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Long non-coding TUG1 accelerates prostate cancer progression through regulating miR-128-3p/YES1 axis

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Abstract. – OBJECTIVE: Dysregulation of long non-coding RNAs (lncRNAs) is being found to have relevance to human cancers, including prostate cancer (PCa). Taurine-upregulated gene 1 (TUG1) has been demonstrated to have a potential oncogenic role in PCa. Then the aim of this study was to investigate the molecular mechanisms of TUG1 on PCa progression.

PATIENTS AND METHODS: The expression levels of TUG1, YES proto-oncogene 1 (YES1) mRNA and miR-128-3p were assessed using quantitative real-time polymerase chain reaction. Cell proliferation ability, apoptosis, and migration and invasion capacities were detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, flow cytometry and transwell assay, respectively. Western blot analysis was employed to evaluate the indicated proteins levels. The interaction between miR-128-3p and TUG1 or YES1 was determined using dual-luciferase reporter assay. *In vivo* assay was used to observe the effect of TUG1 on tumor growth *in vivo*.

RESULTS: Our data indicated that TUG1 was upregulated in PCa tissues and cells and predicted poor prognosis. TUG1 knockdown weakened PCa cell proliferation, migration, invasion, epithelial-mesenchymal transition (EMT), and accelerated cell apoptosis *in vitro*. Mechanistically, TUG1 directly interacted with miR-128-3p and miR-128-3p mediated the regulatory effects of TUG1 depletion on PCa cell progression. YES1 was a direct target of miR-128-3p and TUG1 modulated YES1 expression by sponging miR-128-3p. Moreover, TUG1 silencing repressed PCa cell progression *in vitro* through YES1. Additionally, TUG1 silencing mitigated tumor growth *in vivo*.

CONCLUSIONS: Our study suggested that TUG1 silencing retarded PCa cell progression *in vitro* and tumor growth *in vivo* through miR-128-3p/YES1 axis, showing that targeting TUG1 might be a novel therapeutic strategy for PCa management.

Key Words:
Prostate cancer, TUG1, MiR-128-3p, YES1, Cell progression.

Abbreviations
lncRNAs = long non-coding RNAs; PCa = prostate cancer; TUG1 = Taurine-upregulated gene 1; YES1 = YES proto-oncogene 1; EMT = epithelial-mesenchymal transition; miRNAs = MicroRNAs; 3'-UTR = 3'-untranslated region; ceRNAs = competing endogenous RNAs; FCS = fetal calf serum; K-SFM = keratinocyte serum-free medium; si-TUG1 = siRNA targeting TUG1; si-NC = non-targeting siRNA sequence; qRT-PCR = Quantitative real-time polymerase chain reaction; DMSO = dimethyl sulfoxide; PI = propidium iodide; HRP = Horseradish peroxidase; shRNA = short-hairpin RNA; SPF = specific-pathogen-free; ANOVA = analysis of variance; BMI-1 = B lymphoma Mo-MLV insertion region 1 homolog; PHF6 = PHD finger protein 6.

Introduction

Prostate cancer (PCa) was the second most common malignancy and the fifth leading cause of tumor-related deaths among men worldwide in 2018[1]. Owing to obesity and westernized lifestyles, the incidence of PCa is increasing rapidly in China, with an estimated 60,300 new cases in 2015[2]. Although conventional treatments such as prostatectomy and radiochemotherapy have contributed to an improved prognosis for this malignancy, the 5-year survival rate of patients with metastatic PCa is still unsatisfactory[3]. Hence, it is very imperative to identify new effective biomarkers for PCa diagnosis, treatment, and prognosis.

Long non-coding RNAs (lncRNAs), a diverse family of RNA molecules ~200 nucleotides, function as major players in a multitude of pathways involved in life[4]. Their dysregulation has been

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found to have relevance to many human tumors, including PCa[5,6]. Taurine-upregulated gene 1 (TUG1), a 7.1-kb tumor-related lncRNA, is found to be aberrantly expressed in cancer tissues and plays a pivotal role in tumorigenesis of multiple human cancers, such as bladder cancer and colorectal cancer[7,8]. Recent researches reported that TUG1 was tightly associated with poorer prognosis of PCa patients and high level of TUG1 accelerated PCa cell proliferation, metastasis, and epithelial-mesenchymal transition (EMT), eliciting a potential oncogenic role of TUG1 in PCa[9,10]. Nevertheless, the underlying mechanisms of TUG1 on PCa progression remain primarily vague.

MicroRNAs (miRNAs) are a type of endogenous transcripts with ~22 nucleotides long and regulate a wide array of biological processes[11]. The predominant effect of miRNAs is to modulate the gene expression by identifying complementary sequences in target mRNAs 3'-untranslated region (3'-UTR)[12]. Emerging evidence has demonstrated that miRNAs participate in tumorigenesis and progression of human tumors, such as PCa[13,14]. Previous reports manifested that miR-128-3p level was downregulated in PCa and highly expressed miR-128-3p weakened the invasion and promoted cell sensitivity to cisplatin in PCa cells[15,16]. Recently, competing endogenous RNAs (ceRNAs) concept suggests that lncRNAs serve as post-transcriptional gene regulators through sponging specific miRNAs, shedding light on the importance of such networks in cancer biology and therapeutics[17]. Yang et al[10] manifested that TUG1 facilitated PCa progression *in vitro* via sponging and sequestering miR-26a. Nevertheless, the effects of the interplay between TUG1 and miR-128-3p remain uncovered.

In this study, our data supported that the expression level of TUG1 was elevated in PCa, and TUG1 silencing mitigated PCa cell malignant behaviors *in vitro* and tumor growth *in vivo*. Consequently, we further explored the underlying mechanisms of TUG1 in PCa cell progression.

Patients and Methods

Clinical Samples and Ethics Statement

Thirty PCa men patients were recruited from the Zhejiang Integrated Traditional and Western Medicine Hospital/Hangzhou Red Cross Hospital between May 2011 and November 2012. All patients signed the informed consent. Malignant PCa tissues and adjacent normal prostatic tissues were collected from these men who underwent radical prostatectomy. The follow-up information was obtained from these patients' medical records, and the latest follow-up was updated on February 2017. Our study was approved by the Ethical Committee of Zhejiang Integrated Traditional and Western Medicine Hospital/Hangzhou Red Cross Hospital.

Cell Culture and Transfection

Two PCa cells (PC-3 and DU145) and human prostate epithelial RWPE-1 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). PCa cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen, Carlsbad, CA, USA), plus 10% fetal calf serum (FCS, Australian Biosearch, Wangarra, Western Australia), 1% penicillin/streptomycin (Invitrogen). RWPE-1 cells were routinely maintained in keratinocyte serum-free medium (K-SFM, Gibco, Rockville, MD, USA) containing 50 μg/mL bovine pituitary extract (Gibco). All cells were grown at 37°C under 5% CO_2 and humidified conditions.

For TUG1 silencing, PCa cells were introduced with 100 nM of siRNA targeting TUG1 (si-TUG1) or non-targeting siRNA sequence (si-NC) as the negative control. For upregulation of TUG1 and YES proto-oncogene 1 (YES1), PCa cells were transfected with 50 ng of corresponding overexpression plasmid (pcDNA-TUG1 and pcDNA-YES1), and non-targeting plasmid (pcDNA-NC) was used as a negative control. MiR-128-3p expression alteration was conducted using 100 nM of miR-128-3p mimic, inhibitor of miR-128-3p (anti-miR-128-3p) or respective oligonucleotide control (miR-NC mimic or anti-miR-NC). Commercial DharmaFECT-4 transfection reagent was purchased from Perbio Science (Helsingborg, Sweden) and used for each transfection in accordance with the producer's guidance. All oligonucleotides and plasmids were designed and synthesized by GenePharma Co., Ltd. (Shanghai, China).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA isolation and cDNA preparation were carried out, as described previously [18]. For quantification of TUG1 and YES1 mRNA, pre-amplified cDNA was subjected to qRT-PCR using FastStart Universal SYBR Green Master (Roche, Basel, Switzerland) on the LightCycler 480-II Real-time thermal cycler (Roche), with

GAPDH as the internal control. Expression of miR-128-3p was measured using the TaqMan® MicroRNA assay kit (Applied Biosystems, Foster City, CA, USA) with U6 snRNA as an internal control. Relative expression levels of TUG1, YES1 mRNA, and miR-128-3p were calculated by $2^{-\Delta\Delta Ct}$ cycle threshold method. The sequences of PCR primers (5'-3') were as follows: TUG1: TAGCAGTTCCCCAATCCTTG (sense) and CACAAATTCCCATCATTCCC (antisense), YES1: TCCAGAACTTTTTCACTTCAGTC (sense) and TCTACATTTTCCTCTCTGTTCATC (antisense), GAPDH: TCTCTGCTCCTCCCTGTTC (sense) and ACACCGACCTTCACCATCT (antisense), miR-128-3p: CTGGTAGGTCACAGTGAACCG (sense) and TCAACTGGTGTCGTGGAGTC (antisense), U6: TCGCTTCGGCAGCACATATAC (sense) and CGCTTCACGAATTTGCGTG (antisense).

Determination of Cell Proliferation

PCa cells were placed into 96-well plates and transfected with si-NC, si-TUG1, si-TUG1+anti-miR-NC, si-TUG1+anti-miR-128-3p, si-TUG1+pcDNA-NC, or si-TUG1+pcDNA-YES1. At 0, 24, 48 and 72 h after transfection, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (Sigma-Aldrich, St. Louis, MO, USA) was used at a final concentration of 0.5 mg/mL per well followed by the incubation for 3 h at 37°C. Afterwards, 200 μL of dimethyl sulfoxide (DMSO, Solarbio, Beijing, China) was added into each well. The quantity of formazan was measured using a standard spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 490 nm absorbance and was proportional to the number of viable cells.

Cell Apoptosis Assay

Flow cytometry was applied to evaluate the apoptotic rate of transfected PCa cells using the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) referring to the protocols of manufacturers. After transfection, PCa cells were collected, trypsinized, and double-stained with Annexin V-FITC and PI. The percentage of Annexin V^+ cells was evaluated by an LSRFortessa flow cytometer (BD Biosciences).

Transwell Migration and Invasion Assay

Cell migration ability was detected on 24-Transwell chambers with 8-μm pore size filters (Corning Life Sciences, Corning, NY, USA), and cell invasion capacity was determined on Matrigel-coated Transwell membranes (Corning Life Sciences) as described previously [19]. In both assays, PCa cells were seeded into the upper compartment of 24-Transwell chambers and starved in serum-free media before transfection for 12 h. Then, the media was replaced with medium containing 1% FCS, and the grown medium containing 10% FCS was added into the lower compartment. After additional 24 h, the penetrated cells through the pores of filters were stained with 0.25% crystal violet. Images were photographed using a Leica DMI 600B microscope (Leica Microsystems, Wetzlar, Germany), and the average number of migrated or invaded cells was calculated in 10 random fields.

Western Blot

Total protein was extracted, separated, and transferred as described previously [7]. The following antibodies were used: anti-Vimentin (1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-N-cadherin (1:1000, Cell Signaling Technology), anti-E-cadherin (1:400, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-YES1 (1:300, Santa Cruz Biotechnology), and anti-GAPDH (1:1000, Cell Signaling Technology). Horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG (1:2000-5000, Santa Cruz Biotechnology) was used as secondary antibodies. Protein bands were visualized using a chemiluminescence detection kit (Ab Frontier, San Diego, CA, USA).

Bioinformatics

Bioinformatics analyses for the interacted miRNAs of TUG1 and the targets of miR-128-3p were performed using starBase v.3.0 software at http://starbase.sysu.edu.cn/.

Dual-Luciferase Reporter Assay

TUG1 and YES1 3'-UTR luciferase reporter (TUG1-WT and YES1 3'-UTR-WT) harboring the miR-128-3p-binding sequence, and site-directed mutants of the target sequence (TUG1-MUT and YES1 3'-UTR-MUT) were obtained from GenePharma. Binding aptitude of miR-128-3p to TUG1 was evaluated using TUG1-WT and TUG1-MUT and transfected into PCa cells, respectively, together with miR-128-3p mimic or miR-NC mimic. YES1 3'-UTR-WT and YES1 3'-UTR-MUT were used to assess the target interaction between YES1 and miR-128-3p in PCa

cells, and miR-128-3p mimic or miR-NC mimic. At 48 h post-transfection, the luciferase activities were measured using the Dual-luciferase® reporter assay kit (Promega, Madison, WI, USA).

Lentiviral Vector Transduction

Lentivirus-delivered short-hairpin RNA (shRNA) targeting TUG1 (sh-TUG1) and non-targeting shRNA (sh-NC) were purchased from GeneCopoeia Inc. (Guangzhou, China). DU145 cells were transduced by sh-TUG1 or sh-NC with different multiplicities of infection in medium containing 8 μg/mL polybrene (Sirion Biotech, Planegg-Martinsried, Germany). 24 h later, the cells with positive transduction were selected by puromycin (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 10 μg/mL.

In Vivo Assay

Male BALB/c mice (6-8 weeks, n=20) were obtained from Hubei Research Center of Laboratory Animal (Wuhan, China) and housed under a specific-pathogen-free (SPF) environment. Approximately 5.0×10^6 sh-NC or sh-TUG1-transduced DU145 cells were subcutaneously inoculated into the nude mice (n=10). After implantation for 7 days, tumor volume was measured every 4 days. 27 days later, all mice were sacrificed to remove xenograft tumors for weight and detection of TUG1, YES1, and miR-128-3p levels. Animal experimental processes were implemented following the Council of Agriculture Guidebook for the Care and Use of Laboratory Animals, and the animal study was approved by the Institutional Ethics Committee of Zhejiang Integrated Traditional and Western Medicine Hospital/Hangzhou Red Cross Hospital.

Statistical Analysis

Experimental results were presented as mean ± SD from 3 biological replicates × 2 technical replicates. Comparisons between groups were conducted using a two-tail Student's *t*-test and one-way analysis of variance (ANOVA). Kaplan-Meier survival assay and log-rank test were used to analyze the relationship between overall survival and TUG1 level. Correlation between miR-128-3p and TUG1 or YES1 was calculated using Spearman's test. *p*-values < 0.05 in all cases were considered statistically significant.

Results

TUG1 Expression was Increased in PCa Tissues and Cells, and Predicted Poor Prognosis

Firstly, the expression of TUG1 was determined in PCa tissues and cells. As shown by qRT-PCR, TUG1 expression was remarkably elevated in PCa tissues compared with normal prostate tissues (Figure 1A). In parallel, the expression of TUG1 was higher in PCa cells than that of control (Figure 1B). Then, we observed the correlation between TUG1 expression and prognosis of PCa patients. Kaplan-Meier survival analysis revealed that PCa patients had shorter overall survival time in high TUG1 expression group than those in low TUG1 expression group (Figure 1C).

TUG1 Silencing Mitigated the Proliferation, Migration, Invasion, EMT, and Enhanced the Apoptosis in PCa Cells

Then, the detailed role of TUG1 on PCa cell progression *in vitro* was determined by loss-of-

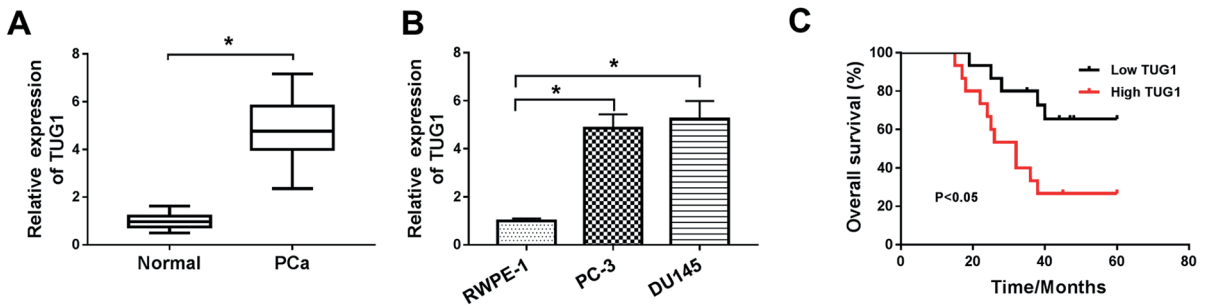

Figure 1. TUG1 was upregulated in PCa tissues and cells and predicted poor prognosis. (A) The expression of TUG1 was assessed by qRT-PCR in 30 pairs of PCa tissues and corresponding normal prostate tissues. (B) TUG1 level was detected by qRT-PCR in PCa cells (PC-3 and DU145) and human prostate epithelial cells (RWPE-1). (C) According to the median of TUG1 expression, the 30 PCa patients were divided into two groups: high TUG1 expression group (n=15) and low TUG1 expression group (n=15). Correlation between TUG1 expression and prognosis of PCa patients was analyzed using Kaplan-Meier survival assay and log-rank test.

function experiments. In contrast to a scrambled negative sequence, transient transfection of si-TUG1 strikingly inhibited the expression of TUG1 in both PC-3 and DU145 cells (Figure 2A). Subsequent MTT assays revealed that compared with negative group, cell proliferation ability was prominently repressed by TUG1 silencing in the two cells (Figure 2B and 2C). Flow cytometry analysis showed that TUG1 knockdown triggered a clear enhancement of cell apoptosis (Figure 2D and 2E). Moreover, cell migration and invasion capacities were markedly mitigated when TUG1 depletion using transwell assays (Figure 2F and 2G). Additionally, TUG1 silencing resulted in retarded EMT in the two cells, as evidenced by a distinct increase of E-cadherin expression, as well as a markedly decrease of Vimentin and N-cadherin levels (Figure 2H and 2I).

TUG1 Directly Interacted with miR-128-3p

To further investigate the molecular mechanism by which TUG1 regulated PCa cell progression *in vitro*, we used a computational method to help to predict the interacted miRNAs of TUG1. Using starBase v.3.0 software, these predicted data showed a putative binding site for miR-128-3p in TUG1 (Figure 3A). To confirm this, dual-luciferase reporter assays were performed using TUG1 wild-type luciferase reporter (TUG1-WT) harboring the miR-128-3p-binding site or site-directed mutant of the target sequence (TUG1-MUT). TUG1-WT and miR-128-3p overexpression in the two PCa cells caused lower luciferase activity compared to miR-NC mimic (Figure 3B and 3C). However, the luciferase activity of TUG1-MUT influenced by miR-128-3p overexpression (Figure 3B and 3C). Additionally, our results revealed that miR-128-3p level was downregulated and inversely correlated with TUG1 expression in PCa tissues (Figure 3D and 3E). Likewise, in contrast to normal control, the expression of miR-128-3p was highly reduced in PCa cells (Figure 3F). Then, we determined whether the miR-128-3p-binding site was functional in PCa cells. As expected, in comparison to their counterparts, miR-128-3p expression was significantly elevated by TUG1 silencing, while it was manifestly reduced when transfection of pcDNA-TUG1 in the two cells (Figure 3G). These data together strongly suggested that TUG1 directly interacted with miR-128-3p and repressed miR-128-3p expression.

MiR-128-3p Mediated the Regulatory Effects of TUG1 Silencing on PCa Cell Proliferation, Migration, Invasion, EMT, and Apoptosis

Given our data that TUG1 directly interacted with miR-128-3p, we further explored whether miR-128-3p served as a potential molecular mediator of TUG1 knockdown in PCa cell progression *in vitro*. As demonstrated by qRT-PCR, the cotransfection of anti-miR-128-3p, but not anti-miR-NC control, remarkably abolished si-TUG1-mediated increasing miR-128-3p expression in both PC-3 and DU145 cells (Figure 4A). Subsequent experiments data showed that in comparison to the negative control, si-TUG1-mediated pro-proliferation (Figure 4B and 4C) and anti-apoptosis (Figure 4D) effects were significantly reversed by restored expression of miR-128-3p. Moreover, the repressive effects of TUG1 silencing on cell migration (Figure 4E), invasion (Figure 4F) and EMT (Figure 4G and 4H) were highly abated by miR-128-3p expression restoration in the two cells. All these results strongly pointed out a notion that TUG1 knockdown exerted its regulatory effects on PCa cell proliferation, migration, invasion, EMT, and apoptosis by miR-128-3p.

TUG1 Regulated YES1 Expression by Acting as a Molecular Sponge of miR-128-3p

MiRNAs function as important regulators in biological processes by suppressing target mRNAs expression. Hence, we carried out a detailed analysis for the targets of miR-128-3p using starBase v.3.0 software. Of interest, these predicted data revealed that the 3'-UTR of YES1 harbored a putative complementary sequence for miR-128-3p (Figure 5A). When we performed the dual-luciferase reporter assay, cotransfection of wild-type luciferase reporter and miR-128-3p mimic into the two PCa cells produced lower luciferase activity than in cells cotransfected with miR-NC mimic (Figure 5B and 5C). However, a little change was observed in the luciferase activity of site-directed mutant of the seed region in the presence of miR-128-3p mimic (Figure 5B and 5C). Our data also demonstrated that compared with normal control, the mRNA and protein levels of YES1 were significantly increased in PCa tissues (Figure 5D and 5E). Moreover, YES1 mRNA level was inversely correlated with miR-128-3p expression in PCa tissues (Figure 5F). In line with PCa tissues, the mRNA and protein levels of YES1 were higher in PCa cells than those in normal control (Figure

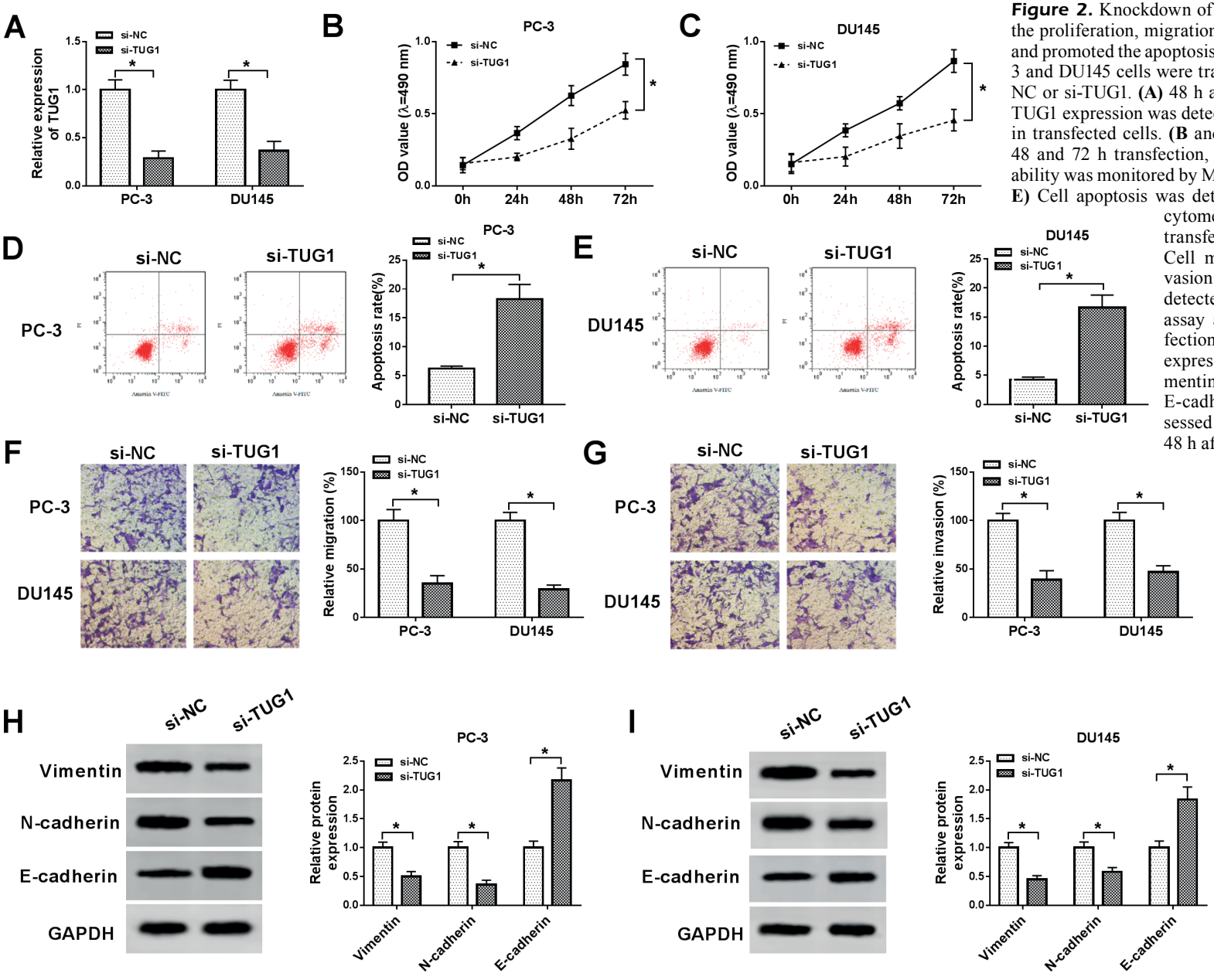

Figure 2. Knockdown of TUG1 hampered the proliferation, migration, invasion, EMT, and promoted the apoptosis in PCa cells. PC-3 and DU145 cells were transfected with si-NC or si-TUG1. **(A)** 48 h after transfection, TUG1 expression was detected by qRT-PCR in transfected cells. **(B** and **C)** After 0, 24, 48 and 72 h transfection, cell proliferation ability was monitored by MTT assay. **(D** and **E)** Cell apoptosis was determined by flow cytometry 48 h after transfection. **(F** and **G)** Cell migration and invasion capacities were detected by transwell assay after 36 h transfection. **(H** and **I)** The expression levels of Vimentin, N-cadherin and E-cadherin were assessed by Western blot 48 h after transfection.

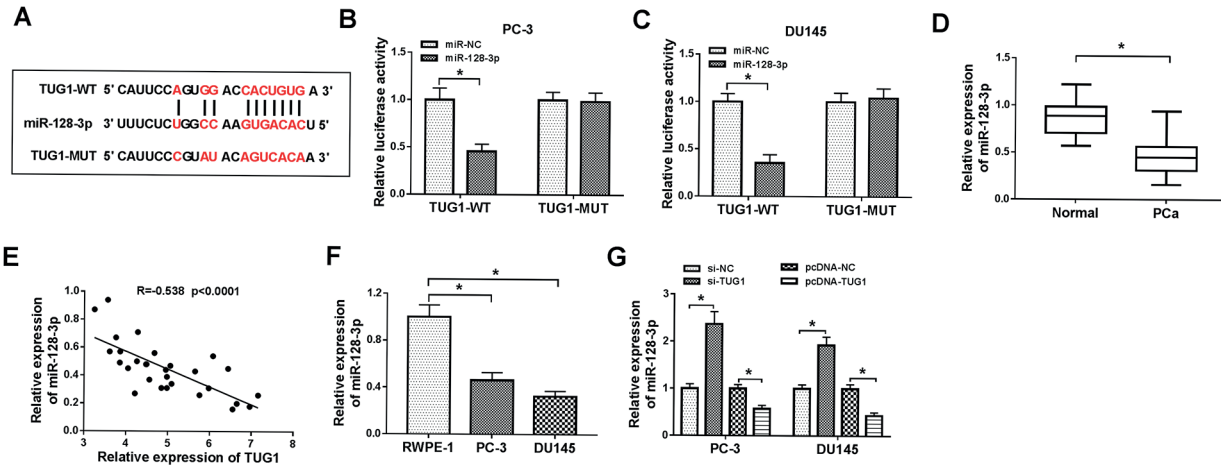

Figure 3. TUG1 directly interacted with miR-128-3p and negatively regulated miR-128-3p expression. **(A)** A putative miR-128-3p binding site was predicted in TUG1 and mutated miR-128-3p binding sequence. **(B** and **C)** The luciferase activities were detected in PC-3 and DU145 transfected with TUG1-WT or TUG1-MUT, together with miR-NC mimic or miR-128-3p mimic. **(D)** MiR-128-3p expression was assessed by qRT-PCR in 30 pairs of PCa tissues and corresponding normal prostate tissues. **(E)** Correlation between TUG1 level and miR-128-3p expression in PCa tissues was analyzed using Spearman's test. **(F)** MiR-128-3p expression was evaluated by qRT-PCR in PC-3, DU145 and RWPE-1 cells. **(G)** PC-3 and DU145 cells were transfected with si-NC, si-TUG1, pcDNA-NC or pcDNA-TUG1, followed by the measurement of miR-128-3p expression.

5G and 5H). To investigate whether miR-128-3p regulated YES1 expression in PCa cells, PC-3 and DU145 cells were transfected with miR-128-3p mimic or anti-miR-128-3p. As expected, the mRNA and protein levels of YES1 were significantly decreased by miR-128-3p mimic transfection, while they were remarkably increased with the introduction of anti-miR-128-3p (Figure 5I and 5J). These data together implied that YES1 was directly targeted and repressed by miR-128-3p.

Next, we determined whether and, if so, how TUG1 modulated YES1 expression in the two PCa cells. In comparison to the negative group, the mRNA and protein levels of YES1 were highly reduced by TUG1 silencing (Figure 5K and 5L). Nevertheless, si-TUG1-mediated decreased YES1 expression was prominently reversed by cotransfection of anti-miR-128-3p in the two cells (Figure 5K and 5L). All these results strongly suggested that TUG1 regulated YES1 expression by acting as a molecular sponge of miR-128-3p.

TUG1 Silencing Exerted its Regulatory Effects on PCa Cell Proliferation, Migration, Invasion, EMT, and Apoptosis by YES1

Our above data revealed that TUG1 modulated YES1 expression by acting as a molecular sponge of miR-128-3p. Further, we observed whether YES1 was involved in the regulatory mechanism of TUG1 on PCa cell progression. Thus, PC-3 and DU145 cells were cotransfected with si-TUG1 and pcDNA-YES1. As demonstrated by qRT-PCR, the cotransfection of pcDNA-YES1 significantly abolished the inhibition of si-TUG1 on YES1 expression in the two cells (Figure 6A and 6B). Subsequent experiment results revealed that compared with negative control, si-TUG1-mediated anti-proliferation (Figure 6C and 6D) and pro-apoptosis (Figure 6E) effects were strikingly reversed by restored YES1 expression. Additionally, the repressive effects of TUG1 knockdown on cell migration (Figure 6F), invasion (Figure 6G), and EMT (Figure 6H and 6I) were substantially abated by YES1 expression restoration. Together, these results established that YES1 mediated the regulatory effects of TUG1 silencing on PCa cell proliferation, migration, invasion, EMT, and apoptosis.

TUG1 Knockdown Retarded Tumor Growth In Vivo

Given our data that TUG1 silencing mitigated PCa cell progression *in vitro*, we further explored the effect of TUG1 knockdown on PCa cell growth *in vivo* using xenograft model. These data revealed that in contrast to the nega-

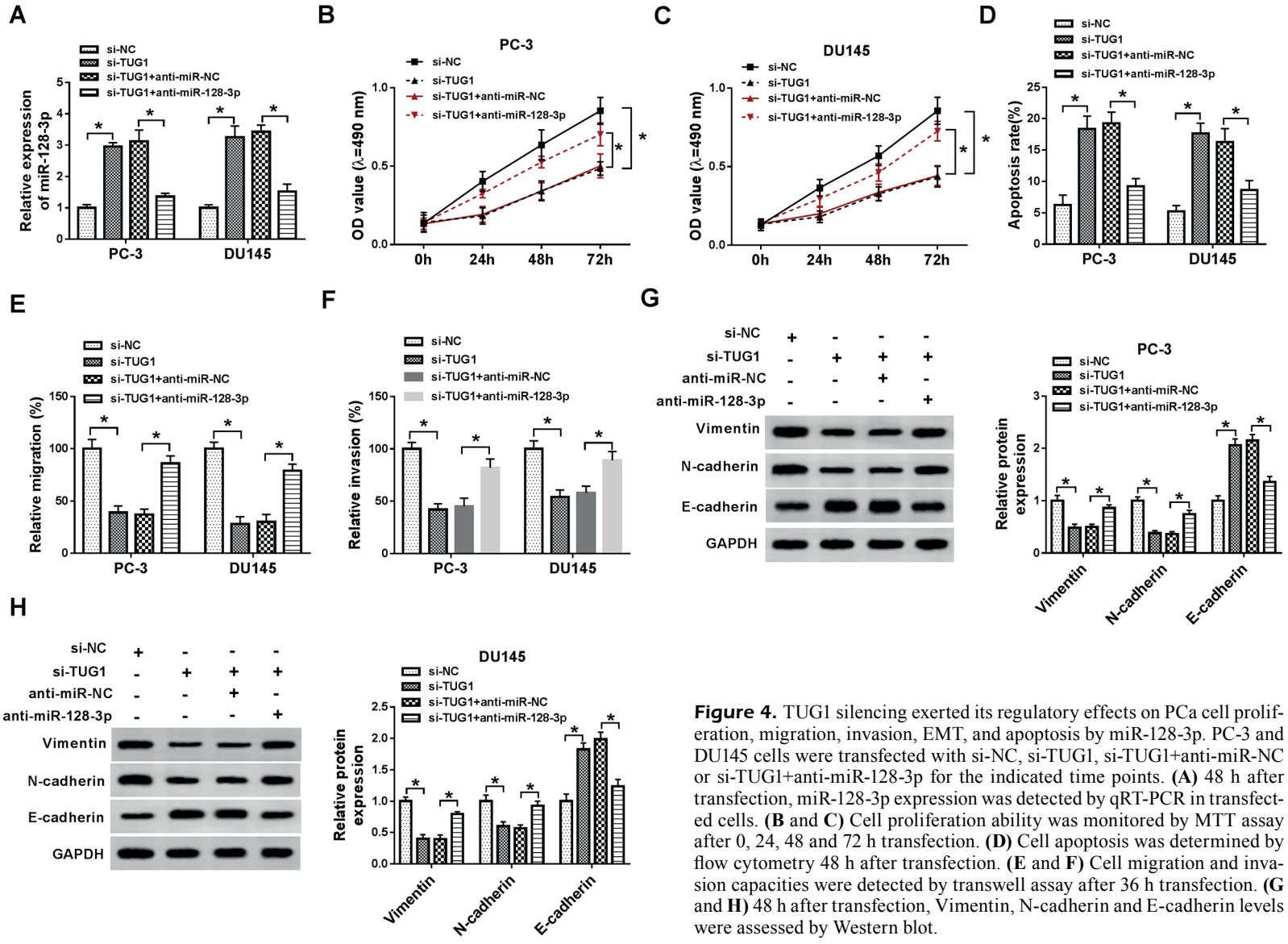

Figure 4. TUG1 silencing exerted its regulatory effects on PCa cell proliferation, migration, invasion, EMT, and apoptosis by miR-128-3p. PC-3 and DU145 cells were transfected with si-NC, si-TUG1, si-TUG1+anti-miR-NC or si-TUG1+anti-miR-128-3p for the indicated time points. **(A)** 48 h after transfection, miR-128-3p expression was detected by qRT-PCR in transfected cells. **(B** and **C)** Cell proliferation ability was monitored by MTT assay after 0, 24, 48 and 72 h transfection. **(D)** Cell apoptosis was determined by flow cytometry 48 h after transfection. **(E** and **F)** Cell migration and invasion capacities were detected by transwell assay after 36 h transfection. **(G** and **H)** 48 h after transfection, Vimentin, N-cadherin and E-cadherin levels were assessed by Western blot.

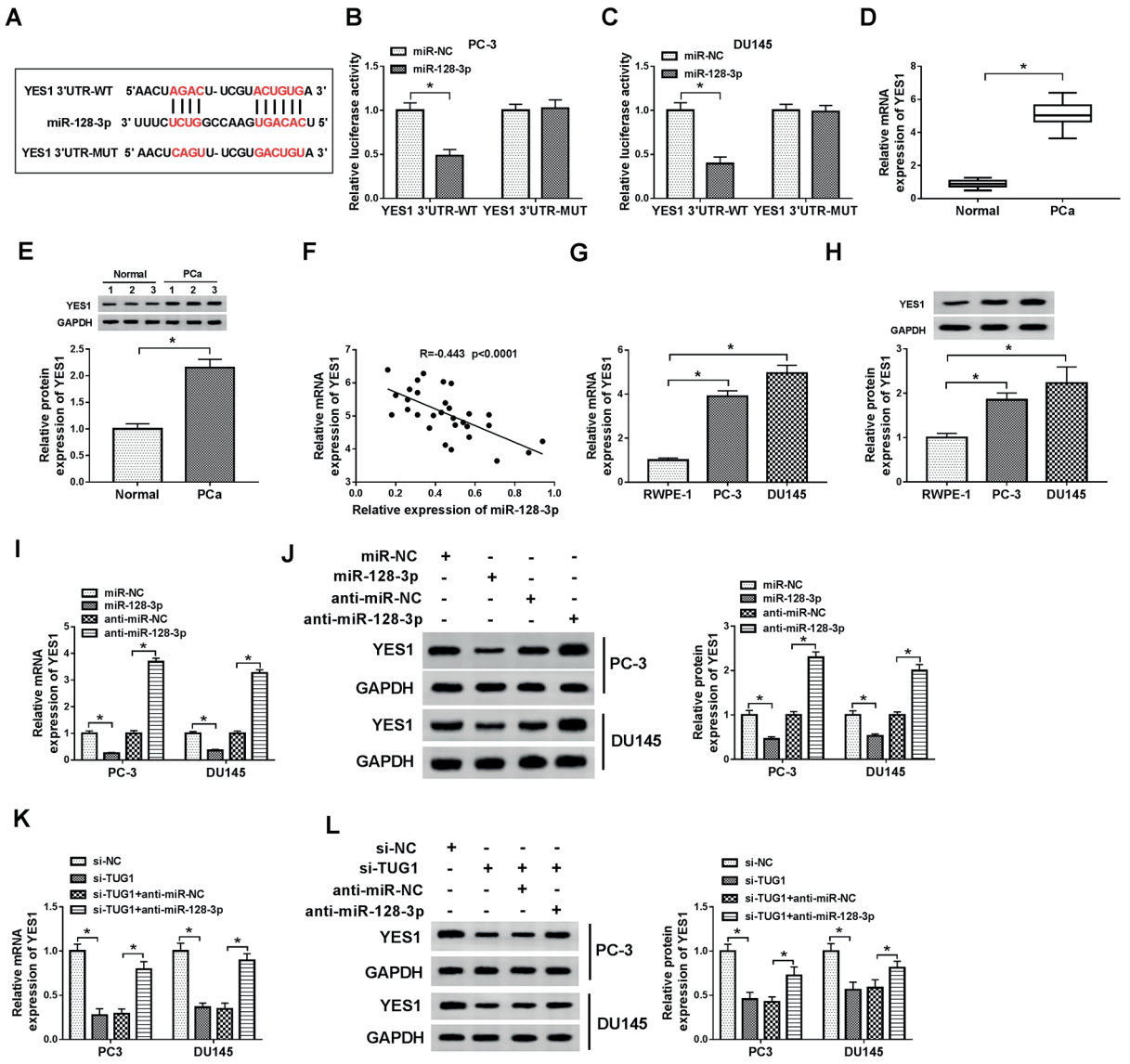

Figure 5. TUG1 regulated YES1 expression by sponging miR-128-3p. **(A)** A putative miR-128-3p complementary sequence was predicted in the 3'-UTR of YES1 and the mutant in the target sequence. **(B** and **C)** The luciferase activity was determined in PC-3 and DU145 cells cotransfected with YES1 3'-UTR-WT or YES1 3'-UTR-MUT and miR-128-3p mimic or miR-NC mimic. **(D** and **E)** The mRNA and protein levels of YES1 were assessed in PCa tissues and adjacent normal prostate tissues. **(F)** Correlation between YES1 level and miR-128-3p expression was analyzed in PCa tissues using Spearman's test. **(G** and **H)** The mRNA and protein levels of YES1 were detected in PC-3, DU145 and RWPE-1 cells. **(I** and **J)** The mRNA and protein levels of YES1 were determined in PC-3 and DU145 cells transfected with miR-NC mimic, miR-128-3p mimic, anti-miR-NC or anti-miR-128-3p. **(K** and **L)** PC-3 and DU145 cells were transfected with si-NC, si-TUG1, si-TUG1+anti-miR-NC or si-TUG1+anti-miR-128-3p, followed by the measurement of YES1 mRNA and protein levels.

tive group, the transduction of sh-TUG1 significantly weakened the tumor cell growth *in vivo*, as evidenced by the reduction of tumor volume and weight (Figure 7A and 7B). Moreover, qRT-PCR results showed that the TUG1 level was evidently reduced, while the miR-128-3p expression was manifestly elevated in tumors derived from sh-TUG1-transduced DU145 cells (Figure 7C and 7D). Western blot analysis demonstrated that YES1 protein level was drastically decreased following TUG1 knockdown in tumor tissues (Figure 7E).

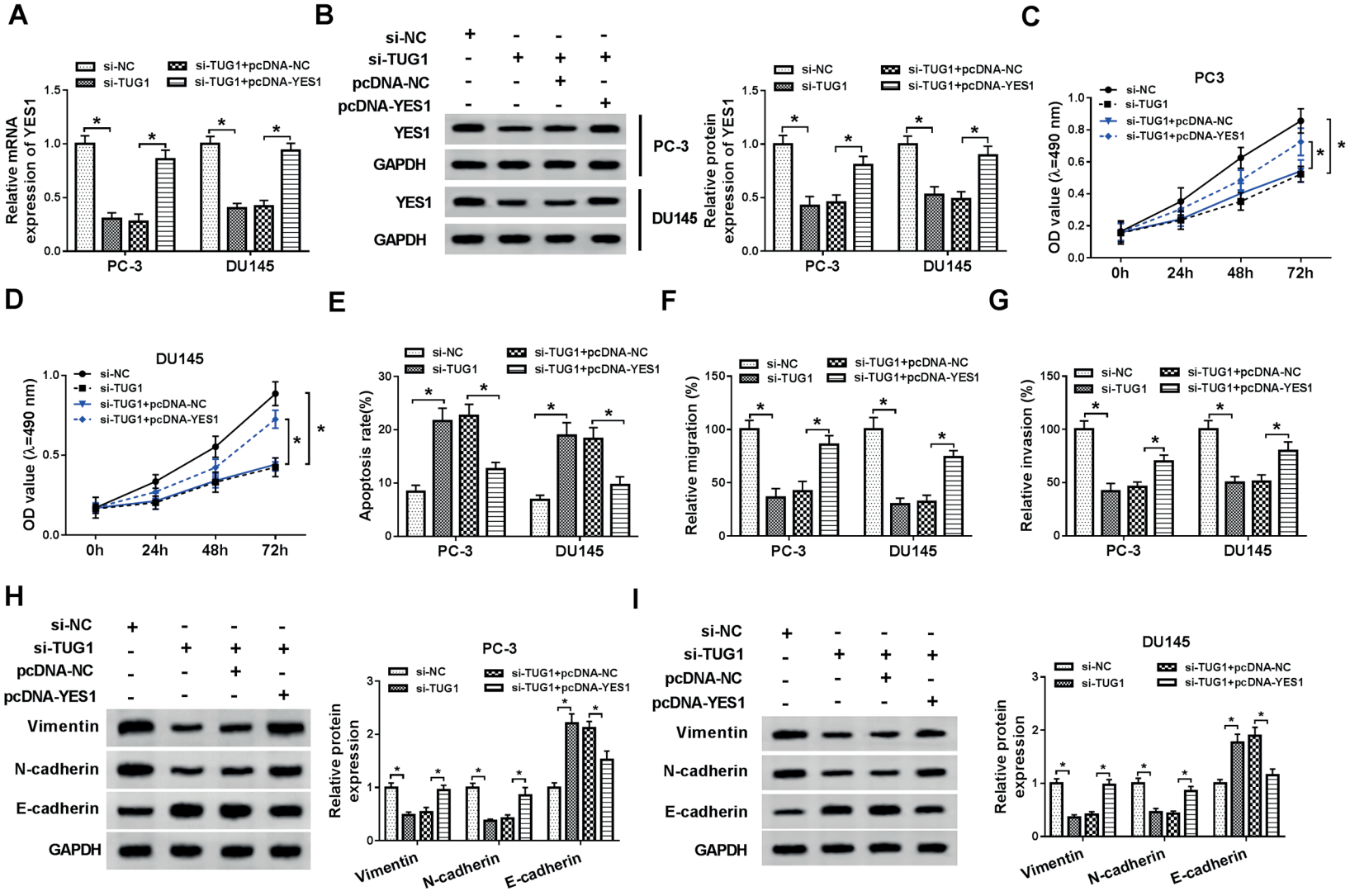

Figure 6. TUG1 silencing exerted its regulatory effects on PCa cell proliferation, migration, invasion, EMT, and apoptosis by YES1. PC-3 and DU145 cells were transfected with si-NC, si-TUG1, si-TUG1+pcDNA-NC or si-TUG1+pcDNA-YES1. **(A** and **B)** The mRNA and protein levels of YES1 were detected in transfected cells 48 h after transfection. **(C** and **D)** After 0, 24, 48 and 72 h transfection, cell proliferation ability was monitored by MTT assay. **(E)** Cell apoptosis was assessed by flow cytometry. **(F** and **G)** 36 h after transfection, cell migration and invasion capacities were determined by transwell assay. **(H** and **I)** 48 h after transfection, Vimentin, N-cadherin and E-cadherin levels were assessed by Western blot.

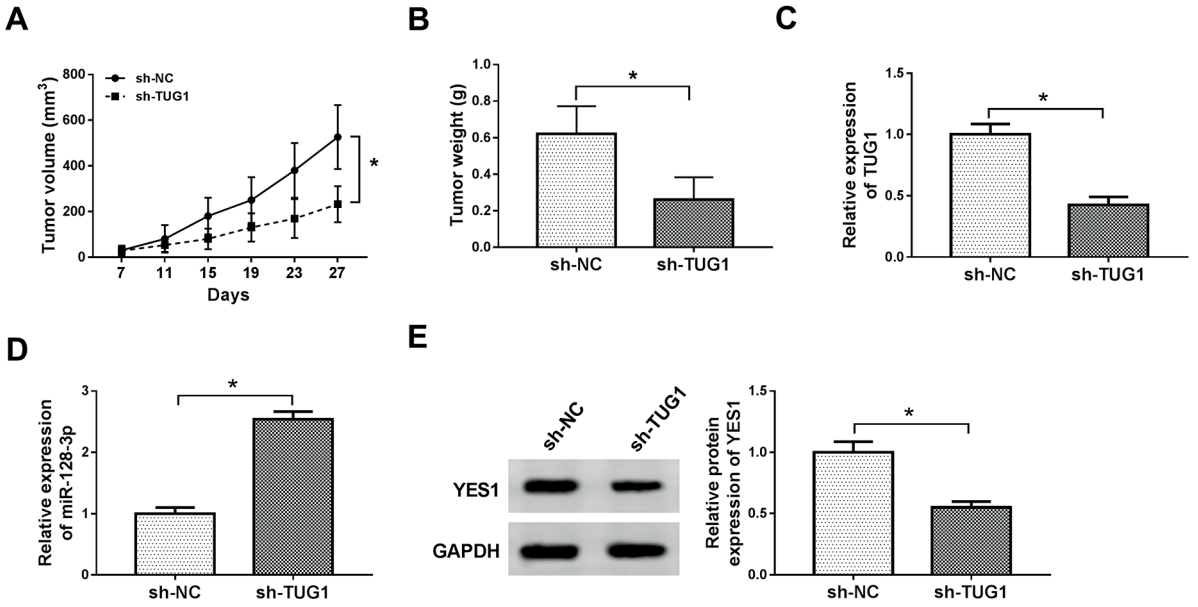

Figure 7. TUG1 knockdown retarded tumor growth *in vivo*. Approximately 5.0 × 106 DU145 cells transduced with sh-NC or sh-TUG1 were subcutaneously inoculated into the nude mice. 27 days later, all mice were euthanized to remove tumor tissues. **(A)** After 7 days implantation, tumor volume was measured with a caliper every 4 days. **(B)** Tumor weight was detected. **(C)** TUG1 expression was assessed by qRT-PCR in resected tumor tissues. **(D)** The expression of miR-128-3p was evaluated by qRT-PCR in xenograft tissues. **(E)** YES1 protein level was determined by Western blot in excised tumors.

Discussion

Recently, TUG1 has been established to play a potential oncogenic role in a series of human cancers, highlighting a possibility of TUG1 for cancer diagnosis, treatment, and prognosis. For example, Sun et al[8] reported that TUG1 enhanced colorectal cancer cell migration, invasion, and EMT by sponging miR-600. Hu et al[20] manifested that TUG1 overexpression accelerated the proliferation and migration abilities in cervical cancer cells. Niu et al[21] demonstrated that TUG1 knockdown weakened cell viability and facilitated the chemosensitivity of small cell lung cancer cells via modulating enhancer of Zesta homologue 2. Moreover, TUG1 was manifested to facilitate PCa progression *in vitro* through modulating Drosha-DGCR8 (DGCR8) and ring finger LIM domain-interacting protein (RLIM)[9,22]. In this study, our results indicated that TUG1 expression was increased in PCa tissues and cells, and associated with poor prognosis of PCa patients, consistent with earlier works[9,23]. Moreover, we validated that TUG1 silencing repressed the proliferation, migration, invasion, EMT, and promoted the apoptosis of PCa cells, in accordance with prior studies[10,22]. In a word, TUG1 performed a potential oncogenic effect in PCa progression. Additionally, accumulating evidence has suggested that EMT plays a crucial role in PCa metastasis[24,25]. For instance, Byles et al[24] demonstrated that the promotion of EMT resulted in increased capacities of PCa cell migration and invasion. Jang et al[25] reported that EMT enhancement trigged a significant increase in PCa metastasis. Thus, in the present work, TUG1 knockdown weakened PCa cell migration and invasion, possibly via inhibiting EMT.

Then, starBase v.3.0 software was used to help to identify the interacted miRNAs of TUG1. Among these predicted candidates, miR-128-3p was of particular interest in this study, considering its tumor-suppressive role in multiple human cancers, such as breast cancer, anaplastic thyroid cancer, and esophageal squamous cell cancer[24-26]. Also, downregulated miR-128-3p was found to be a new biomarker for the early diagnosis of lung cancer[27]. Additionally, previous researches manifested that miR-128-3p mitigated PCa cell progression *in vitro* and limited cancer stem-like cell properties mediated by B lympho-

ma Mo-MLV insertion region 1 homolog (BMI-1), highlighting its role as a tumor suppressor in PCa[15,16,28]. These researches already described above prompted us to examine miR-128-3p as a potential molecular mediator of TUG1 knockdown on PCa cell behaviors. In this study, our data firstly verified that miR-128-3p directly interacted with TUG1 in PCa cells. More importantly, our findings substantiated that TUG1 knockdown exerted its regulatory effects on the proliferation, migration, invasion, EMT, and apoptosis of PCa cells by miR-128-3p. Nevertheless, a previous document manifested that PHD finger protein 6 (PHF6), a tumor suppressor, was targeted and inhibited by miR-128-3p in T-cell acute lymphoblastic leukemia cells, providing evidence for miR-128-3p as a novel oncomiR[29]. These converse conclusions might be attributed to different types of human tumors.

MiRNAs are widely accepted to regulate a wide array of biological processes through modulating gene expression at the post-transcriptional level. Therefore, we carried out a detailed analysis for the targets of miR-128-3p using starBase v.3.0 software. Among these predicted targets, YES1 was selected for further research, because it was manifested to be a targetable oncogene in human cancers[30-32]. In fact, Garmendia et al[33] demonstrated that highly expressed YES1 was a potential predictor for poor prognosis in patients with non-small cell lung cancer and YES1 facilitated NSCLC progression. Fang et al[34] reported that YES1 depletion mediated the repressive effects of miR-140-5p on gastric cancer cell proliferation and metastasis. Besides, YES1 was underscored to be a direct target of miR-199a and miR-140, and thus involved in PCa progression and chemoresistance development[35,36]. In this study, we firstly validated that YES1 was a target of miR-128-3p in PCa cells. Furthermore, our data substantiated that TUG1 regulated YES1 expression via sponging and sequestering miR-128-3p. More intriguingly, our results firstly shed light on the fact that YES1 mediated the regulatory effects of TUG1 depletion on the proliferation, migration, invasion, EMT, and apoptosis in PCa cells. Previous researches had reported the role of TUG1 as a ceRNA of several miRNAs in PCa cells[10,37,38]. Yang et al[10] manifested that TUG1 enhanced the progression of PCa through acting as a ceRNA of miR-26a. Wang et al[37] reported that TUG1 acted as a ceRNA of miR-197-3p to regulate the chemoresistance in colorectal cancer through regulating thymidylate synthetase (TYMS) expression. Liu et al[38] demonstrated that TUG1 promoted the tumorigenesis of oral squamous cell carcinoma by acting as a ceRNA of miR-524-5p and upregulating distal-less homeobox 1 (DLX1).

Lastly, *in vivo* assays demonstrated that TUG1 knockdown retarded tumor growth and resulted in an increase of miR-128-3p level and a decrease of YES1 protein level. These results indicated that TUG1 silencing hindered tumor growth *in vivo*, possibly via modulating miR-128-3p/YES1 axis.

Conclusions

We proved evidence that the depletion of TUG1 hindered PCa cell progression *in vitro* and *in vivo* at least partly via sponging miR-128-3p and modulating YES1 expression. This study highlighted that targeting TUG1 might be a promising therapeutic agent for PCa treatment.

Conflict of Interests

The Authors declare that they have no conflict of interests.

Declarations

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Zhejiang Integrated Traditional and Western Medicine Hospital/Hangzhou Red Cross Hospital. The methods used in this study were performed in accordance with relevant guidelines and regulations. Written consent was obtained from the participants or guardians of participants under 16 years old.

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References

1) Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68: 394-424.

2) Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. CA Cancer J Clin 2016; 66: 115-132.

3) Miller KD, Siegel RL, Lin CC, Mariotto AB, Kramer JL, Rowland JH, Stein KD, Alteri R, Jemal A. Cancer treatment and survivorship statistics, 2016. CA Cancer J Clin 2016; 66: 271-289.
4) Fatica A, Bozzoni I. Long non-coding RNAs: new players in cell differentiation and development. Nat Rev Genet 2014; 15: 7-21.
5) Martens-Uzunova ES, Böttcher R, Croce CM, Jenster G, Visakorpi T, Calin GA. Long noncoding RNA in prostate, bladder, and kidney cancer. Eur Urol 2014; 65: 1140-1151.
6) Jin G, Sun J, Isaacs SD, Wiley KE, Kim ST, Chu LW, Zhang Z, Zhao H, Zheng SL, Isaacs WB, Xu J. Human polymorphisms at long non-coding RNAs (lncRNAs) and association with prostate cancer risk. Carcinogenesis 2011; 32: 1655-1659.
7) Yu G, Zhou H, Yao W, Meng L, Lang B. lncRNA TUG1 promotes cisplatin resistance by regulating CCND2 via epigenetically silencing miR-194-5p in bladder cancer. Mol Ther Nucleic Acids 2019; 16: 257-271.
8) Sun J, Hu J, Wang G, Yang Z, Zhao C, Zhang X, Wang J. LncRNA TUG1 promoted KIAA1199 expression via miR-600 to accelerate cell metastasis and epithelial-mesenchymal transition in colorectal cancer. J Exp Clin Cancer Res 2018; 37: 106.
9) Xu T, Liu CL, Li T, Zhang YH, Zhao YH. LncRNA TUG1 aggravates the progression of prostate cancer and predicts the poor prognosis. Eur Rev Med Pharmacol Sci 2019; 23: 4698-4705.
10) Yang B, Tang X, Wang Z, Sun D, Wei X, Ding Y. TUG1 promotes prostate cancer progression by acting as a ceRNA of miR-26a. Biosci Rep 2018; 38. pii: BSR20180677.
11) Iwakawa HO, Tomari Y. The functions of microRNAs: mRNA decay and translational repression. Trends Cell Biol 2015; 25: 651-665.
12) Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ. miRBase: tools for microRNA genomics. Nucleic Acids Res 2008; 36: D154-158.
13) Brase JC, Johannes M, Schlomm T, Falth M, Haese A, Steuber T, Beissbarth T, Kuner R, Sultmann H. Circulating miRNAs are correlated with tumor progression in prostate cancer. Int J Cancer 2011; 128: 608-616.
14) Ozen M, Creighton CJ, Ozdemir M, Ittmann M. Widespread deregulation of microRNA expression in human prostate cancer. Oncogene 2008; 27: 1788-1793.
15) Sun X, Li Y, Yu J, Pei H, Luo P, Zhang J. miR-128 modulates chemosensitivity and invasion of prostate cancer cells through targeting ZEB1. Jpn J Clin Oncol 2015; 45: 474-482.
16) Sun X, Yang Z, Zhang Y, He J, Wang F, Su P, Han J, Song Z, Fei Y. Prognostic implications of tissue and serum levels of microRNA-128 in human prostate cancer. Int J Clin Exp Pathol 2015; 8: 8394-8401.
17) Sanchez-Mejias A, Tay Y. Competing endogenous RNA networks: tying the essential knots for cancer biology and therapeutics. J Hematol Oncol 2015; 8: 30.
18) Carlsson J, Davidsson S, Helenius G, Karlsson M, Lubovac Z, Andren O, Olsson B, Klinga-Levan K. A miRNA expression signature that separates between normal and malignant prostate tissues. Cancer Cell Int 2011; 11: 14.
19) Ghosh A, Dasgupta D, Ghosh A, Roychoudhury S, Kumar D, Gorain M, Butti R, Datta S, Agarwal S, Gupta S, Krishna Dhali G, Chowdhury A, Schmittgen TD, Kundu GC, Banerjee S. MiRNA199a-3p suppresses tumor growth, migration, invasion and angiogenesis in hepatocellular carcinoma by targeting VEGFA, VEGFR1, VEGFR2, HGF and MMP2. Cell Death Dis 2017; 8: e2706.
20) Hu Y, Sun X, Mao C, Guo G, Ye S, Xu J, Zou R, Chen J, Wang L, Duan P, Xue X. Upregulation of long noncoding RNA TUG1 promotes cervical cancer cell proliferation and migration. Cancer Med 2017; 6: 471-482.
21) Niu Y, Ma F, Huang W, Fang S, Li M, Wei T, Guo L. Long non-coding RNA TUG1 is involved in cell growth and chemoresistance of small cell lung cancer by regulating LIMK2b via EZH2. Mol Cancer 2017; 16: 5.
22) Guo BH, Zhao Q, Li HY. TUG1 promotes the development of prostate cancer by regulating RLIM. Eur Rev Med Pharmacol Sci 2019; 23: 1926-1933.
23) Yang XL, Wei C, Zhang YB, Guo HQ. Long noncoding RNA TUG1 promotes progression via upregulating DGCR8 in prostate cancer. Eur Rev Med Pharmacol Sci 2019; 23: 2391-2398.
24) Byles V, Zhu L, Lovaas JD, Chmilewski LK, Wang J, Faller DV, Dai Y. SIRT1 induces EMT by cooperating with EMT transcription factors and enhances prostate cancer cell migration and metastasis. Oncogene 2012; 31: 4619-4629.
25) Jang MJ, Baek SH, Kim JH. UCH-L1 promotes cancer metastasis in prostate cancer cells through EMT induction. Cancer Lett 2011; 302: 128-135.
26) Zhao J, Li D, Fang L. MiR-128-3p suppresses breast cancer cellular progression via targeting LIMK1. Biomed Pharmacother 2019; 115: 108947.
27) Chen J, Zhao D, Meng Q. Knockdown of HCP5 exerts tumor-suppressive functions by up-regulating tumor suppressor miR-128-3p in anaplastic thyroid cancer. Biomed Pharmacother 2019; 116: 108966.
28) Zhao L, Li R, Xu S, Li Y, Zhao P, Dong W, Liu Z, Zhao Q, Tan B. Tumor suppressor miR-128-3p inhibits metastasis and epithelial-mesenchymal transition by targeting ZEB1 in esophageal squamous-cell cancer. Acta Biochim Biophys Sin (Shanghai) 2018; 50: 171-180.
29) Pan J, Zhou C, Zhao X, He J, Tian H, Shen W, Han Y, Chen J, Fang S, Meng X, Jin X, Gong Z. A two-miRNA signature (miR-33a-5p and miR-128-3p) in whole blood as potential biomarker for early diagnosis of lung cancer. Sci Rep 2018; 8: 16699.

30) Jin M, Zhang T, Liu C, Badeaux MA, Liu B, Liu R, Jeter C, Chen X, Vlassov AV, Tang DG. miRNA-128 suppresses prostate cancer by inhibiting BMI-1 to inhibit tumor-initiating cells. Cancer Res 2014; 74: 4183-4195.

31) Mets E, Van Peer G, Van der Meulen J, Boice M, Taghon T, Goossens S, Mestdagh P, Benoit Y, De Moerloose B, Van Roy N, Poppe B, Vandesompele J, Wendel HG, Van Vlierberghe P, Speleman F, Rondou P. MicroRNA-128-3p is a novel oncomiR targeting PHF6 in T-cell acute lymphoblastic leukemia. Haematologica 2014; 99: 1326-1333.

32) Hamanaka N, Nakanishi Y, Mizuno T, Horiguchi-Takei K, Akiyama N, Tanimura H, Hasegawa M, Satoh Y, Tachibana Y, Fujii T, Sakata K, Ogasawara K, Ebiike H, Koyano H, Sato H, Ishii N, Mio T. YES1 is a targetable oncogene in cancers harboring YES1 gene amplification. Cancer Res 2019 Aug 7. doi: 10.1158/0008-5472.CAN-18-3376. [Epub ahead of print]

33) Garmendia I, Pajares MJ, Hermida-Prado F, Ajona D, Bertolo C, Sainz C, Lavin A, Remirez AB, Valencia K, Moreno H, Ferrer I, Behrens C, Cuadrado M, Paz-Ares L, Bustelo XR, Gil-Bazo I, Alameda D, Lecanda F, Calvo A, Felip E, Sanchez-Cespedes M, Wistuba, II, Granda-Diaz R, Rodrigo JP, Garcia-Pedrero JM, Pio R, Montuenga LM, Agorreta J. YES1 drives lung cancer growth and progression and predicts sensitivity to dasatinib. Am J Respir Crit Care Med 2019; 200: 888-899.

34) Fang Z, Yin S, Sun R, Zhang S, Fu M, Wu Y, Zhang T, Khalio J, Li Y. miR-140-5p suppresses the proliferation, migration and invasion of gastric cancer by regulating YES1. Mol Cancer 2017; 16: 139.

35) Chen L, Cao H, Feng Y. MiR-199a suppresses prostate cancer paclitaxel resistance by targeting YES1. World J Urol 2018; 36: 357-365.

36) Zhao S, Jie C, Xu P, Diao Y. MicroRNA-140 inhibit prostate cancer cell invasion and migration by targeting YES proto-oncogene 1. J Cell Biochem 2019 Jul 16. doi: 10.1002/jcb.29231. [Epub ahead of print]

37) Wang M, Hu H, Wang Y, Huang Q, Huang R, Chen Y, Ma T, Qiao T, Zhang Q, Wu H, Chen Q, Han D, Wang G, Wang X. Long non-coding RNA TUG1 mediates 5-fluorouracil resistance by acting as a ceRNA of miR-197-3p in colorectal cancer. J Cancer 2019; 10: 4603-4613.

38) Liu S, Liu LH, Hu WW, Wang M. Long noncoding RNA TUG1 regulates the development of oral squamous cell carcinoma through sponging miR-524-5p to mediate DLX1 expression as a competitive endogenous RNA. J Cell Physiol 2019; 234: 20206-20216.