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ORIGINAL RESEARCH

Long noncoding RNA *XIST* promotes proliferation and invasion by targeting miR-141 in papillary thyroid carcinoma

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Background: The long noncoding RNA X-inactive specific transcript (*XIST*) was reported to play vital roles in tumor progression. In the present study, we determined the regulatory function of *XIST* in papillary thyroid carcinoma (PTC).

Materials and methods: *XIST* expression was determined in PTC tissues and cell lines by quantitative real-time polymerase chain reaction (PCR) (qRT-PCR). Cellular proliferation, migration, and invasion were measured using the Cell Counting Kit-8 (CCK-8) assay, wound-healing assay, and transwell invasion assay, respectively. Western blotting was used to determine protein expression. The downstream target miRNAs for *XIST* were identified by luciferase reporter assay and qRT-PCR.

Results: Relative expression of *XIST* was upregulated in PTC tissues and cell lines. High *XIST* expression was positively correlated with TNM stage and lymph node metastasis. Function assay demonstrated that knockdown of *XIST* significantly decreased cell proliferation, migration, and invasion in PTC cells. Moreover, we showed that the effects of *XIST* on PTC cell progression were mediated by miR-141.

Conclusion: Our results demonstrated that *XIST* functioned as an oncogene in PTC progression by regulating miR-141, suggesting that *XIST* might be a promising therapeutic target for PTC treatment.

Keywords: papillary thyroid carcinoma, *XIST*, miR-141, proliferation

Introduction

Thyroid cancer is the most common malignancy of the endocrine system, and it has shown a steadily increasing incidence over the past 40 years.[1] Papillary thyroid carcinoma (PTC) is the most common histotype of thyroid cancer, accounting for 85%–90% of all thyroid cancers.[1,2] Most patients with PTC can be effectively treated by surgical removal, followed by adjuvant radioactive iodine (RAI) therapy. However, 10%–15% of patients with PTC do not respond to RAI therapy or they progress to metastatic disease, which has poor prognosis.[3] Therefore, it is urgent to identify potential biomarkers and therapeutic targets that correlate with tumorigenesis and progression in PTC.

Long noncoding RNAs (lncRNAs) constitute a newly identified class of RNAs, whose transcripts are >200 nucleotides in length without protein-encoding capabilities.[4] As a new class of modulators, lncRNAs have been shown to be involved in regulating various biological processes and diseases in humans.[5] Increasing evidence has suggested that lncRNAs play vital roles in progression of cancers, including PTC.[6] A number of lncRNAs have been identified as oncogenes or tumor suppressors in

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PTC,[7,8] suggesting that lncRNAs could serve as diagnostic markers and therapeutic agents for PTC.

Recently, researchers have paid more attention to the lncRNA X-inactive specific transcript (*XIST*). XIST, a product of the *XIST* gene, has been reported to participate in the differentiation, proliferation, and genome maintenance of human cells.[9] *XIST* has been found to be upregulated and to function as an oncogene in multiple cancers, such as breast cancer,[10] non-small-cell lung cancer,[11] pancreatic cancer,[12] bladder cancer,[13] hepatocellular carcinoma,[14] gastric cancer,[15] and colorectal cancer.[16] However, the expression levels and exact role of *XIST* in PTC remain unclear. In this study, we investigated the expression level, biological function, and the underlying mechanisms of *XIST* action in PTC using a series of experiments.

Materials and methods

Clinical specimens and ethics

A total of 36 patients with PTC who had undergone thyroidectomy and node dissection without chemotherapy or radiotherapy at the China–Japan Union Hospital of Jilin University (Changchun, China) were enrolled in this study. After surgery, PTC samples and paired adjacent noncancerous samples were harvested and confirmed by two independent pathologists. All specimens were immediately preserved in liquid nitrogen until RNA extraction. Before the tissue samples were collected, written informed consent was obtained from all patients. This study was approved by the ethics review board of the China–Japan Union Hospital of Jilin University.

Cell lines and transfection

Human thyroid follicular epithelial cell line Nthy-ori 3-1 and human thyroid cancer cell lines (TPC-1, HTH83, 8505C, and SW1736) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/mL penicillin (Sigma-Aldrich, St Louis, MO, USA), and 100 µg/mL streptomycin (Sigma-Aldrich) at 37°C in a humidified incubator with 5% CO_2.

miR-141 mimic (miR-141), mimic negative control (miR-NC), miR-141 inhibitor (anti-miR-141), inhibitor scrambled control (anti-miR-NC), siRNA specifically targeting *XIST* (si-*XIST*), and scrambled negative control siRNA (si-NC) were chemically synthesized by GenePharma Co, Ltd (Shanghai, China). TPC-1 cells in logarithmic phase were transfected with these molecular products using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Further experiments were performed at 24 h posttransfection.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were isolated from tissues or cultured cells using Trizol reagent (Invitrogen) and subsequently reverse-transcribed into cDNAs using the PrimeScript RT reagent Kit (Promega, Madison, WI, USA) according to the protocol of the manufacturer. Relative gene expression levels of miR-141 and *XIST* were measured using TaqMan miRNA assay (Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Kit (Takara Biochemicals, Kyoto, Japan) under the ABI 7900 Fast Real-Time PCR system (Applied Biosystems), respectively. U6 snRNA and *GAPDH* were used as the internal control for miR-141 and *XIST*, respectively. The relative quantification of *XIST* and miR-141 expression levels was achieved by the $2^{-\Delta\Delta Ct}$ method. The primers for qRT-PCR were as follows: *XIST* – forward, 5′-CTC TCC ATT GGG TTC AC-3′, reverse, 5′-GCG GCA GGT CTT AAG AGA TGA G-3′; GAPDH – forward, 5′-CAC CCACTCCTCCACCTTTG-3′, reverse, 5′-CCACCACCC TGTTGCTGTAG-3′; miR-141 – forward, 5′-AGACCTCA CCTGGCCTGTGGCC-3′, reverse 5′-GAACCCACCC GGGAGCCATCTT-3′; U6 – forward, 5′-CTC GCT TCG GCAGCA CA-3′, reverse, 5′-AAC GCT TCA CGA ATT TGC GT-3′.

Cell proliferation, cell cycle, migration, and invasion assays

Cell proliferation was determined using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. Briefly, logarithmically growing cells (5×10^3 cells per well) were inoculated into 96-well culture plates. At the indicated time points (24 h, 48 h, and 72 h), a volume of 10 µL of CCK-8 solution was added into each well and cultured for 4 h at 37°C. The absorbance at 450 nm was measured using enzyme immunoassay analyzer (Thermo Fisher Scientific, Inc, Waltham, MA, USA).

Flow cytometric analysis was conducted to analyze the cell cycle. Forty-eight hours after transfection, the cells were collected and fixed in 70% ethanol overnight. Then, cells were incubated with RNase (50 µg/mL) and propidium iodide (PI) (50 µg/mL, Sigma) for 30 min in the dark.

Cell cycle analysis was conducted using a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA, USA).

Cell migration capacity was determined by the scratch assay. In brief, 2×10^5 transfected cells were plated into six-well plates and grown to reach 100% confluence. The cells were then wounded by scraping with a 100 µL tip, followed by three washes in PBS and incubation in serum-free medium. Wounds were observed at 0 h and 24 h with a light microscope (IX71; Olympus, Tokyo, Japan) at 200× magnification. The cell migration distances were calculated by subtracting the wound width at each time point from the wound width at the 0 h time point.

Cell invasion was measured using transwell chambers (8 µm pore size; EMD Millipore, Billerica, MA, USA). The transfected cells (5×10^4) suspended in 150 µL serum-free medium were plated into the upper chamber coated with Matrigel (BD, Franklin Lakes, NJ, USA), whereas the lower chamber was filled with culture medium containing 20% (v/v) FBS as a chemoattractant. After incubation for 48 h, the noninvading cells were scraped off with a cotton swab, and the invaded cells on the lower chamber were fixed with 4% formaldehyde for 10 min and stained with 0.1% crystal violet for 5 min. The cells were counted in five independent fields using an inverted fluorescence microscope (Olympus Corp) at 200× magnification.

Dual luciferase reporter assay

XIST was predicted to be a directly regulated target of miR-141 by Starbase2.0 bioinformatics tools (http://starbase.sysu.edu.cn). *XIST* 3′-untranslated region (UTR) fragment was amplified and cloned into psiCHECK-2 vectors, and the product was named as WT-*XIST*. The *XIST*-mutant construct was obtained using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), named as MUT-*XIST*. For reporter assays, TPC-1 cells were co-transfected with 100 nM miR-141 mimic or miR-NC mimic (GenePharma, Suzhou, China) and 100 ng of WT-*XIST* or MUT-*XIST* reporter plasmid using Lipofectamine 2000. After 48 h transfection, luciferase activity was measured using the Dual Luciferase Reporter Assay Kit (Promega).

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software (SPSS Inc, Chicago, IL, USA). All data are represented as mean ± SD. Significant differences in the continuous data between groups were compared using two-tailed Student's *t*-test and one-way analysis of variance (ANOVA). The relationship between *XIST* and miR-141 expressions was tested using Pearson's correlation assay. A value of $P<0.05$ was considered statistically significant.

Results

XIST expression is upregulated in PTC tissues and cell lines

To determine the role of *XIST* in PTC progression, we first determined its expression levels in 36 pairs of PTC tissues and adjacent normal tissues by qRT-PCR. We found that *XIST* expression was upregulated in PTC tissues compared with the expression in adjacent normal tissues (Figure 1A). In addition, we verified the expression of *XIST* in four PTC cell lines, namely, TPC-1, HTH83, 8505C, and SW1736, and in human thyroid follicular epithelial cell line Nthy-ori 3-1.

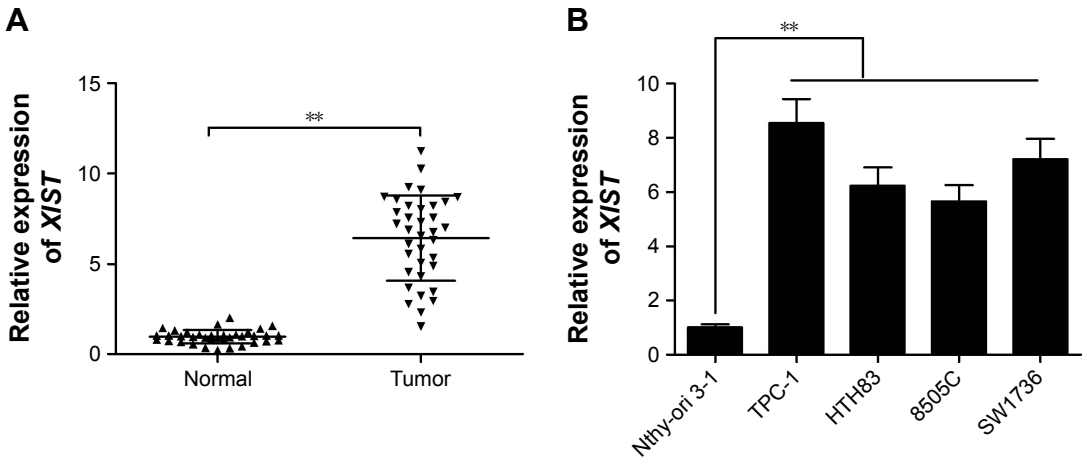

Figure 1 *XIST* was upregulated in PTC tissues and cell lines.
Notes: (**A**) qRT-PCR was performed to examine the expression levels of *XIST* in 36 PTC tissues and adjacent normal tissues. (**B**) qRT-PCR was applied to estimate the expression levels of *XIST* in four human thyroid cancer cell lines (TPC-1, HTH83, 8505C, and SW1736) and a thyroid follicular epithelial cell line Nthy-ori 3-1. **$P<0.01$. Data are expressed as the mean ± SD of at least three independent experiments.
Abbreviations: *XIST*, X-inactive specific transcript; PTC, papillary thyroid carcinoma; qRT-PCR, quantitative real-time polymerase chain reaction.

Table 1 Correlation between clinicopathological features and *XIST* expression in 36 patients with PTC

Variables	No. of cases	*XIST* expression		*P*-value
		High, n (%)	Low, n (%)	
Age, years				$P>0.05$
<60	15	9 (60.0)	6 (40.0)	–
≥60	21	11 (52.3)	10 (47.7)	–
Gender				$P>0.05$
Male	13	7 (53.8)	6 (46.2)	–
Female	23	13 (56.5)	10 (42.5)	–
TNM stage				$P<0.01$
I–II	28	12 (42.9)	16 (57.1)	–
III–IV	8	8 (100)	0 (0)	–
Tumor size				$P>0.05$
<5 cm	26	14 (53.8)	12 (46.2)	–
≥5 cm	10	6 (60.0)	4 (40.0)	–
Lymph node metastasis				$P<0.01$
No	27	12 (44.4)	15 (55.6)	–
Yes	9	8 (88.9)	1 (10.1)	–

Abbreviations: PTC, papillary thyroid carcinoma; *XIST*, X-inactive specific transcript.

XIST was clearly elevated in the four PTC cell lines compared to Nthy-ori 3-1 (Figure 1B). We also investigated the potential associations between *XIST* expression and the clinicopathological features of patients with PTC (Table 1). High *XIST* expression was significantly positively correlated with a late TNM stage and lymph node metastasis (Table 1). These results revealed that *XIST* has potential oncogenicity in PTC.

Knockdown of *XIST* inhibits cell proliferation of PTC cells

To test the biological function of *XIST* in PTC, we knocked down the expression of the lncRNA *XIST* using a specific siRNA (si-*XIST*), while using a nonsilencing siRNA (si-NC) as the negative control. We found that transfection of si-*XIST* significantly decreased *XIST* expression in TPC-1 cells compared with cells transfected with si-NC (Figure 2A). CCK-8 assay revealed that knockdown of *XIST* significantly decreased cell proliferation in TPC-1 cells (Figure 2B). Moreover, flow cytometry was utilized to test the effect of *XIST* on the cell cycle. The results showed that knockdown of *XIST* in TPC-1 cells induced a significant increase in the percentage of cells in the G1/G0 phase and a decrease in the percentage of cells in the S phase (Figure 2C). These results suggested that downregulation of *XIST* suppressed PTC cell proliferation in vitro.

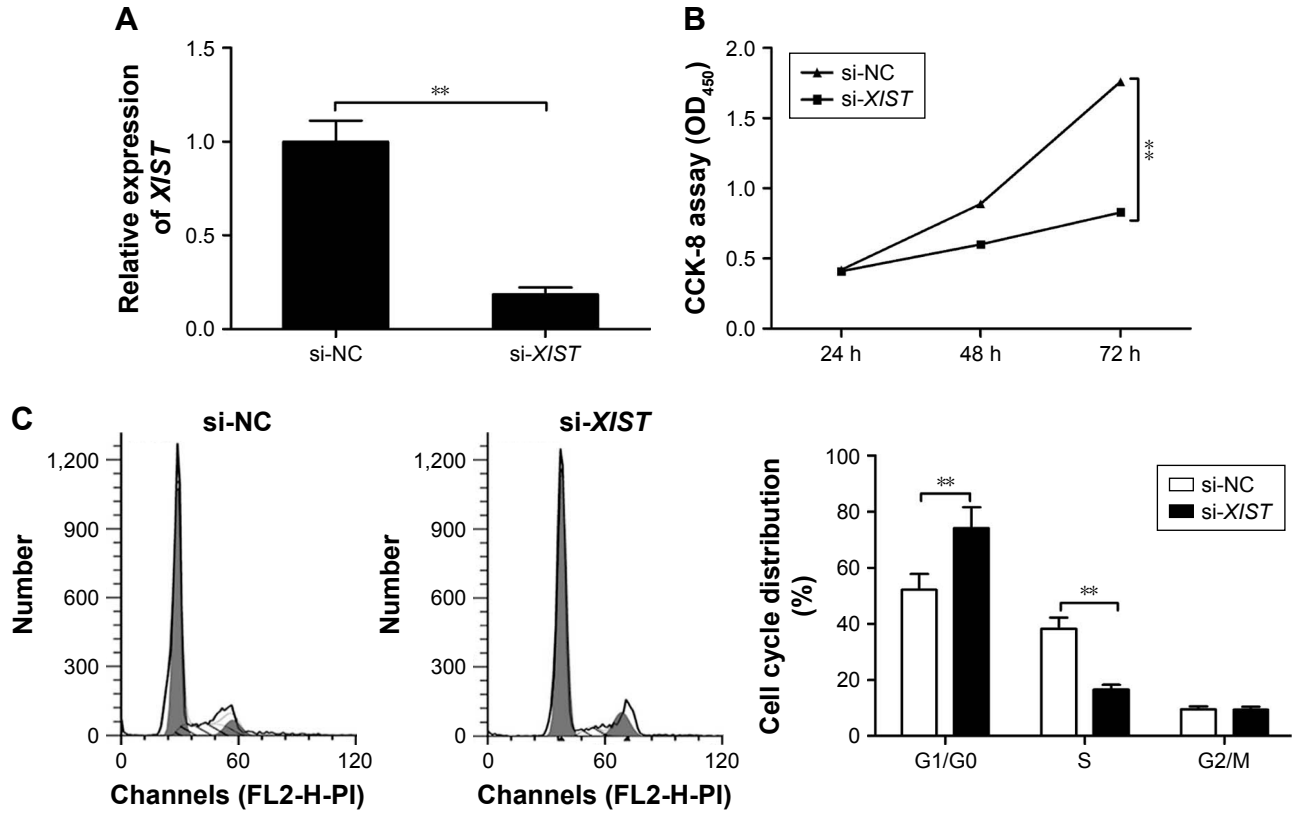

Figure 2 Knockdown of *XIST* inhibits cell proliferation of PTC cells.

Notes: (**A**) qRT-PCR was performed to examine the expression levels of *XIST* in TPC-1 cells transfected with si-*XIST* or si-NC. (**B**) CCK-8 assay was performed to examine cell proliferation in TPC-1 cells transfected with si-*XIST* or si-NC. (**C**) Flow cytometric analysis was conducted to analyze the cell cycle arrest in TPC-1 cells transfected with si-*XIST* or si-NC. $^{**}P<0.01$. Data are expressed as the mean ± SD of at least three independent experiments.

Abbreviations: *XIST*, X-inactive specific transcript; PTC, papillary thyroid carcinoma; qRT-PCR, quantitative real-time polymerase chain reaction; NC, negative control; CCK, Cell Counting Kit; OD, optical density.

Knockdown of *XIST* inhibits cell migration and invasion of PTC cells

Furthermore, we explored whether *XIST* was involved in cell migration and invasion in TPC-1 cells. Scratch assay showed that downregulation of *XIST* remarkably decreased migration ability in TPC-1 cells (Figure 3A). The transwell assay showed that knockdown of *XIST* in TPC-1 cells resulted in much weaker abilities to invade through Matrigel than for cells transfected with si-NC (Figure 3B). These results suggested that downregulation of *XIST* suppressed cell migration and invasion of PTC cells in vitro.

miR-141 expression is directly regulated by *XIST*

It has been elucidated that lncRNA can competitively bind to miRNAs and function as a competing endogenous RNA (ceRNA), thus participating in the pathogenesis and development of cancers.[17] To better reveal the underlying molecular mechanism by which lncRNA *XIST* exerts its biological function in PTC progression, we used the online bioinformatics tool Starbase2.0 (http://starbase.sysu.edu.cn) to predict the downstream target of *XIST*. miR-141 was predicted to be a target of *XIST* with a potential binding site (Figure 4A). To verify that *XIST* can bind to miR-141 directly, the luciferase reporter assay was performed. Our result demonstrated that luciferase activity was remarkably decreased in TPC-1 cells co-transfected with WT-*XIST* and miR-141 mimics (Figure 4B; $P<0.05$). Moreover, our results also showed that knockdown of *XIST* clearly increased miR-141 expression level in TPC-1 cells (Figure 4C), whereas transfection of miR-141 mimic significantly inhibited the expression of *XIST* in TPC-1 cells, and transfection with miR-141 inhibitor (anti-miR-141) increased *XIST* expression in TPC-1 cells (Figure 4D). We also found that miR-141 expression was downregulated in PTC tissues (Figure 4E) and was inversely correlated with *XIST* expression (Figure 4F). These results suggested that *XIST* directly binds to miR-141 in PTC cells.

miR-141 inhibitor reversed the inhibitory effect of *XIST* knockdown on PTC cell proliferation, migration, and invasion

To determine whether *XIST* exerts its biological functions through miR-141, we performed rescue experiments by inhibiting miR-141 expression using miR-141 inhibitor in *XIST*-knockdown cells (Figure 5A). CCK-8 assay showed that cell proliferation was decreased in *XIST*-knockdown TPC-1 cells, whereas miR-141 inhibitor partially reversed the reduction of proliferation (Figure 5B; $P<0.05$). Furthermore, our result also revealed that miR-141 inhibitor reversed the cell cycle arrest, migration, and invasion in TPC-1 cells induced by *XIST* knockdown (Figure 5C–E).

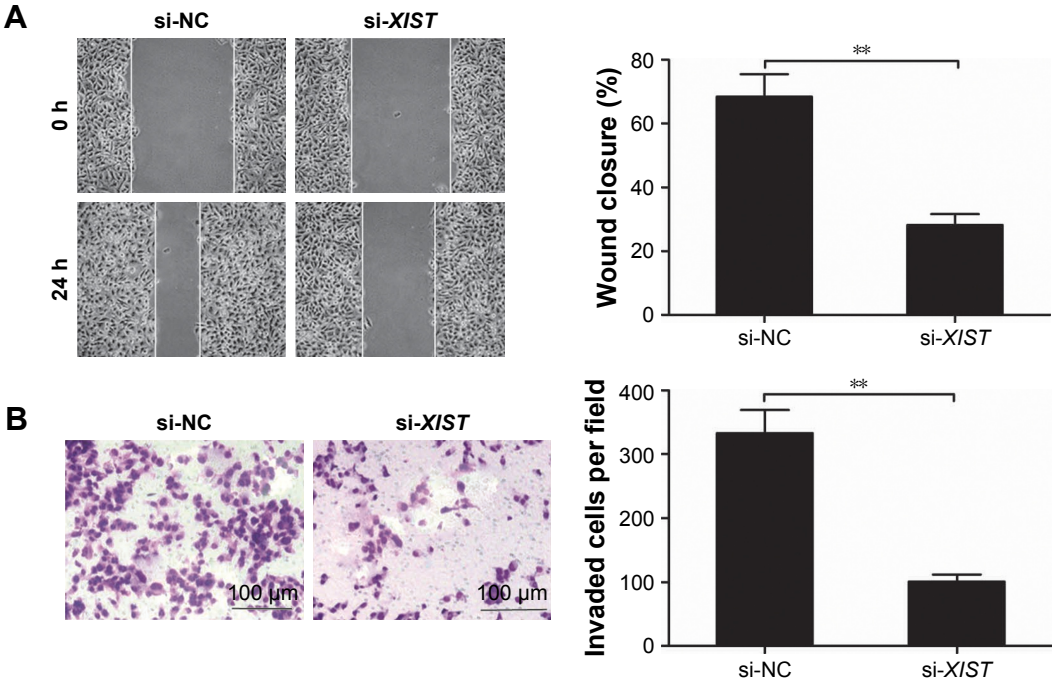

Figure 3 Knockdown of *XIST* inhibits cell migration and invasion of PTC cells.
Notes: (**A**) Wound-healing assay to examine cell migration in TPC-1 cells transfected with si-*XIST* or si-NC. (**B**) Transwell invasion assay was performed to examine cell invasion in TPC-1 cells transfected with si-*XIST* or si-NC. **$P<0.01$. Data are expressed as the mean ± SD of at least three independent experiments.
Abbreviations: *XIST*, X-inactive specific transcript; PTC, papillary thyroid carcinoma; NC, negative control.

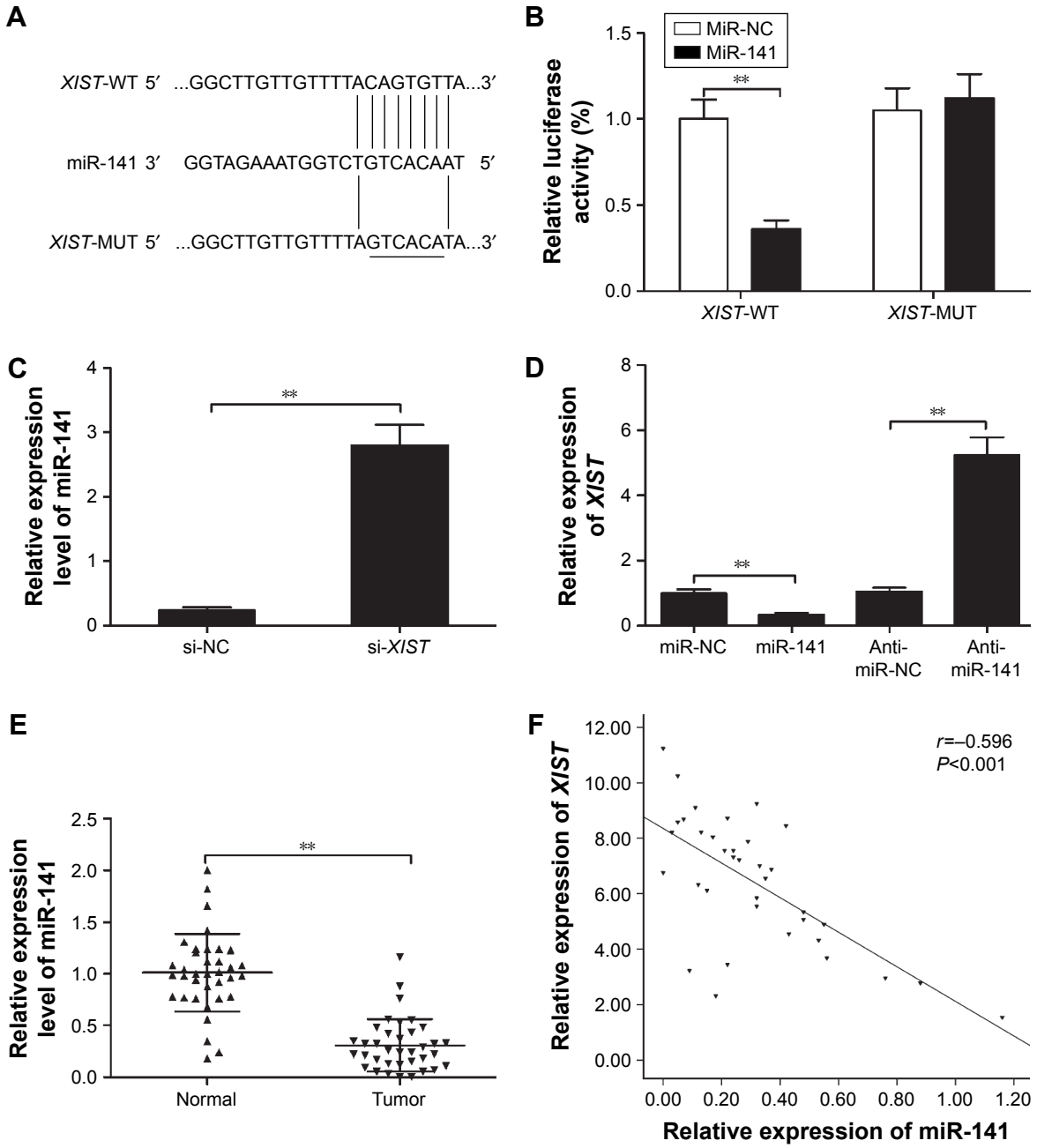

Figure 4 miR-141 expression is directly regulated by *XIST*.

Notes: (**A**) Bioinformatics analysis showed that miR-141 could directly target the 3′-UTR of the *XIST* wild-type (WT-*XIST*) sequence. *XIST*-mutant (MUT-*XIST*) indicates the mutation of the binding sites in the 3′-UTR of *XIST*. (**B**) Luciferase activity was determined in TPC-1 cells co-transfected with the miR-141 mimic or miR-NC and WT-*XIST* or MUT-*XIST* reporter plasmid. (**C**) qRT-PCR was performed to examine the expression levels of miR-141 in TPC-1 cells transfected with si-*XIST* or si-NC. (**D**) qRT-PCR was performed to examine the expression levels of *XIST* in TPC-1 cells transfected with miR-141 mimic, miR-NC, miR-141 inhibitor (anti-miR-141), and anti-miR-NC. (**E**) qRT-PCR was performed to examine the expression levels of miR-141 in 36 PTC tissues and adjacent normal tissues. (**F**) The negative association between *XIST* and miR-141 expression levels was analyzed in PTC tissues (n=36). $^{**}P<0.01$. Data are expressed as the mean ± SD of at least three independent experiments.

Abbreviations: *XIST*, X-inactive specific transcript; UTR, untranslated region; NC, negative control; qRT-PCR, quantitative real-time polymerase chain reaction.

Discussion

Accumulating evidence has revealed that lncRNAs play crucial critical roles in regulating many physiological and pathological processes of various cancers, including PTC.[6–8] For example, Yuan et al reported that lncRNA HOTTIP knockdown downregulated Akt1 expression and suppressed cell proliferation, invasion, and migration in PTC cells by regulating miR-637.[18] Chen et al demonstrated that lncRNA CNALPTC1 promotes PTC progression via sponging miR-30 family.[19] Wang et al showed that lncRNA H19 inhibited cell viability, migration, and invasion via downregulation of insulin receptor substrate (IRS)-1 in thyroid cancer cells.[20]

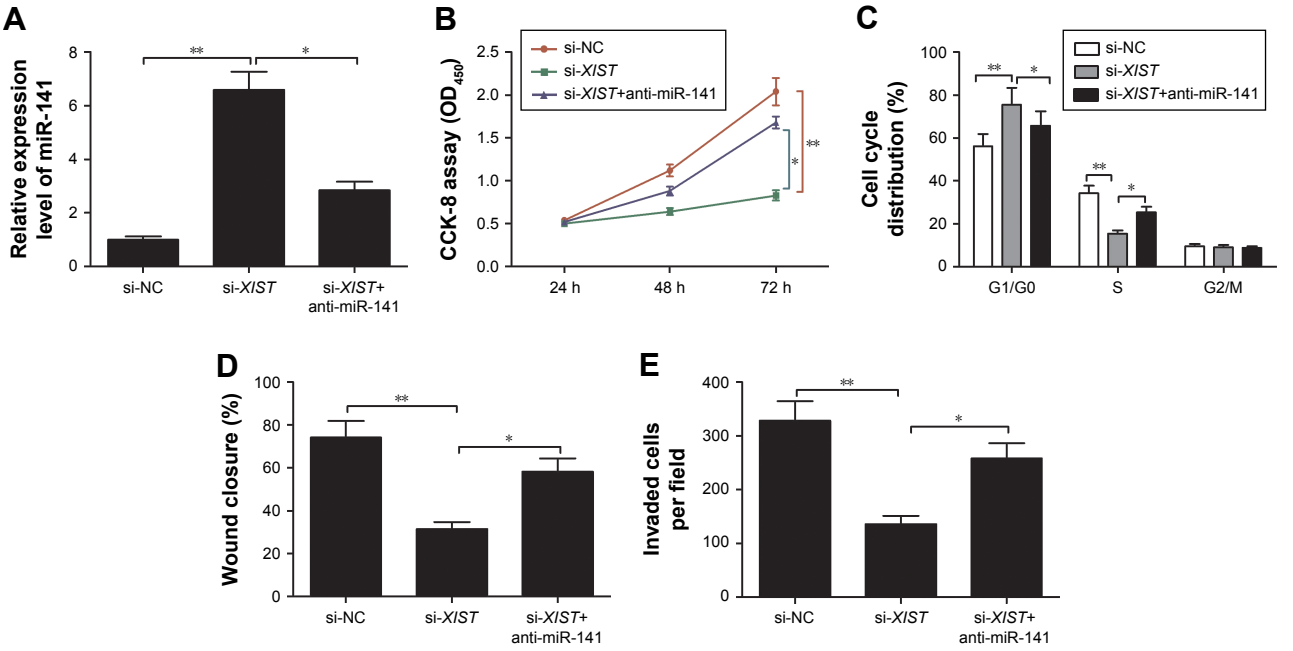

Figure 5 miR-141 inhibitor reverses the inhibitory effect of *XIST* knockdown on PTC cell proliferation, cycle, migration, and invasion.
Notes: (**A**) qRT-PCR assay showed that *XIST* knockdown increased miR-141 expression, whereas miR-141 inhibition reversed it in the meantime. (**B**) CCK-8 assay showed that *XIST* knockdown inhibited TPC-1 cell proliferation, whereas miR-141 inhibition reversed it. (**C–E**) *XIST* knockdown increased cell cycle arrest at G1/G0 stage and decreased cell migration and invasion, whereas miR-141 inhibition in the meantime reversed these trends. *$P<0.05$, **$P<0.01$. Data are expressed as the mean ± SD of at least three independent experiments.
Abbreviations: *XIST*, X-inactive specific transcript; PTC, papillary thyroid carcinoma; qRT-PCR, quantitative real-time polymerase chain reaction; CCK, Cell Counting Kit; NC, negative control.

Zhang et al found that lncRNA NEAT1 regulates PTC progression by modulating miR-129-5p/KLK7 expression.[21] Here, we found that *XIST* expression is upregulated in PTC tissues and cell lines, and its expression was associated with advanced clinical stage and lymph node metastasis. Knockdown of *XIST* inhibited cell proliferation, migration, and invasion of PTC cells. Mechanistically, we demonstrated that *XIST* acted as a ceRNA to sponge miR-141 in PTC. These findings suggested that *XIST* might act as a novel target for PTC treatment.

Recently, the ceRNA hypothesis showed that lncRNAs implicated in the pathogenesis and development of cancers act as miRNA sponges to modulate the expression of miRNA target genes.[17] To explore how *XIST* regulates PTC development and progression, we proposed to search the target microRNAs of *XIST* with an online tool (Starbase2.0). There are potential binding sites of miR-141 in *XIST;* thus, miR-141 was predicted as a potential target of *XIST*. Moreover, miR-141 was identified to act as a downstream target of *XIST* in non-small-cell lung cancer.[22] Luciferase reporter assays demonstrated that luciferase activity was remarkably decreased in TPC-1 cells co-transfected with *XIST*-WT and miR-141 mimic, suggesting that *XIST* can bind to miR-141. Moreover, we also found that knockdown of *XIST* clearly increased miR-141 expression level in TPC-1 cells, whereas transfection of miR-141 mimic significantly inhibited the expression of *XIST* in TPC-1 cells. These results suggested that *XIST* directly binds to miR-141 in PTC cells.

miR-141, a member of the miR-200 family, has attracted much attention because it is aberrantly downregulated and acts as an important tumor suppressor in multiple types of cancer, such as nasopharyngeal carcinoma,[23] prostate cancer,[24] breast cancer,[25] non-small-cell lung cancer,[26] colorectal cancer,[27] and hepatocellular carcinoma.[28] Interestingly, miR-141 was reported to be significantly downregulated in PTC patients,[29] and overexpression of miR-141 could inhibit cell proliferation, migration, and invasion in PTC cells by targeting IRS-2.[30] The present study convincingly demonstrated that miR-141 expression was downregulated in PTC tissues compared to adjacent normal tissues. We also found that miR-141 expression was inversely correlated with *XIST* expression in PTC tissues. In addition, rescue experiments revealed that miR-141 inhibitor could reverse the cell proliferation, migration, and invasion ability of *XIST*-knockdown TPC-1 cells. These studies implied that *XIST* exerted its oncogene role in PTC via sponging miR-141.

Some limitations were present in this study. First, the biological function of *XIST* was evaluated in a loss-of-function model in PTC; gain-of-function studies via upregulation of *XIST* in PTC are needed to further confirm our findings.

Second, to investigate the clinical significance of *XIST*, large enough samples are needed. Third, two or more PTC cell lines should be adopted to test the biological function of *XIST* in PTC.

Conclusion

We found that *XIST* expression level was upregulated in PTC tissues and cell lines. Knockdown of lncRNA *XIST* inhibits PTC cell proliferation, migration, and invasion through regulation of miR-141. Our findings elucidated the potential mechanism underlying the oncogenic role of *XIST* in PTC and suggested that *XIST* might be a potential target for PTC.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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