

lncRNA DUXAP8 inhibits papillary thyroid carcinoma cell apoptosis via sponging the miR-20b-5p/SOS1 axis

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Abstract. Papillary thyroid carcinoma (PTC) is the most common type of cancer in the endocrine system. Long non-coding RNAs (lncRNAs) are associated with PTC progression. Therefore, the present study aimed to identify a novel lncRNA involved in PTC. Herein, dysregulated lncRNAs were analyzed in The Cancer Genome Atlas (TCGA)-thyroid cancer (THCA) data. Furthermore, the association between double homeobox A pseudogene 8 (DUXAP8) gene expression and disease stage, and prognosis of patients with PTC was evaluated using the GEPIA online database, while the correlation between DUXAP8 expression and the clinicopathological characteristics of patients with PTC was analyzed by Chi-square test. In addition, the biological effect of DUXAP8 expression on cell proliferation and apoptosis was also investigated. The protein and mRNA/microRNA (miRNA)/lncRNA expression levels were assessed by western blot analysis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR), respectively. The interaction between miR-20b-5p and DUXAP8 was verified using bioinformatics analysis, RNA RIP assay, dual luciferase reporter assay, western blot analysis and RT-qPCR. The analysis of the TCGA-THCA data revealed that DUXAP8 was one of the most significantly upregulated lncRNAs in PTC. This finding was further confirmed in tissues from patients with PTC. Increased DUXAP8 expression was associated with higher grade and poorer prognosis in patients with PTC. In PTC cell lines, silencing of DUXAP8 expression with small interfering RNA-DUXAP8 (si-DUXAP8) induced cell apoptosis and attenuated cell proliferation. Additionally, transfection of PTC cells with si-DUXAP8 decreased the phosphorylation levels of MEK1/2 and ERK1/2, as well as downregulated the expression of son of sevenless 1 (SOS1), cyclin D1 (CCND1) and c-Myc. The results of the present study

also revealed that miR-20b-5p could directly target DUXAP8. DUXAP8 expression was positively associated with that of SOS1, c-Myc and CCND1 in the TCGA-THCA data, and DUXAP8 level was positively correlated with that of SOS1 in PTC tumor tissues. Finally, transfection of PTC cells with the SOS1 over-expression plasmid, pcDNA3.1-SOS1, rescued the effects of si-DUXAP8 on cell proliferation and apoptosis. The present study was the first to identify DUXAP8 as a novel upregulated lncRNA in PTC, and provided new insights in understanding the effect of the lncRNA-miRNA-mRNA network in PTC.

Introduction

Thyroid cancer, the most frequent type of cancer of the endocrine system, originates from parafoollicular thyroid cells or follicular thyroid cells (1). There are several different histologic subtypes of thyroid cancer, such as anaplastic thyroid carcinoma, papillary thyroid carcinoma (PTC), and follicular thyroid carcinoma (2). PTC, which stems from thyroid follicular cells, accounts for the majority of thyroid cancer cases (3). The overall five-year survival rate in patients at early stages of PTC is as high as 97%, whereas, in those in advanced stages, the overall five-year survival rate is estimated to be approximately 59% (4). Therefore, identifying the molecular mechanisms underlying the progression of PTC is of great importance in order to optimize the prognosis in patients with PTC.

Long non-coding RNAs (lncRNAs) are a subtype of non-coding transcripts resulting from the lack of open reading frames (ORFs). Their length is >200 nt and are encoded by the mammalian genome (5). It has been reported that the dysregulation of lncRNAs is associated with carcinogenesis. For example, lncRNA HOTAIR mediated the metastasis of breast cancer cells via targeting genome-wide retargeting of polycomb repressive complex 2 (PRC2) (6). Additionally, the dysregulation of lncRNA KIAA0125 was involved in the development of gallbladder carcinoma via regulating β -catenin and vimentin (7). Several lncRNAs have been identified to serve an important role in the development of thyroid cancer. For instance, H19, which is overexpressed in thyroid cancer tissues and cells, induced cell growth, migration, and invasion by serving as a competitive endogenous RNA (ceRNA) for miR-17-5p and regulating the miR-17-5p/YES1 axis (8). Furthermore, the expression of actin filament-associated protein 1-antisense RNA 1 (AFAP1-AS1) was revealed to be increased in thyroid cancer tissues, and its downregulation attenuated cell proliferation and invasion, and

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induced cell apoptosis (9). In 2018, a study demonstrated that the lncRNA double homeobox A pseudogene 8 (DUXAP8) was differentially expressed in human thyroid cancer using genome-wide analysis (10). lncRNA DUXAP8 has been identified to promote the growth of numerous cancers, including non-small cell lung cancer (11), pancreatic carcinoma (12), and renal cell carcinoma (13). However, the role of lncRNA DUXAP8 in PTC has not been investigated.

Mechanistically, some studies have demonstrated that lncRNAs serve as microRNA (miRNA) sponges through ceRNA activity, thus promoting the expression of the downstream mRNAs (14,15); and some studies have indicated that lncRNAs and miRNAs interact (16,17). miRNAs are a group of small, non-coding, and single-chain molecules, which regulate the translation or degradation of their target mRNAs (18). Notably, miRNAs play important roles in regulating several biological behaviors, including cell proliferation, apoptosis, and differentiation (19). However, the function of the lncRNA DUXAP8-miRNA network in the pathogenesis of PTC remains elusive.

Materials and methods

Sample collection. Tumor tissues and the adjacent normal tissues (>5 cm away from the tumor) from 60 patients (31 males and 29 females; aged 25–66) with PTC were collected at the First Hospital of Jilin University, between January 2016 and December 2018. The inclusion/exclusion criteria were as follows: No chemotherapy or radiotherapy received before the surgery. The clinical stage of each patient after surgery was evaluated based on the 7th edition of the American Joint Committee on Cancer (AJCC) tumor-node-metastasis staging system (20). The adjacent normal tissues served as matched controls for the PTC tissues. Written informed consent was provided by all patients prior to enrollment in this study. All experimental procedures were approved by the Ethics Committee of the First Hospital of Jilin University.

Bioinformatics analysis. The dysregulated lncRNAs were analyzed in The Cancer Genome Atlas (TCGA)-thyroid carcinoma (THCA) data. TCGA-THCA data were retrieved from the Broad Institute Genome Data Analysis Center (GDAC) Firehose as previously reported (21). The interaction between miR-20b-5p and DUXAP8 was predicted by the miRDB (<http://mirdb.org/>) bioinformatics analysis tool. The association between DUXAP8 and disease stage as well as with the prognosis of patients with PTC was evaluated with the Gene Expression Profiling Interactive Analysis (GEPIA) database (22). GEPIA downloaded level 3 gene expression data (RNA sequencing data after alignment and standardization by RSEM method) and patient information from TCGA database. All patients from TCGA-THCA (with survival information and gene expression data) were used to investigate the association between DUXAP8 expression and overall survival by Kaplan-Meier Plot analysis with log-rank testing on GEPIA database, which was employed to divide the patients into a high-DUXAP8 expression group and a low-DUXAP8 expression group according to the median expression of DUXAP8. The correlation between DUXAP8 and son of sevenless 1 (SOS1), c-Myc, or cyclin D1 (CCND1) was analyzed in the TCGA-THCA data.

Cell culture. The human PTC cell lines, CGTH W-3 and TPC1, and the human thyroid follicular epithelial cell line, Nthy-ori 3-1, were obtained from the American Type Culture Collection (ATCC). Cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.), supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified incubator at 37°C with 5% CO₂.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from PTC tissues and cells using TRIzol (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA with the TaqMan microRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). Subsequently, RT-qPCR was carried out using Bio-Rad cFX96 Real-Time PCR System (Bio-Rad Laboratories, Inc.) and SYBR Premix Ex Taq kit (Takara Bio, Inc.) according to the manufacturer's instructions. The thermocycling conditions were as follows: 95°C for 5 min, followed by 45 cycles at 95°C for 10 sec, 55°C for 30 sec, and a melting curve every 0.2°C from 55 to 95°C for 2 min. The expression levels of DUXAP8 and SOS1 were normalized to GAPDH, and those of miR-20b-5p to U6 expression with the 2^{-ΔΔC_q} method (23). The primers were as follows: DUXAP8F forward, 5'-AGGATGGAGTCTCGCTGTATTGC-3' and reverse, 5'-GGAGGTTTGTCTTCTCTTTT-3'; SOS1 forward, 5'-ACCACAGATGTTTGCAGTGTATTTG-3' and reverse, 5'-GCAGATTCTGGTCGTCTTCGTG-3'; GAPDH forward, 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse, 5'-TGGTGAAGACGCCAGTGGA-3'; miR-20b-5p forward, 5'-CAAAGTGCTCATAGTGCAGGT-3' and reverse, Uni-miR qPCR (Takara Biotechnology Co., Ltd.); U6 forward, 5'-CTCGCTTCGGCAGCACAC-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'.

Western blot analysis. Total proteins were extracted from PTC tissues and cells using RIPA lysis buffer (Roche Diagnostics) containing protease inhibitors, following the manufacturer's instructions. The supernatant was harvested by centrifugation at 13,282 × g for 10 min at 4°C. The protein concentration was measured with a BCA Protein Assay kit (Beyotime Institute of Biotechnology). The protein extracts (20 µg) were separated by SDS-PAGE (8%) and were then blotted onto a polyvinylidene fluoride (PVDF) membrane. Subsequently, the proteins on the PVDF membrane were blocked with 5% non-fat milk for 2 h at 25°C prior to incubation with primary antibodies against SOS1 (1:1,000; product no. 12409), extracellular signal-regulated kinase 1/2 (ERK1/2; 1:1,000; product no. 9101) and GAPDH (1:1,000; cat. no. 5174; all from Cell Signaling Technology, Inc.) overnight at 4°C. The membrane was then incubated with a horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 2 h at 25°C. The blots were visualized using an ECL reagent (Beyotime Institute of Biotechnology) and analyzed with ImageJ software 1.48u (National Institutes of Health). The target-protein expression was normalized to that of GAPDH.

Downregulation of DUXAP8. The small interfering RNA (siRNA) technology was applied for DUXAP8 downregulation

in PTC cells. The DUXAP8 siRNAs (si-DUXAP8; 5'-AAG AUA AAG GUG GUU UCC ACA AGA ATT-3') and control siRNA (siRNA-NC; 5'-AGCUUGAUACGACAAAGCUTT-3') were purchased from Shanghai GenePharma Co., Ltd. PTC cells were transfected with DUXAP8 siRNAs or siRNA-NC (50 nM) using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol, and were then incubated for 48 h according to the manufacturer's instructions.

Overexpression of *SOS1*. The pcDNA3.1 and pcDNA3.1-SOS1 plasmids (2 µg) were purchased from GenePharma Co., Ltd. PTC cells were transfected with pcDNA3.1 or pcDNA3.1-SOS1 using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) following manufacturer's protocol, and incubated for 48 h following the manufacturer's protocol.

Cell proliferation assay. A Cell Counting Kit-8 (CKK-8) assay was carried out to measure cell proliferation, following the manufacturer's protocol. Briefly, cells (density, 1×10^4 cells/well) were seeded into 96-well plates, and were supplemented with 10 µl of CKK-8 reagent (Dojindo Molecular Technologies, Inc.) for 2 h at 37°C. The optical density at 450 nm was measured in each well at 0, 24, 48 and 72 h using a microplate reader (Thermo Fisher Scientific, Inc.).

Cell apoptosis assay. Briefly, PTC cells (density, 1×10^6 cells/ml) were first treated with 100 µl of 1X binding buffer. The mixture was supplemented with 2 µl of Annexin V-FITC and maintained for 15 min at room temperature. Subsequently, 400 µl of PBS and 1 µl propidium iodide (PI) were added and cells were incubated for 5 min at room temperature. Following treatment with Annexin V-FITC/PI, cells were subjected to flow cytometric analysis on a FACSCalibur flow cytometer (BD Biosciences) with the CellQuest software (version 6.0; BD Biosciences). Finally, the data were analyzed using the FlowJo software (version 10.2; FlowJo, LLC).

RNA immunoprecipitation (RIP) assay. To verify the association between DUXAP8 and miR-20b-5p a RIP assay was carried out using the EZMagna RIP RNA-binding protein immunoprecipitation kit (EMD Millipore) following the manufacturer's instructions. Briefly, PTC cells were lysed with RNA lysis buffer supplemented with protease and RNase inhibitors. The cell lysate was then treated with magnetic beads conjugated with human Ago2 antibody (cat. no. 10686-1-AP; ProteinTech Group, Inc.) or a negative control IgG (product code ab133470; Abcam). Following incubation at 4°C overnight, the co-precipitated RNAs were subjected to RT-qPCR analysis as previously described.

Dual luciferase reporter assay. The wild-type or mutant DUXAP8 3'untranslated region (3'UTR; Shanghai GeneChem Co., Ltd.) was sub-cloned into the pGL3 vector (Promega Corporation). Subsequently, the plasmids were transfected into PTC cells for 24 h at 37°C using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). Finally, the luciferase activity was measured using the dual-luciferase reporter assay system (Promega Corporation), and was normalized to that of *Renilla*.

Statistical analysis. Data are expressed as the mean \pm standard deviation (SD). The GraphPad Prism 6 (GraphPad Software,

Table I. Association between DUXAP8 expression level and clinicopathological characteristics of patients with PTC.

Characteristics	Cases	DUXAP8 expression		P-value
		Low	High	
Sex				0.606
Male	31	14	17	
Female	29	16	13	
Age (years)				0.204
≤45	31	13	18	
>45	29	17	12	
Extrathyroidal extension				0.438
Yes	28	12	16	
No	32	18	14	
Lymph node metastasis				0.601
N0	25	11	14	
N1	35	19	16	
Tumor size (cm)				0.019
<2	32	21	11	
2-4	28	9	19	
TNM stage				0.017
I-II	24	17	7	
III-IV	26	13	23	

Mean expression level of DUXAP8 was used as the cut-off. DUXAP8, double homeobox A pseudogene 8.

Inc.) software was applied for all statistical analyses. All the experiments were repeated 3 times. The comparisons between two groups were carried out using unpaired Student's t-test, while one-way analysis of variance (ANOVA) followed by Bonferroni's correction as a post hoc test was performed for the comparisons among multiple groups. The association between DUXAP8 and the clinicopathological characteristics of PTC patients was evaluated by Chi-square test. The association between the expression of DUXAP8 and that of *SOS1*, c-Myc and cyclin D1 (*CCND1*) in the TCGA-THCA dataset or PTC tumor tissues was analyzed by Spearman's correlation test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

DUXAP8 is upregulated in PTC tissues. The results from the TCGA-THCA revealed that DUXAP8 was one of the most significantly upregulated lncRNAs in patients with PTC (Fig. 1A). In addition, it was confirmed that DUXAP8 was increased in our collected PTC samples compared with the adjacent normal tissues (Fig. 1B). Furthermore, the data from the GEPIA online software revealed that the DUXAP8 expression levels were higher in the high-grade (IV) PTC samples compared with those in low-grade (I) samples (Fig. 1C). In addition, the GEPIA database revealed that high DUXAP8 expression was associated with poor overall survival in patients with PTC (Fig. 1D).

As revealed in Table I, the expression of DUXAP8 was associated with tumor size and TNM stage, but not with sex,

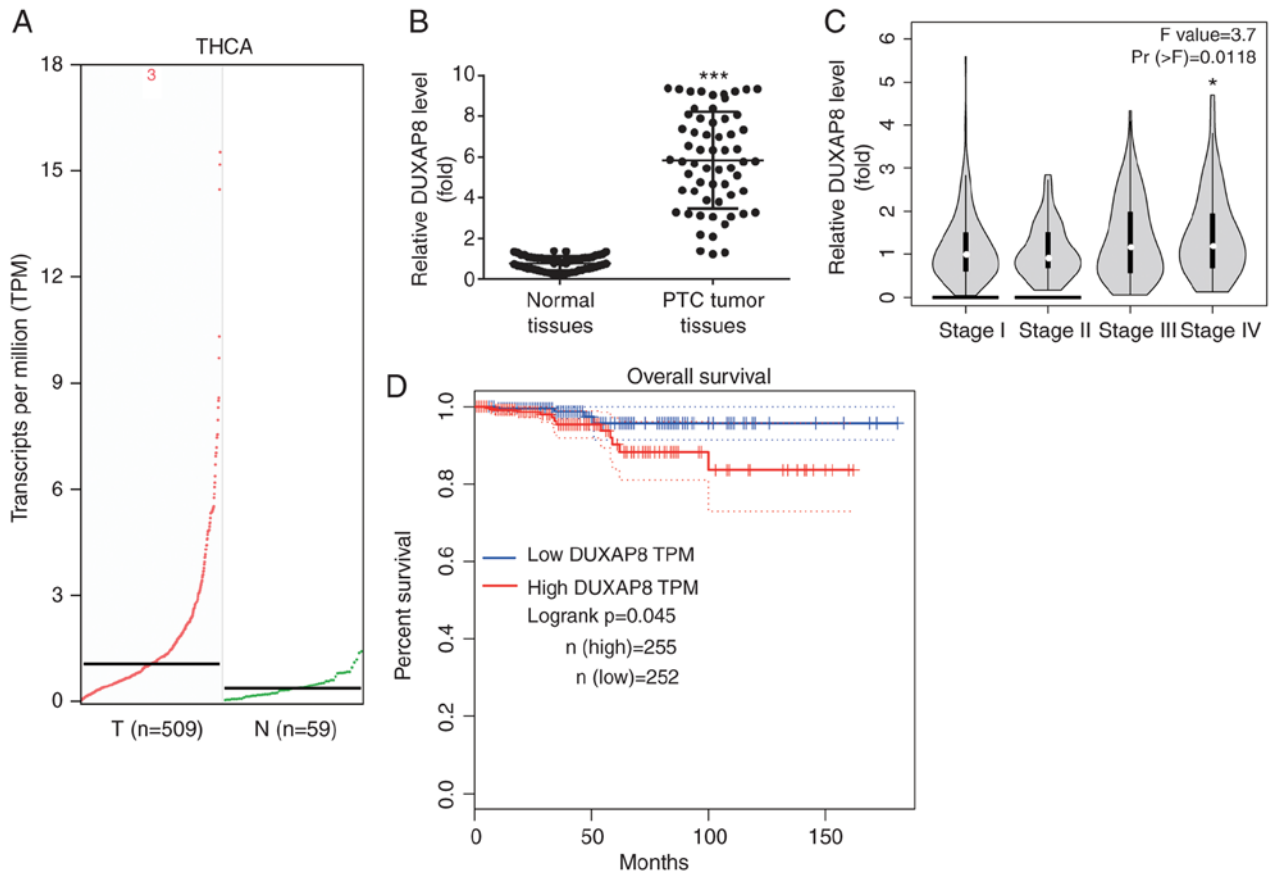


Figure 1. DUXAP8 is upregulated in PTC tissues. (A) TCGA-THCA data were used for the analysis of DUXAP8 level in PTC. The data included 509 tumors (T) and 59 normal tissues (N). (B) RT-qPCR was applied for the examination of DUXAP8 expression level in PTC samples and the normal tissues (B). The online software GEPIA was used to analyze the correlation between DUXAP8 and (C) disease stage as well as (D) the prognosis of patients with PTC. * $P < 0.05$, stage IV compared with stage I. *** $P < 0.001$, PTC tumor tissues compared with the normal tissues. DUXAP8, double homeobox A pseudogene 8; PTC, papillary thyroid carcinoma; TCGA, The Cancer Genome Atlas; THCA, thyroid carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GEPIA, Gene Expression Profiling Interactive Analysis; TPM, transcripts per million.

age, extrathyroidal extension or lymph node metastasis in patients with PTC.

DUXAP8 knockdown inhibits cell proliferation and induces cell apoptosis. DUXAP8 was also upregulated in the PTC cell lines, CGTH W-3 and TPC1, compared with the human thyroid follicular epithelial cells, Nthy-ori 3-1 (Fig. 2A). Subsequently, the expression of DUXAP8 was silenced in PTC cell lines using the si-RNA technology in order to investigate the effect of DUXAP8 in PTC. The results revealed that compared with the si-normal control (si-NC) group, transfection of CGTH W-3 and TPC1 PTC cell lines with si-DUXAP8 significantly reduced DUXAP8 expression levels (Fig. 2B). Furthermore, DUXAP8 silencing significantly attenuated CGTH W-3 and TPC1 cell proliferation compared with the si-NC group (Fig. 2C and D). However, compared with the si-NC group, transfection with si-DUXAP8 significantly induced CGTH W-3 and TPC1 cell apoptosis (Fig. 2E and F).

DUXAP8 knockdown inactivates the MAPK/ERK pathway. It has been reported that the constitutive activation of the MAPK/ERK pathway contributes to the malignant progression of PTC (22). However, the effects of DUXAP8 on the PTC-associated signaling pathways and molecules have not been studied. Therefore, the results of the present study

demonstrated that DUXAP8 downregulation reduced the phosphorylation levels of mitogen-activated protein kinase kinase 1/2 (p-MEK1/2) and ERK1/2 (p-ERK1/2) in the CGTH W-3 and TPC1 PTC cell lines (Fig. 3A-C). Additionally, the mRNA and protein expression levels of CCND1 and c-Myc, two downstream target genes of the MAPK/ERK pathway (24), were also downregulated following silencing of DUXAP8 in PTC cell lines (Fig. 3A and D-G).

DUXAP8 negatively regulates miR-20b-5p. Subsequently, the present study investigated whether specific miRNAs could be sponged by DUXAP8. Screening in the miRDB online database revealed numerous miRNAs complementary to DUXAP8. Among them, miR-20b-5p attracted our attention. It has been reported that miR-20b-5p is downregulated in PTC, and inhibits the progression of the disease (25) (Fig. 4A). Therefore, the association between DUXAP8 and miR-20b-5p was further verified. Transfection of the CGTH W-3 and TPC1 PTC cell lines with miR-20b-5p mimic significantly increased miR-20b-5p expression levels (Fig. 4B), indicating that the transfection procedure was efficiently performed. miR-20b-5p overexpression reduced the luciferase activity of DUXAP8 (Fig. 4C and D) and downregulated the expression of DUXAP8 in PTC cells (Fig. 4E), which was consistent with the reciprocal suppression between miRNA and lncRNA

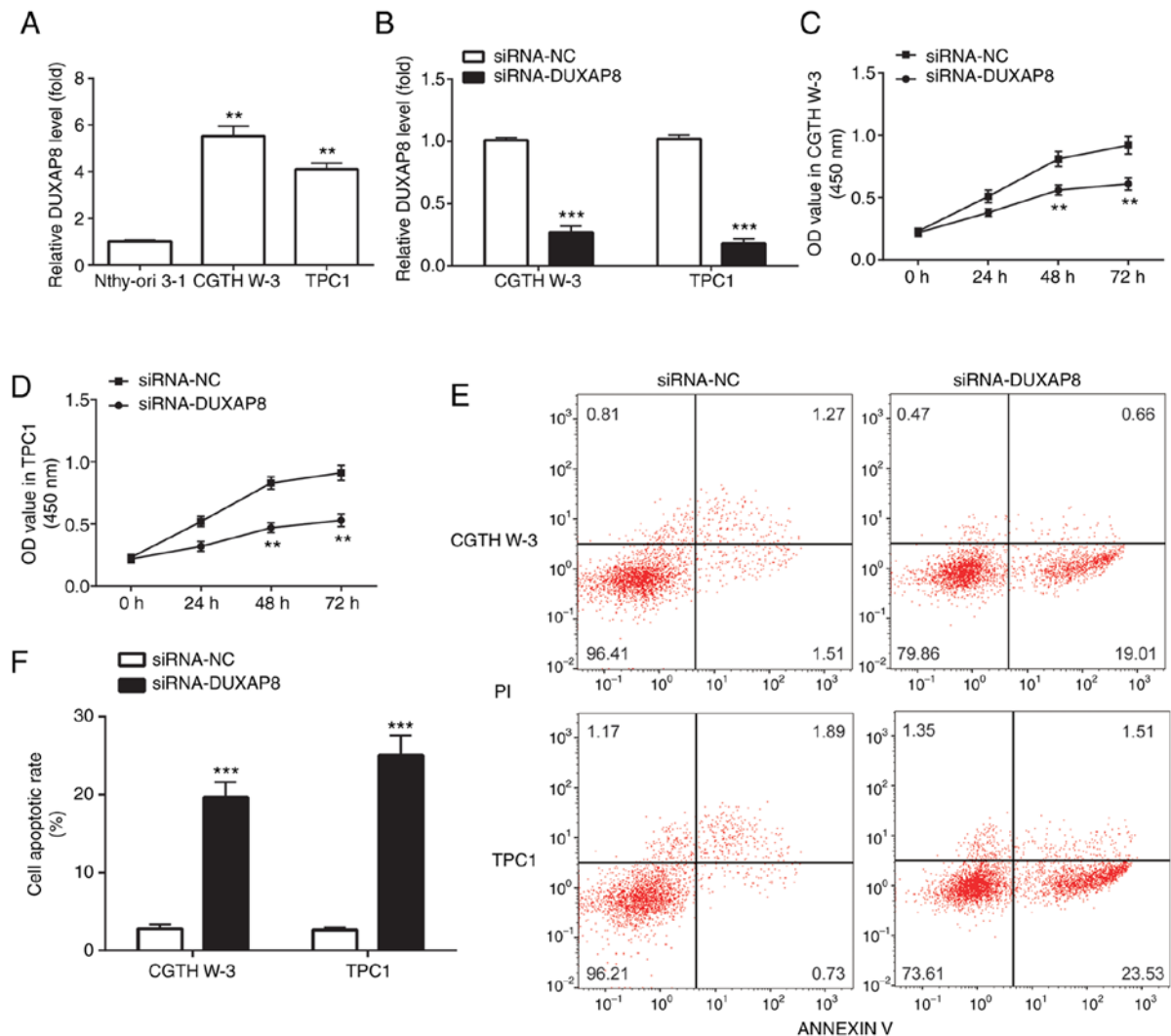


Figure 2. DUXAP8 knockdown inhibits cell proliferation and induces cell apoptosis. (A and B) RT-qPCR was applied for the examination of DUXAP8 expression level. (C and D) CCK-8 assay was used to detect cell proliferation, and (E and F) flow cytometric assay was used to detect cell apoptosis. ** $P < 0.01$, CGTH W-3 and TPC1 compared with Nthy-ori 3-1; and siRNA-DUXAP8 compared with siRNA-NC. *** $P < 0.001$, siRNA-DUXAP8 compared with siRNA-NC. DUXAP8, double homeobox A pseudogene 8; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CCK-8, Cell Counting Kit-8; siRNA, small interfering RNA; NC, negative control; PI, propidium iodide.

as recently reported (26). Furthermore, DUXAP8 knockdown significantly increased the expression of miR-20b-5p in CGTH W-3 and TPC1 cells (Fig. 4F). The RIP assay results in the PTC cell lines revealed that DUXAP8 and miR-20b-5p were enriched in the Ago2-pulled down protein complex (Fig. 4G and H). The abovementioned findings suggested that DUXAP8 could directly target miR-20b-5p in PTC cells.

DUXAP8 is positively associated with miR-20b-5p-related target genes. It has been reported that miR-20b-5p regulates the MAPK/ERK signaling and PTC cell proliferation via targeting SOS1 (25). In the TCGA-THCA data, Spearman's correlation analysis demonstrated that the expression of DUXAP8 was positively associated with the SOS1 mRNA levels (Fig. 5A). In addition, in the TCGA-THCA dataset, the expression of DUXAP8 was strongly associated with the downstream target genes of the MAPK/ERK signaling, including c-Myc and CCND1 (Fig. 5B and C). Moreover, in our collected tissues, SOS1 was increased in PTC tumor

tissues compared with the adjacent normal tissues (Fig. 5D), and was positively correlated with DUXAP8 level in the PTC tumor tissues (Fig. 5E).

As revealed in Fig. 5F SOS1 could share the same binding sites with DUXAP8 on miR-20b-5p. Subsequently, the effect of DUXAP8 on the expression of SOS1 was evaluated. As anticipated, DUXAP8 knockdown downregulated the mRNA expression levels of SOS1 (Fig. 5G) in PTC cells.

DUXAP8 knockdown inhibits cell proliferation and induces cell apoptosis via regulating SOS1. To explore the effect of DUXAP8 and SOS1 on the progression of PTC, SOS1 was overexpressed in PTC cell lines. Therefore, transfection of CGTH W-3 and TPC1 cells with pcDNA3.1-SOS1, increased the mRNA and protein levels of SOS1 (Fig. 6A-C). In addition, si-DUXAP8 inhibited PTC cell proliferation (Fig. 6D and E) and induced apoptosis (Fig. 6F and G). These effects were reversed following transfection of CGTH W-3 and TPC1 cells with the SOS1 overexpression plasmid pcDNA3.1-SOS1.

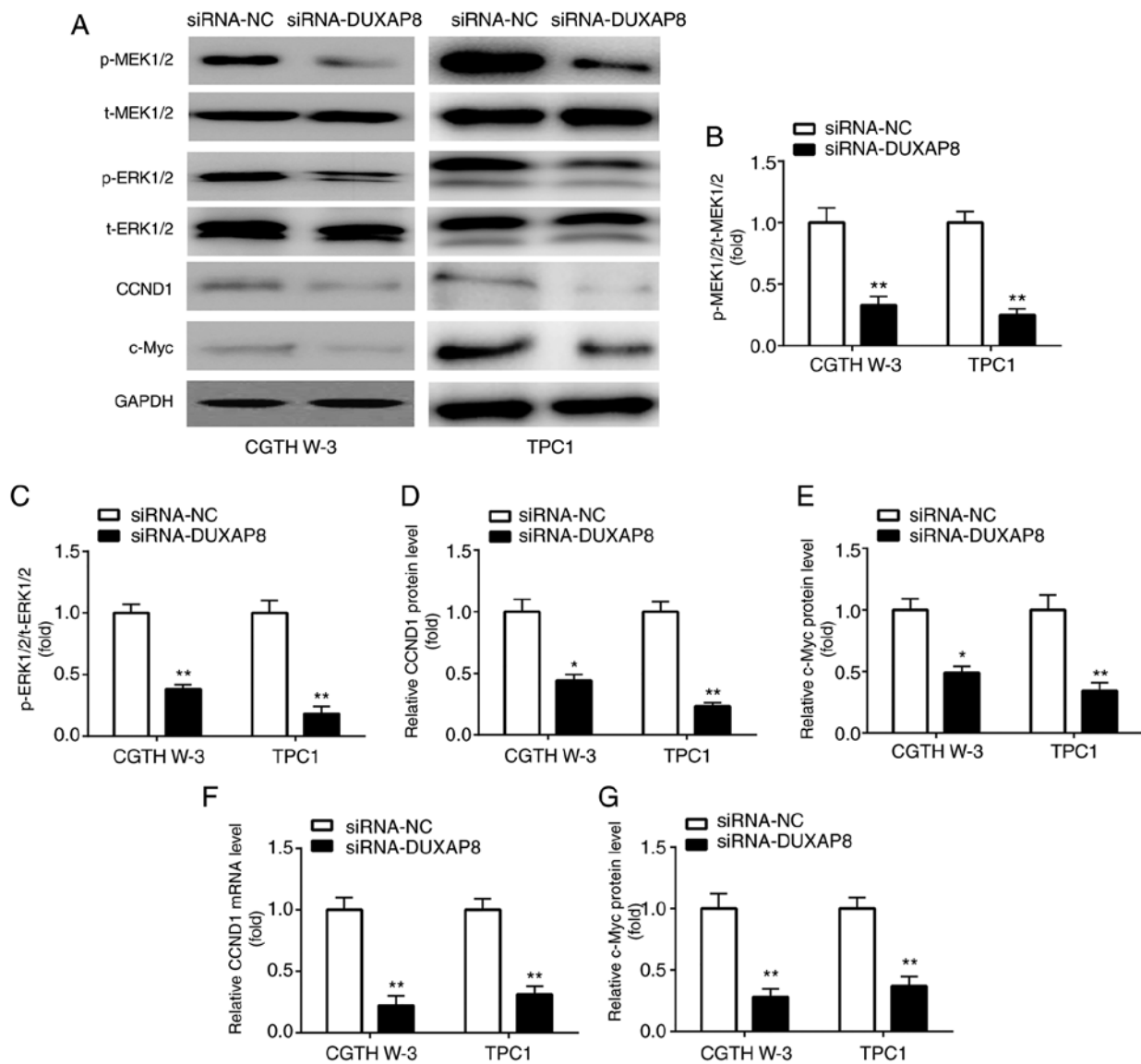


Figure 3. DUXAP8 knockdown inactivates the MAPK/ERK pathway. (A-E) Western blotting was applied for the detection of p-MEK1/2, p-ERK1/2, CCND1 and c-Myc level in PTC cells. (F and G) RT-qPCR was applied for the detection of mRNA expression of (F) CCND1 and (G) c-Myc. * $P < 0.05$ and ** $P < 0.01$, siRNA-DUXAP8 compared with siRNA-NC. DUXAP8, double homeobox A pseudogene 8; p-, phosphorylated; MEK1/2, mitogen-activated protein kinase 1/2; ERK1/2, extracellular signal-regulated kinase 1/2; cyclin D1, CCND1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; t-, total; siRNA, small interfering RNA; NC, negative control.

Discussion

The incidence of PTC is growing globally (27). Several non-coding RNAs, including lncRNAs and miRNAs, have been identified in recent years (28). It has been reported that lncRNA DUXAP8 promotes the growth of non-small cell lung cancer (11), pancreatic carcinoma (12), and renal cell carcinoma (13). However, the effect of lncRNA DUXAP8 on PTC remains elusive. In 2018, a genome-wide analysis revealed that lncRNA DUXAP8 was upregulated in human thyroid cancer (10). Consistently, the present study demonstrated that the expression of DUXAP8 was increased in the PTC TCGA-THCA data. This finding was further confirmed in tumor samples from patients with PTC. Furthermore, transfection of PTC cell lines with si-DUXAP8 inhibited cell proliferation and induced cell apoptosis. This finding was also consistent with previous studies on the role of DUXAP8 in

other types of cancer (11-13). The abovementioned results supported the promotional effects of DUXAP8 on the progression of PTC.

The MAPK/ERK signaling regulates cell viability and apoptosis via phosphorylating numerous substrates (29), and serves a crucial role during the tumorigenesis of multiple cancers, including PTC (30). ERK2 regulates several biological cell processes via regulating the expression of downstream substrates of the MAPK/ERK signaling, such as c-Myc and CCND1 (27). Consistently, in the present study, transfection of PTC cells with si-DUXAP8 decreased the levels of p-MEK1/2 and p-ERK1/2 and downregulated CCND1 and c-Myc expression, resulting in the inactivation of the MAPK/ERK pathway.

As acknowledged, miRNAs regulate various biological processes, including cell proliferation and apoptosis (19). However, the effect of the lncRNA DUXAP8-miRNA

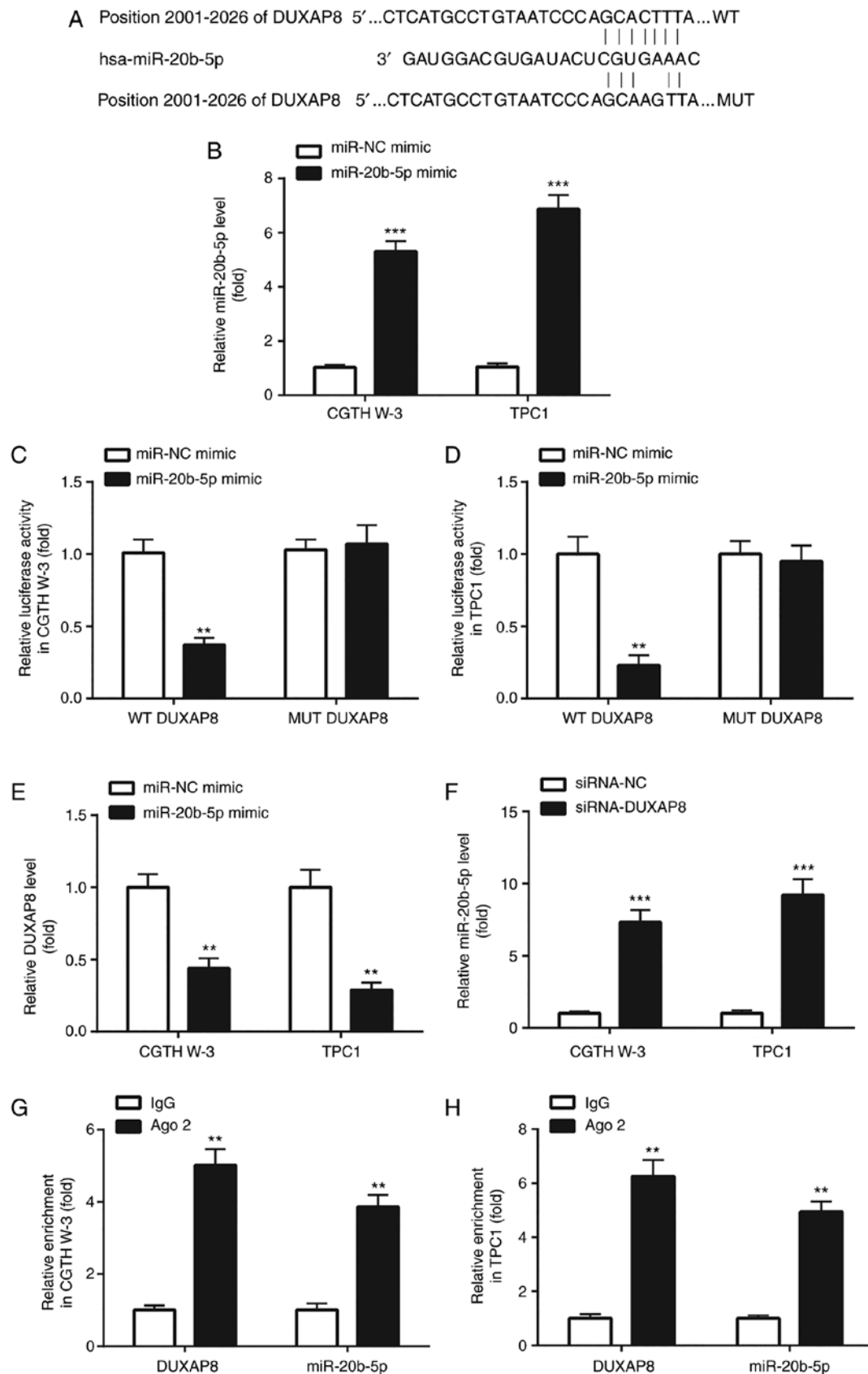


Figure 4. DUXAP8 negatively regulates miR-20b-5p. (A) The online database miRDB (<http://mirdb.org/>) was used to predict the complementary sites between DUXAP8 and miR-20b-5p. (B) RT-qPCR was applied for the detection of miR-20b-5p in PTC cells. (C and D) Dual luciferase reporter assay was used to detect the association between miR-20b-5p and DUXAP8 in PTC cells. (E and F) RT-qPCR was applied for the detection of DUXAP8 and miR-20b-5p in PTC cells. (G and H) RIP assay was used to further confirm the interaction between miR-20b-5p and DUXAP8 in PTC cells. ** $P < 0.01$, miR-20b-5p mimic compared with miR-NC mimic; siRNA-DUXAP8 compared with siRNA-NC; and Ago 2 compared with IgG. *** $P < 0.001$, miR-20b-5p mimic compared with miR-NC mimic; siRNA-DUXAP8 compared with siRNA-NC. DUXAP8, double homeobox A pseudogene 8; miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; PTC, papillary thyroid carcinoma; RIP, RNA immunoprecipitation; siRNA, small interfering RNA; NC, negative control; WT, wild-type; MUT, mutant.

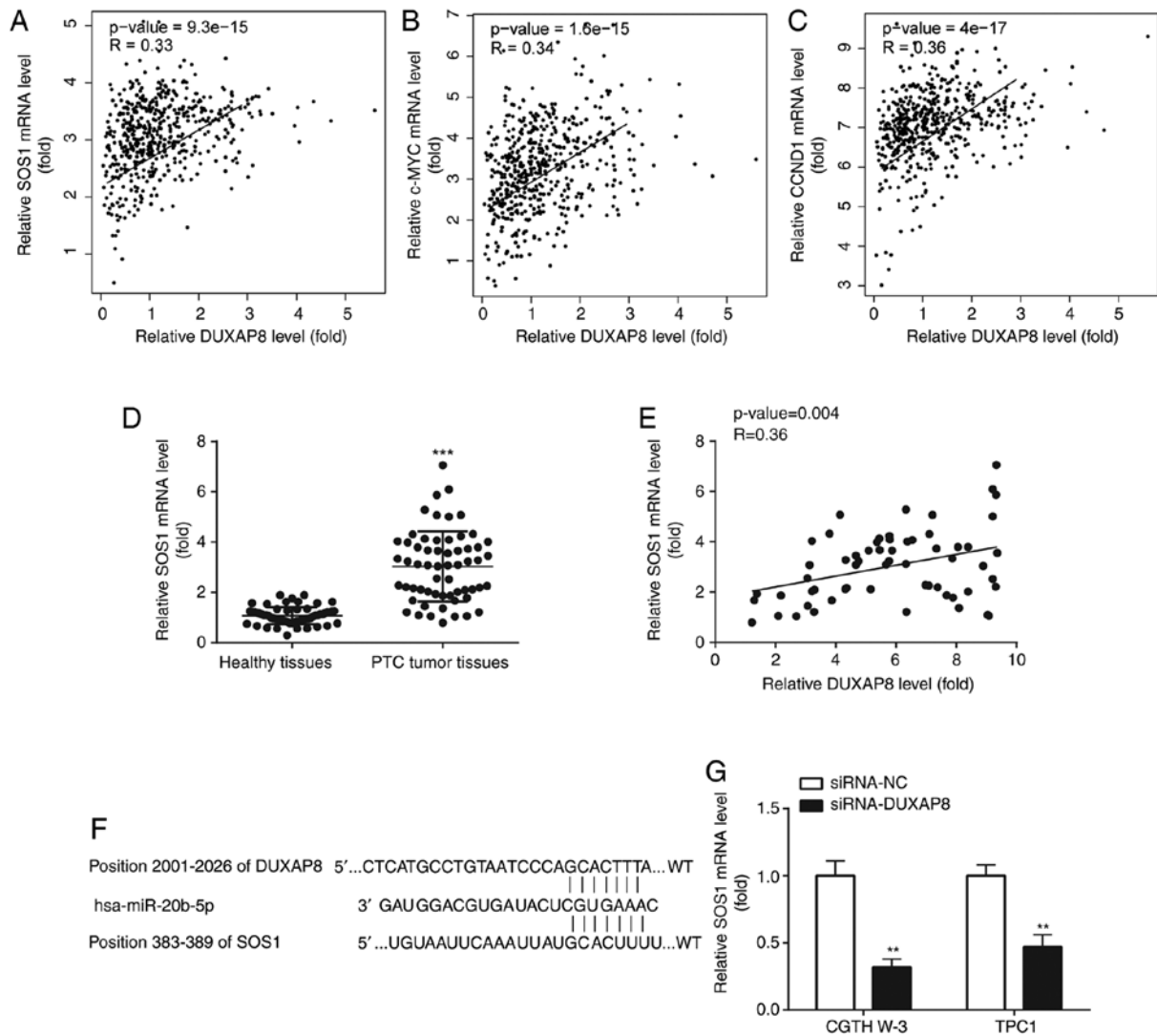


Figure 5. DUXAP8 is positively correlated with miR-20b-5p-related target genes. TCGA-THCA dataset was used to exhibit the association between DUXAP8 level and mRNA level of (A) SOS1, (B) c-Myc, and (C) CCND1. (D) RT-qPCR was applied for the examination of SOS1 mRNA level in PTC tumor tissues and the normal tissues. (E) Spearman's correlation analysis was used to analyze the correlation between SOS1 mRNA level and DUXAP8 level in the PTC tumor tissues. (F) The binding sites among DUXAP8, miR-20b-5p and SOS1 are presented. (G) RT-qPCR was applied for the detection of mRNA expression of SOS1. ** $P < 0.01$, siRNA-DUXAP8 compared with siRNA-NC. *** $P < 0.001$, PTC tumor tissues compared to healthy tissues. DUXAP8, double homeobox A pseudogene 8; miR, microRNA; TCGA, The Cancer Genome Atlas; THCA, thyroid carcinoma; SOS1, son of sevenless 1; cyclin D1, CCND1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; PTC, papillary thyroid carcinoma; siRNA, small interfering RNA; NC, negative control; WT, wild-type; MUT, mutant.

network in the pathogenesis of PTC has not been identified. Therefore, miRNAs, acting as tumor suppressors during the progression of PTC, attracted the attention of the present study. Bioinformatics analysis predicted that DUXAP8 was complementary to miR-20b-5p. Regarding the role of miR-20b-5p in PTC, a study demonstrated, using next-generation deep sequencing and microarray analysis, that the expression of miR-20b-5p was decreased in PTC compared with normal thyroid tissues (31). In addition, miR-20b acted as a tumor suppressor in PTC via regulating the MAPK/ERK signaling (25). Furthermore, in 2018, a miRNA profiling analysis revealed that the expression of miR-20b-5p was downregulated and was associated with the aggressive behavior of PTC (32). As acknowledged, a reciprocal suppression between miRNA and lncRNA has been revealed (26), for example, TUSC7 and miR-23b in gastric cancer (33), as well as lncRNA LOC728196 and

miR-513c in glioma (34), and TUG1 and miR-145 in bladder cancer (35). Herein, a reciprocal suppression between DUXAP8 and miR-20b-5p was revealed, i.e., DUXAP8 and miR-20b-5p suppressed the expression of each other, miR-20b-5p decreased the luciferase activity of DUXAP8, and RIP assays revealed that miR-20b-5p could directly target DUXAP8. Therefore, the present study is the first to the best of our knowledge, to identify the effect of the DUXAP8/miR-20b-5p network in PTC.

SOS1, a downstream target for miR-20b-5p in PTC (25), also exerts an oncogenic function in multiple types of cancer (36), such as ovarian cancer (37), glioblastoma (38) and colorectal cancer (39). However, the association between DUXAP8 and SOS1 in PTC has not been identified. Herein, DUXAP8 knockdown downregulated the expression of SOS1. Additionally, the Spearman's correlation analysis revealed that DUXAP8 was positively associated with SOS1, c-Myc and

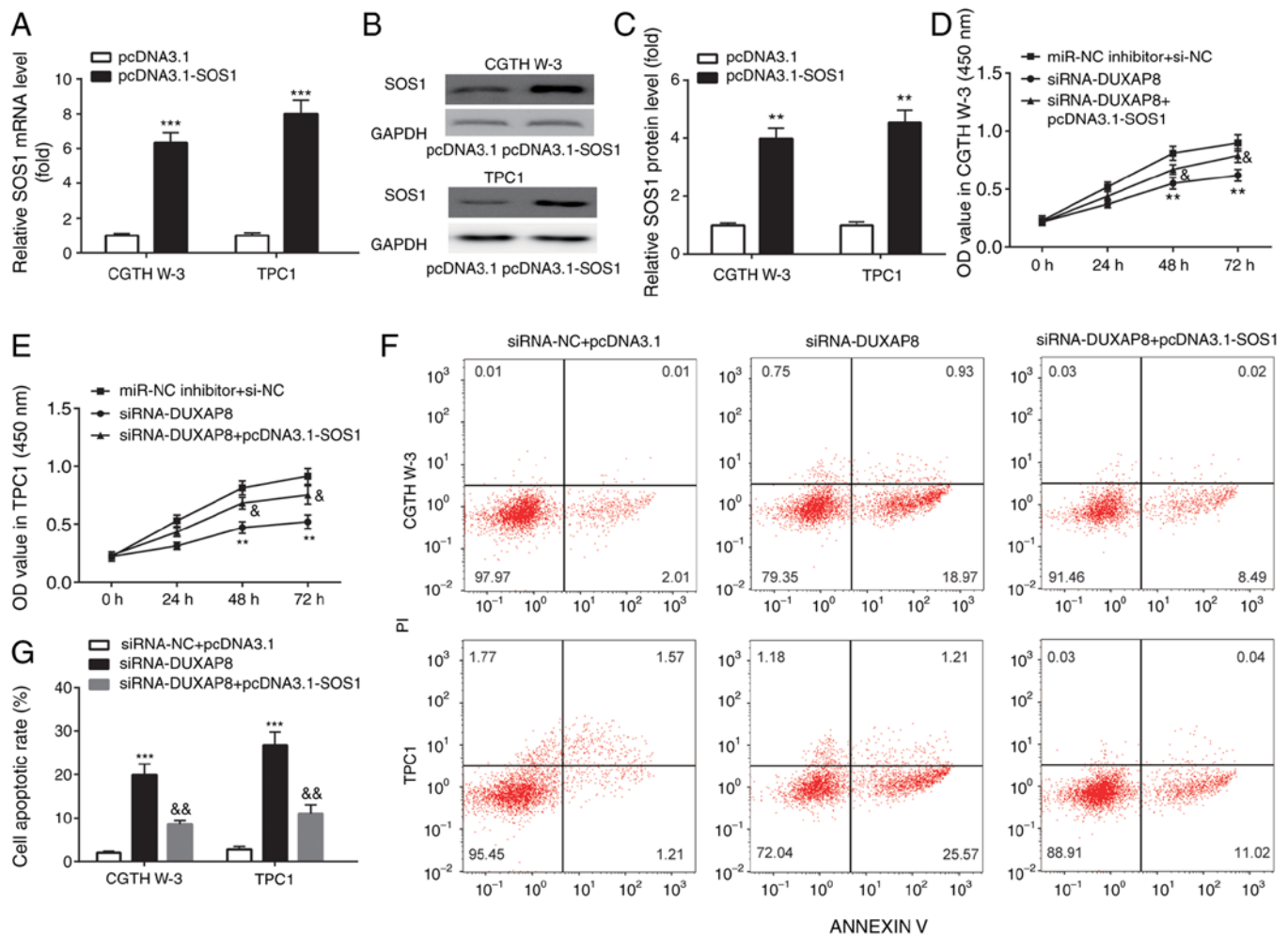


Figure 6. DUXAP8 knockdown inhibits cell proliferation and induces cell apoptosis by regulation of SOS1. (A-C) RT-qPCR and western blotting were applied for the examination of mRNA and protein expression of SOS1 in PTC cell lines. (D and E) CCK-8 assays were used to detect cell proliferation, and (F and G) flow cytometric assays were used to detect cell apoptosis in PTC cell lines. ** $P < 0.01$, pcDNA3.1-SOS1 compared with pcDNA3.1; siRNA-DUXAP8 compared with miR-NC inhibitor+si-NC. *** $P < 0.001$, pcDNA3.1-SOS1 compared with pcDNA3.1; siRNA-DUXAP8 compared with siRNA-NC+pcDNA3.1. & $P < 0.05$ and && $P < 0.01$, siRNA-DUXAP8+pcDNA3.1-SOS1 compared with siRNA-DUXAP8. DUXAP8, double homeobox A pseudogene 8; SOS1, son of sevenless 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; PTC, papillary thyroid carcinoma; CCK-8, Cell Counting Kit-8; miR, microRNA; siRNA, small interfering RNA; NC, negative control; PI, propidium iodide.

CCND1 in the TCGA-THCA data. In addition, DUXAP8 was positively correlated with SOS1 in our collected PTC tumor tissues. Finally, SOS1 overexpression rescued the DUXAP8 downregulation-mediated inhibition of cell proliferation and promotion of cell apoptosis in PTC cells. Therefore, these results were the first to reveal the positive association between DUXAP8 and SOS1 expression in PTC.

Collectively, the present study, to the best of our knowledge, was the first to identify DUXAP8 as a novel upregulated lncRNA in PTC and provided new insights in understanding the role of the DUXAP8/miR-20b-5p/SOS1 network in the progression of PTC.

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Availability of data and materials

The data and materials were available from the corresponding author on special request.

Authors' contributions

Both authors, RP and SY performed the experimentation and data analysis. SY designed the study and wrote the manuscript. Both authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental procedures were approved by the Ethics Committee of the First Hospital of Jilin University. Written informed consent was provided by all patients prior to enrollment in this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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