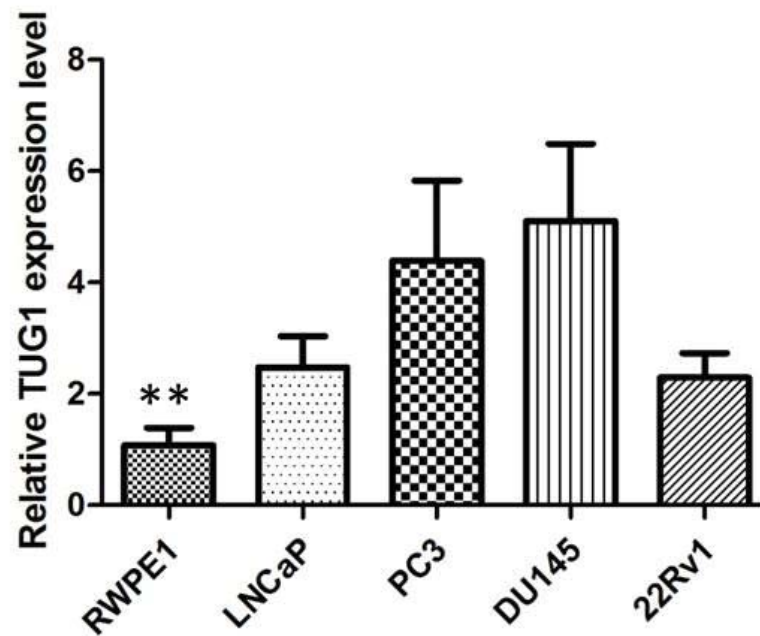
mean \pm SD. *P < 0.05, **P < 0.01

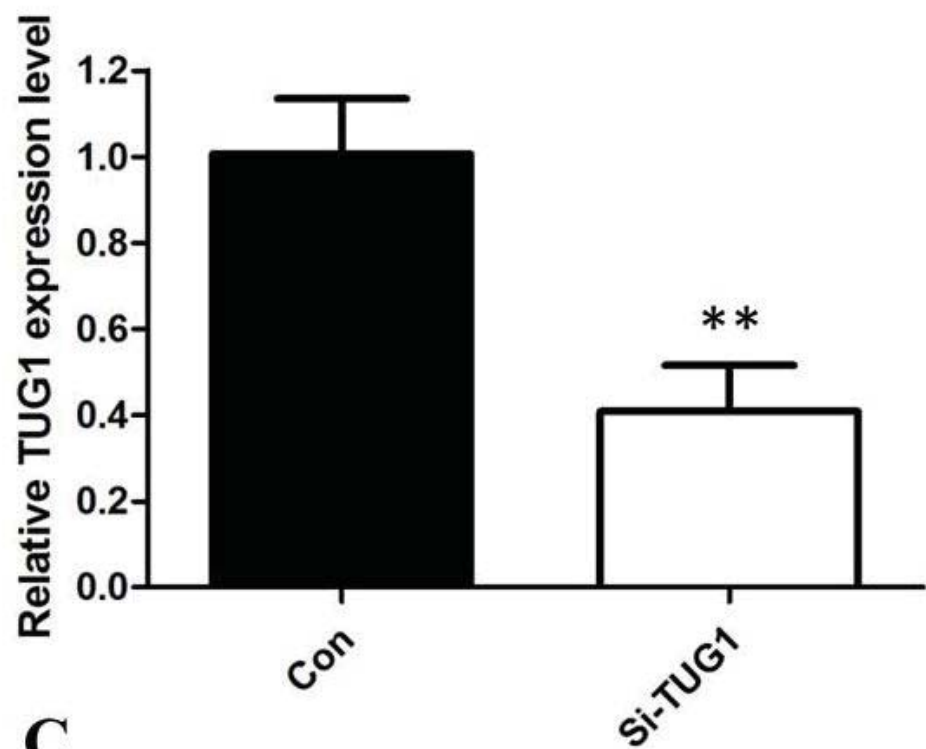
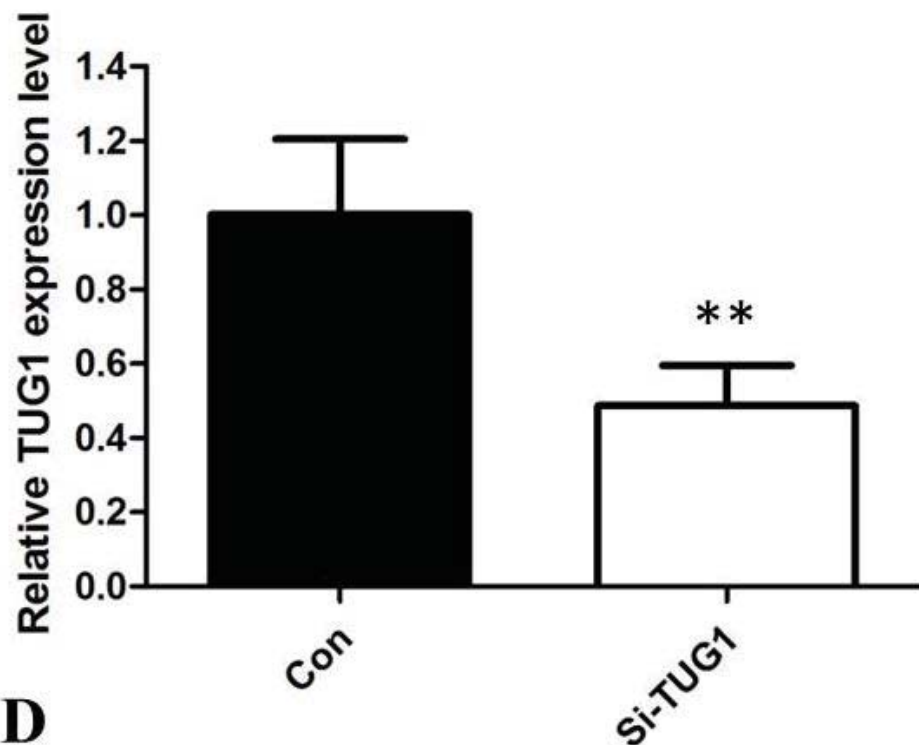
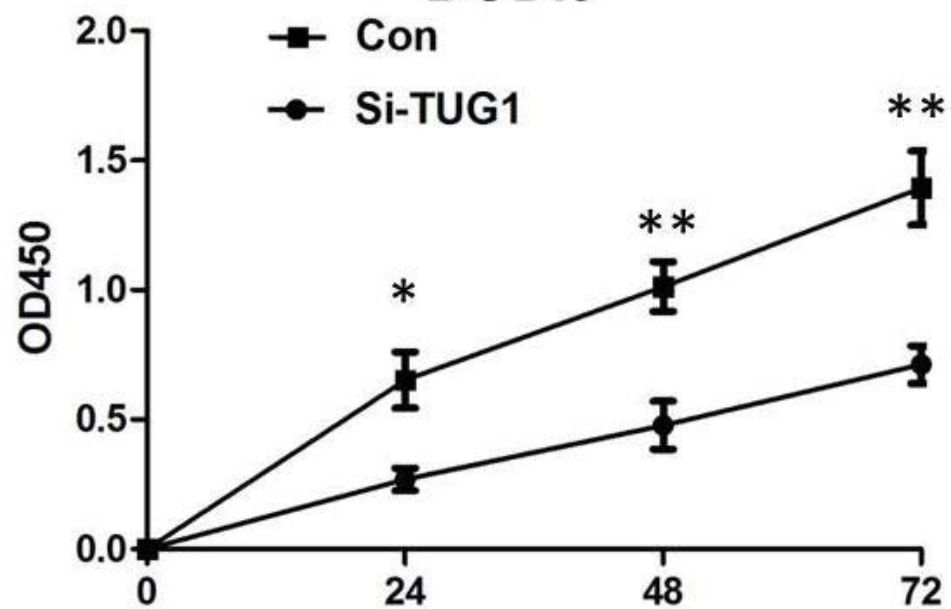
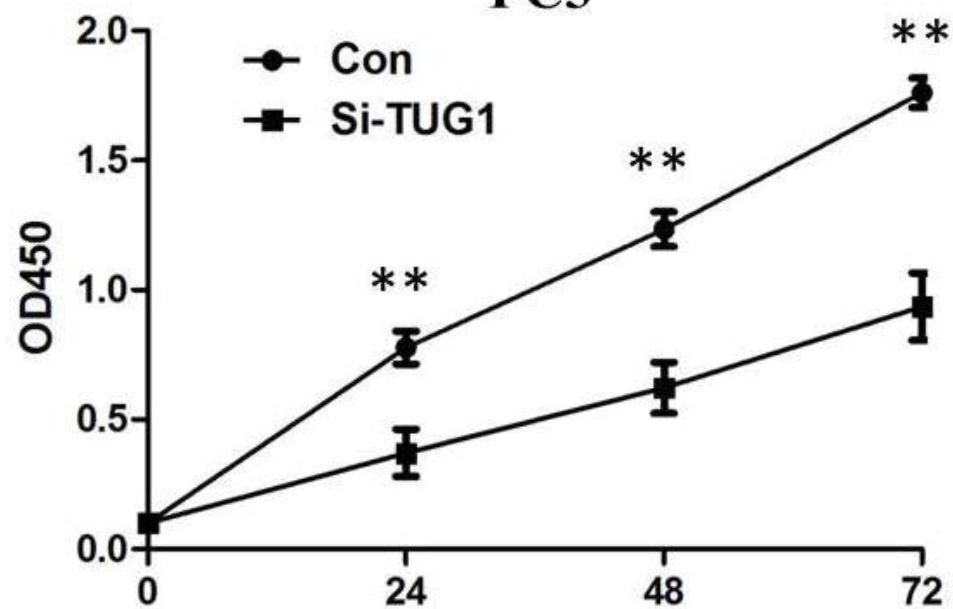
Figure 3 A: flow cytometry showed that DU145 cells transfected with TUG1 siRNA had higher apoptotic rate in comparison with control cells; B: flow cytometry showed that PC3 cells transfected with TUG1 siRNA had higher apoptotic rate in comparison with control cells; C: DU145 cells transfected with si-TUG1 had cell-cycle arrest at the G1-G0 phase; D: PC3 cells transfected with si-TUG1 had cell-cycle arrest at the G1-G0 phase; All tests were at least performed three times. Data were expressed as mean \pm SD. *P < 0.05, **P < 0.01

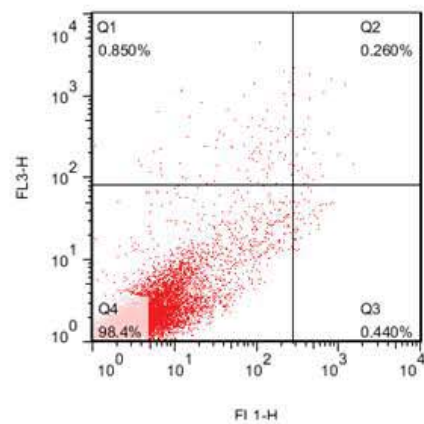
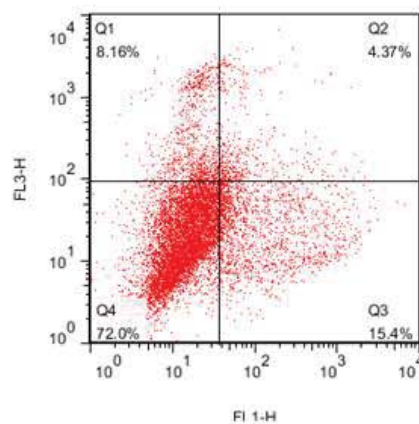
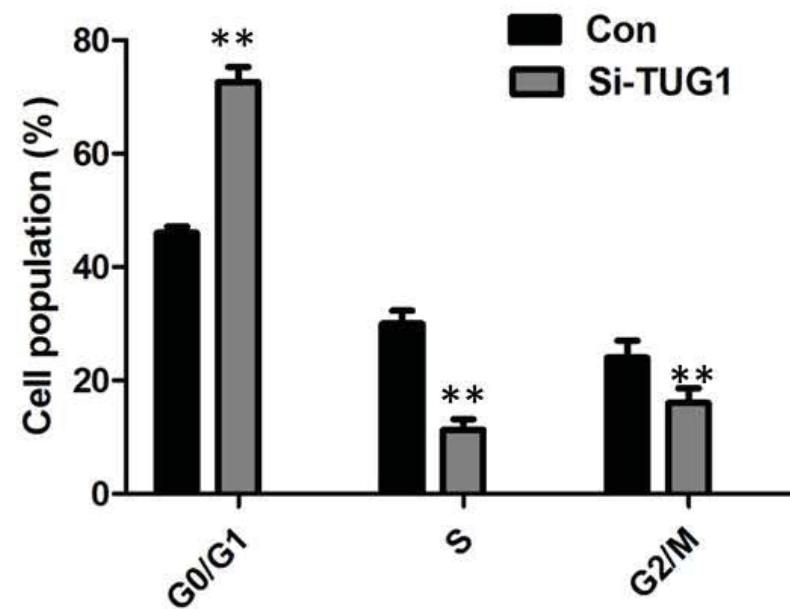
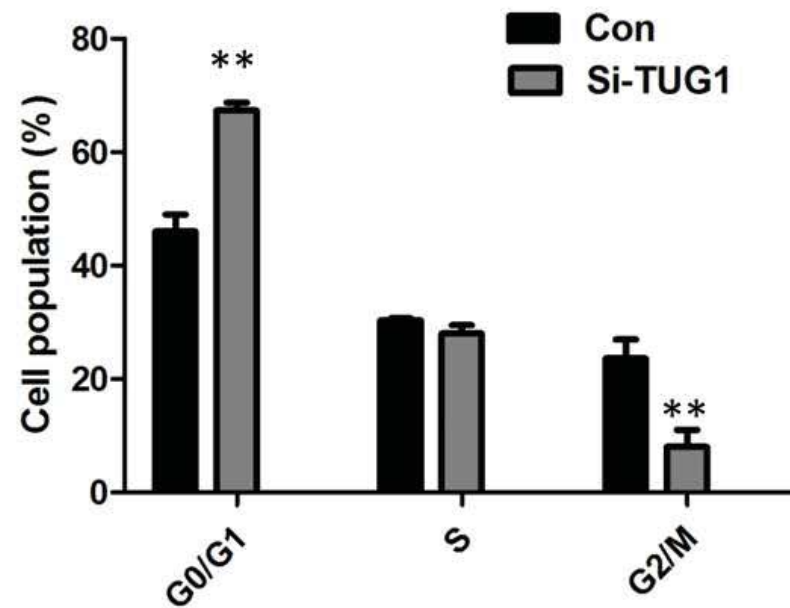
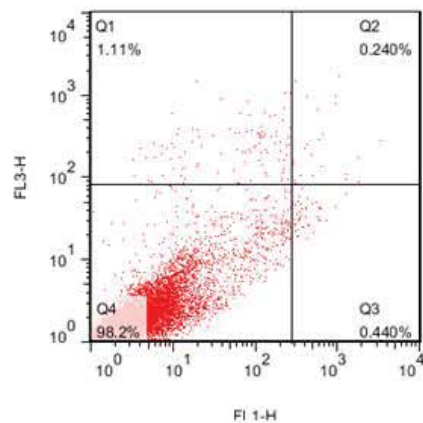
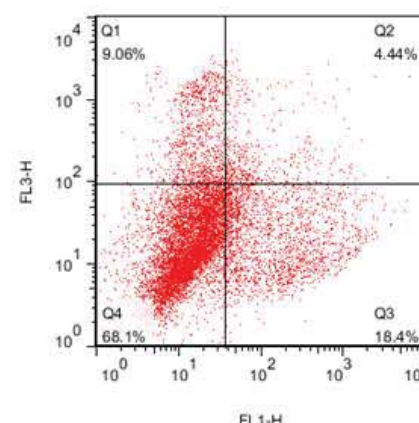
Figure 4 A: Inhibition of Invasion of DU145 cells by TUG1 siRNA; B: Inhibition of Invasion of PC3 cells by TUG1 siRNA; C: Knockdown of TUG1 reverses EMT in DU145 cells; D: Knockdown of TUG1 reverses EMT in PC3 cells; All tests were at least performed three times. Data were expressed as mean \pm SD. *P < 0.05, **P < 0.01

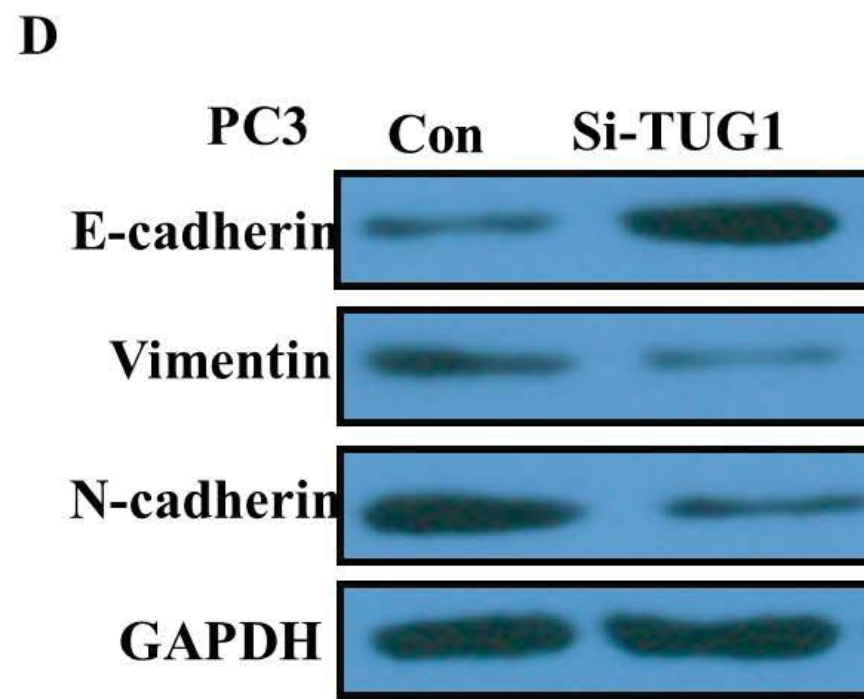
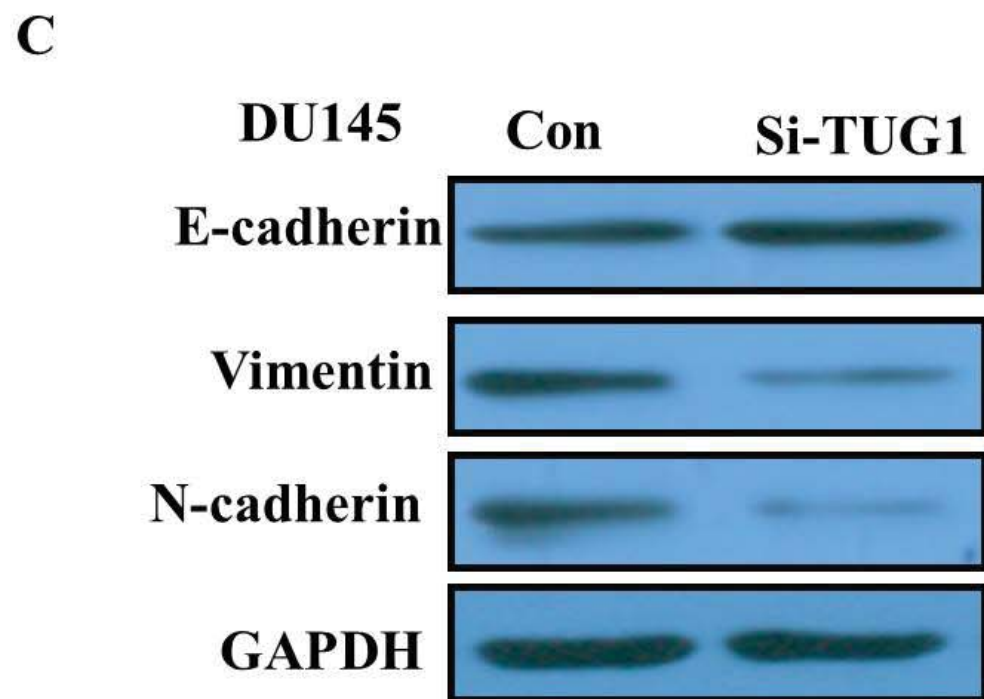
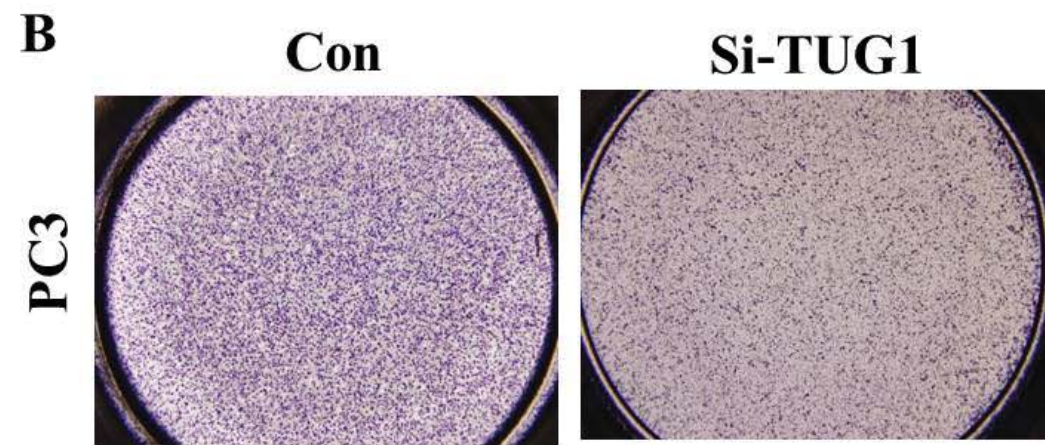
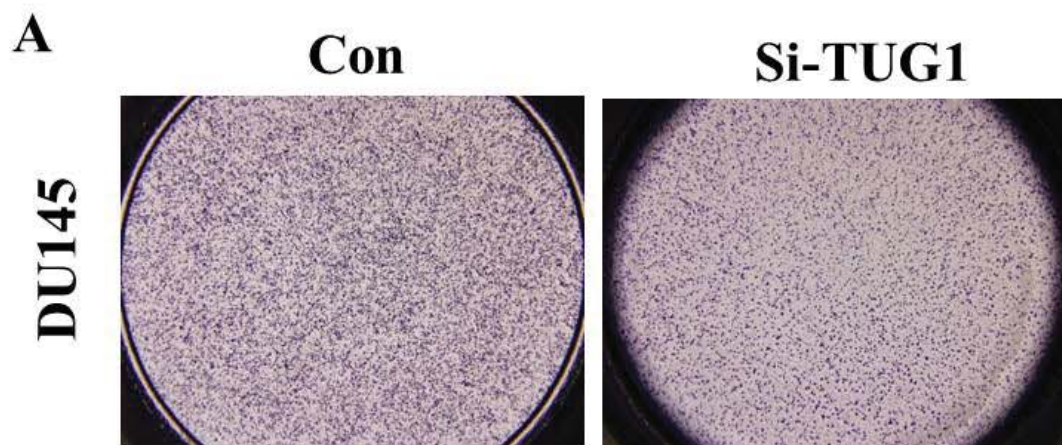
Figure 5 A: The wild-type or mutant miR-26a-binding sites in TUG1 were inserted into pMIR-report luciferase vector. Luciferase activity was detected in PCa cells co-transfected with miR-26a or negative control (miR-control) and reporter plasmids containing WT-TUG1 (wild type) or MUT-TUG1 (mutant type). The normalized luciferase activity in the

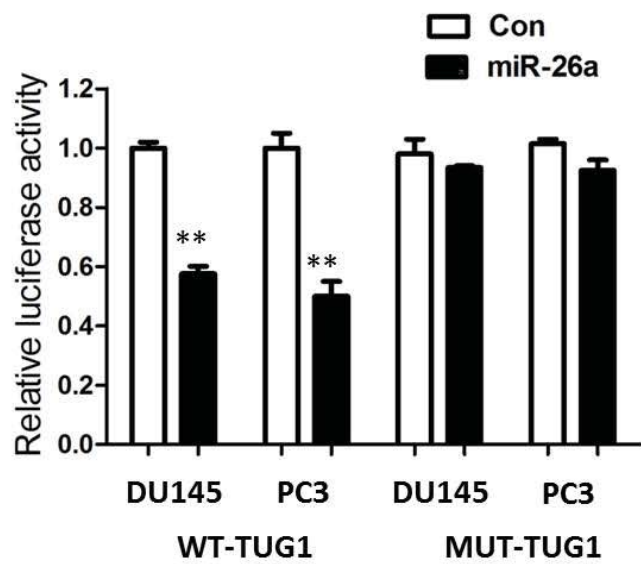
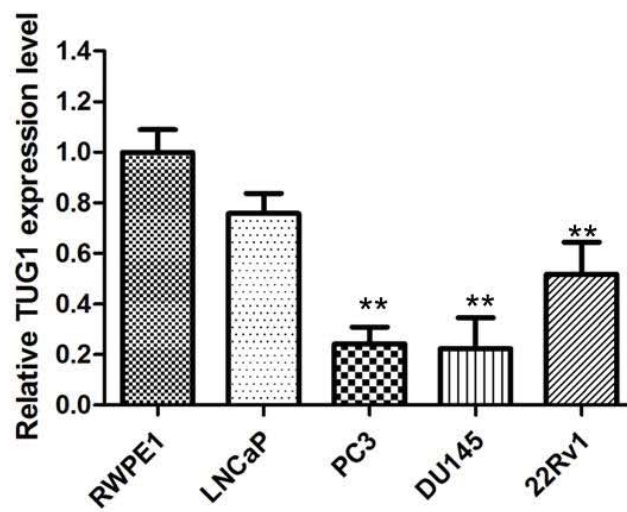
miR-control group was used as the relative luciferase activity. B: Expression levels of miR-26a in different PCa cell lines were determined by qRT-PCR; C: The co-transfection of miR-26A and TUG1 by pcDNA3.1. The expression of miR-26a was detected by qRT-PCR. All tests were at least performed three times. Data were expressed as mean \pm SD. *P < 0.05, **P < 0.01

A**B****C****D**

A**DU145****B****PC3****C****DU145****D****PC3**

A**DU145****Con****si-TUG1****C****DU145****D****PC3****B****PC3****Con****si-TUG1**



A**B****C**