

lncRNA TPTEP1 competitively sponges miR-328-5p to inhibit the proliferation of non-small cell lung cancer cells

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Abstract. Accumulating evidence suggests that lncRNAs are involved in almost all normal physiological processes and that aberrant expression of lncRNAs may be involved in the development of diseases, including non-small cell lung cancer (NSCLC). However, the roles of lncRNA-TPTE pseudogene 1 (TPTEP1) in lung cancer and the underlying molecular mechanisms have remained elusive. In the present study, significant downregulation of TPTEP1 in tumors compared with normal tissues from patients with NSCLC was observed. Overexpression of TPTEP1 inhibited cell proliferation and induced apoptosis in NSCLC cells. A bioinformatics analysis based on miRDB predicted microRNA (miR)-328-5p as a potential binding miRNA for TPTEP1. Using a dual-luciferase reporter assay and western blot analysis, it was further validated that TPTEP1 sponged miR-328-5p to upregulate Src kinase signaling inhibitor 1 (SRCIN1) in NSCLC cells. Through regulation of SRCIN1, TPTEP1 was indicated to inactivate the Src and STAT3 pathways in NSCLC cells. Notably, silencing of SRCIN1 reversed the TPTEP1 overexpression-induced inhibition of cell proliferation and increase of the apoptotic rate in NSCLC cells. Pearson correlation analysis revealed a

significant positive correlation between TPTEP1 and SRCIN1 mRNA levels in NSCLC tumors. The present results provided insight into the roles of TPTEP1 in NSCLC and the underlying mechanisms.

Introduction

Lung cancer remains the leading cause of cancer-associated mortality for males and females worldwide. According to statistics, >2 million new cases were diagnosed in 2018 and ~1.7 million patients succumbed to lung cancer (1). According to the pathological features, lung cancer may be categorized into two major types: Non-small cell lung cancer (NSCLC) and SCLC (2,3). Compared with SCLC, NSCLC is a common lung cancer subtype, accounting for >80% of diagnosed cases (4). NSCLC is insensitive to chemotherapy and the prognosis of patients with NSCLC is unsatisfactory with a five-year overall survival rate of <50% (5,6). Further investigation of the molecular mechanisms of NSCLC is imperative to provide valuable targets for the development of novel therapeutic approaches for patients with NSCLC (7).

Non-coding RNAs are single-stranded RNAs with no protein-coding function (8). Long non-coding RNAs (lncRNAs) are non-coding RNAs of >200 nucleotides in length (9). Previously considered as 'junk' RNAs, recent studies revealed that dysregulation of lncRNAs is relevant to human diseases, including tumorigenesis (10,11). Mechanistically, lncRNAs may interact with micro (mi)RNAs, mRNAs or proteins to regulate gene expression and protein localization (12,13). The competing endogenous (ce)RNA hypothesis suggests that the expression of lncRNAs controls the mRNAs levels via sponging miRNAs (14). Several lncRNAs have been reported to be oncogenes or tumor suppressors in NSCLC (15-17). For instance, Yang *et al* (18) indicated that lncRNA insulin-like growth factor binding protein 4-1 was significantly upregulated in lung cancer and promoted tumor cell metabolism to facilitate cancer cell proliferation. lncRNA-HIT interacted

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with E2F transcription factor 1 to regulate target gene expression and promoted cell proliferation of NSCLC cells (19). lncRNA TPTE pseudogene 1 (TPTEP1) was identified as one of most significantly downregulated lncRNAs in NSCLC via a bioinformatics analysis of The Cancer Genome Atlas (TCGA) dataset (20). However, the roles of TPTEP1 in NSCLC have remained elusive.

Src kinase signaling inhibitor 1 (SRCIN1), also known as p140CAP, is an adapter protein that binds to Src and inactivates Src kinase through C-terminal Src kinase (21). Non-receptor protein tyrosine kinase Src is a well-characterized oncogene and its activity is associated with the progression of cancer (22,23). Src is known to mediate several oncogenic signaling pathways in cancer cells, including the PI3K and STAT3 pathways (24,25). Via inactivation of Src, SRCIN1 functions as a tumor suppressor in multiple cancer types (26,27). However, it has remained elusive how SRCIN1 expression is regulated in NSCLC.

The present study aimed to investigate the clinicopathological significance and prognosis of TPTEP1 as well as its functional role in NSCLC. A bioinformatics analysis, reverse transcription-quantitative (RT-q)PCR, western blot analysis and dual-luciferase reporter assays were performed to explore the molecular mechanisms of TPTEP1 in NSCLC cells. The results demonstrated a tumor suppressor role of TPTEP1 in NSCLC.

Materials and methods

Patients and samples. Human NSCLC tumors and matched normal tissues were collected from 56 patients (41 males and 15 females; age range, 35-76 years) with NSCLC who underwent surgery at Shangqiu First People's Hospital and the First Affiliated Hospital of Henan University between June 2015 and July 2016. The information of sex, age and smoking history was obtained from patients. Written informed consent was obtained from all participants prior to the study. The patients did not receive any chemotherapy or radiotherapy prior to surgery. The NSCLC samples were staged according to surgical and pathological results, which were based on the guidelines described by the 7th edition of the American Joint Committee on Cancer/Union for International Cancer Control (28). All experiments were approved by the Ethics Committee of Shangqiu First People's Hospital and the First Affiliated Hospital of Henan University. Tissues were stored in liquid nitrogen at the time of surgery and stored in a -80°C refrigerator.

Cell lines and culture. Human NSCLC cell lines (A549 and NCI-H1299) and the human lung epithelial cell line BEAS-2B were purchased from the American Type Culture Collection. These cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator with 5% CO₂.

RNA extraction and RT-qPCR. Total RNA was extracted from BEAS-2B, A549, NCI-H1299 cells and tissue samples with the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. The RNA concentration was measured with a NanoDrop 2000 (Thermo Fisher Scientific, Inc.). First-strand complementary

(c) DNA was synthesized with a SuperScript III First-Strand kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Realtime qPCR was performed using TB Green Premix Ex Taq (Takara Bio, Inc.) with the following protocol: Initial pre-denaturation at 98°C for 30 sec, followed by 35 cycles of denaturation at 98°C for 5 sec and elongation/annealing at 60°C for 30 sec. GAPDH and U6 were used as internal controls for mRNA and miRNA, respectively. The relative expression of genes were calculated with the 2^{-ΔΔC_q} method (29). The primer sequences were listed as follows: Stem-loop, 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCCTGA-3'; miR-328-5p-forward, 5'-GCCGAGGGGGGGGAGGAGG-3' and reverse, 5'-CTCAACTGGTGTCGTGGA-3'; TPTEP1 forward, 5'-CTGGGAGAAGTGCCCTTGC-3' and reverse, 5'-CACCTCATCAGTCATTTGCTCA-3'; SRCIN1 forward, 5'-GAGGCTCGCAACGTCTTCTAC-3' and reverse, 5'-GCGATGCGTACACCATCTCTC-3'; GAPDH forward, 5'-TCAACAGCAACTCCCACTCTTCCA-3' and reverse, 5'-ACCCTGTTGCTGTAGCCGTATTCA-3'.

Overexpression of TPTEP1 and silencing of SRCIN1. Full-length TPTEP1 was amplified by PCR (TPTEP1 forward, 5'-GTGAATTCCTCGAGACTAGTTCTGCCTCTCCCGGTACCTGCT-3' and reverse, 5'-GGATCCGCGGCCGCTCTAGCACTAGTTTTTGATGGAATTTTATGTTT-3') from A549 cDNA and ligated into pcDNA3.1 plasmid. pcDNA3.1 or pcDNA3.1-TPTEP1 was transfected into A549 or NCI-H1299 cells with Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. SRCIN1 siRNA and control siRNA were purchased from GenePharma Co., Ltd. SRCIN1 siRNA (5'-GCCCGCUGAGCGCCUCCAGAC-3') or control siRNA (5'-UUCUCCGAACGUGUCACGU-3') was transfected into A549 or NCI-H1299 cells with Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 72 h, the cells were collected and the RNA or proteins were extracted for the subsequent experiments.

Knockdown and overexpression of miR-328-5p. miR-negative control (NC) mimics (5'-UUCUCCGAACGUGUCACGU-3'), miR-328-5p mimics (5'-AGGGGGGGCAGGAGGGGCUAGGG-3'), miR-NC inhibitor (5'-UUCUCCGAACGUGUCACGU-3') and miR-328-5p inhibitor (5'-CCCUGAGCCCCUCCUGCCCCCCCCU-3') were synthesized and provided by GenePharma Co., Ltd. miR-NC mimics, miR-328-5p mimics, miR-NC inhibitor or miR-328-5p inhibitor was transfected into A549 or NCI-H1299 cells with Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. At 72 h after transfection, the cells were collected and subjected to RT-qPCR or western blot analysis.

Dual-luciferase reporter assay. Full-length TPTEP1 and the full length of 3'untranslated region (3'UTR) of SRCIN1 were amplified from A549 cDNA and ligated into pGL3-basic plasmid. Two site mutations were introduced into pGL3-TPTEP1 or pGL3-SRCIN1 3'UTR plasmid with a Quick Site-directed mutation kit (Agilent Technologies, Inc.). For the dual-luciferase reporter assay, cells were transfected with a combination of pGL3-TPTEP1 or pGL3-SRCIN1 3'UTR or pGL3-TPTEP1 mutated (Mut) or pGL3-SRCIN1

3'UTR-Mut with miR-NC mimics or miR-328-5p mimics by using Lipofectamine 3000. At 48 h after transfection, the relative luciferase activity was measured with a Dual-Luciferase Reporter Assay System (Promega Corp.).

Protein extraction and western blot analysis. Primary antibodies of SRCIN1 (product number 3757; dilution 1:1,000), P-SRC (TYR416) (product number 6943; dilution 1:1,000), SRC (product number 2108; dilution 1:1,000) p-STAT3 (product number 9145; dilution 1:1,000) and STAT3 (product number 9139; dilution 1:1,000) were purchased from Cell Signaling Technology, Inc. GAPDH (product code ab8245; dilution 1:5,000) antibody was obtained from Abcam. HRP-conjugated secondary antibodies of mouse (cat. no. SA00001-1; dilution 1:5,000) and rabbit (cat. no. SA00001-2; dilution 1:5,000) were purchased from ProteinTech Group. Radioimmunoprecipitation assay lysis buffer (RIPA; Thermo Fisher Scientific, Inc.) was used to prepare protein lysates from cells following the manufacturer's protocol. Subsequently, the protein concentration was determined with a BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). Lysates containing 25 μ g protein were loaded in each well of 8% SDS gels and separated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (EMD Millipore) and blocked with non-fat milk for 30 min at room temperature. The membrane was then washed and incubated with a primary antibody for 1 h at room temperature. Subsequently, the membranes were washed and incubated with secondary antibodies for 1 h at room temperature. Finally, the blots were developed with ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.). The relative expression of protein was quantified with ImageJ software (v. 1.52r; National Institutes of Health).

Bioinformatics analysis. The expression of TPTEP1 in multiple cancer types and normal tissues of different organs was analyzed using the Gene Expression Profiling Interactive Analysis database (GEPIA; <https://gepia.cancer-pku.cn/>) based on TCGA datasets. To study the association between TPTEP1 expression and prognosis of patients with NSCLC, the gene expression data and patient survival information from the dataset GSE30219 (30) comprising 307 patients with lung cancer were downloaded. The samples were divided into two groups based on the expression of TPTEP1 (above or below the median expression level of TPTEP1 (relative expression of TPTEP1=4.20554) (n=141 for each group) and were analyzed using Kaplan-Meier analysis with log-rank testing. The miRNAs containing putative binding sites for TPTEP1 were predicted with miRDB software (<http://mirdb.org/>). The potential target genes of miR-328-5p were predicted with TargetScan software (http://www.targetscan.org/vert_72/).

Cell proliferation assay. The proliferation ability of cells was determined with a CCK-8 kit (Dojindo Molecular Technologies, Inc.) following the manufacturer's protocol. On the first day, 5,000 cells were seeded in each well of 96-well plates. On the second day, cells were transfected with plasmids and/or miRNA inhibitor. At 0, 24, 48 and 72 h after transfection, 10 μ l CCK-8 solution was added to each well, followed by incubation for 2 h. The medium containing CCK-8 was trans-

ferred to another 96-well plate and the absorbance at 450 nM was detected with a microplate reader (Bio-Rad Laboratories, Inc.) to determine the number of cells.

Cell apoptosis assay. The cell apoptosis rate was detected with a Dead Cell Apoptosis Kit with Annexin V Alexa Fluor™ 488 and propidium iodide (PI) (Thermo Fisher Scientific, Inc.). In brief, at 48 h after transfection, cells were harvested and resuspended in 1X Binding Buffer provided with the kit. Subsequently, 5 μ l PI and 1 μ l Annexin V was added to the cells, followed by incubation at room temperature for 15 min. The cells were immediately subjected to flow cytometric analysis on a MACSQuant Analyzer X (Miltenyi Biotec, Inc.). The data were analyzed using FlowJo 10 software (FlowJo LLC).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc.). Student's t-test (paired test for specimens and unpaired test for cell-based assays) was applied to analyze differences between two groups. For comparison of three groups, one-way analysis of variance followed by Student-Neuman-Keuls analysis was performed. The association between TPTEP1 expression and characteristics of patients was analyzed by Chi-square test. All statistical analyses were two-tailed and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

lncRNA TPTEP1 is downregulated in NSCLC and associated with good prognosis. To investigate the expression pattern of TPTEP1 in NSCLC, the GEPIA database was used to explore its expression in cancer and normal tissues of various origins from TCGA datasets. TPTEP1 was downregulated in a majority of cancer types, including bladder urothelial carcinoma and breast invasive carcinoma. Notably, TPTEP1 was significantly downregulated in lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) (Fig. 1A). For confirmation, RT-qPCR was performed to detect TPTEP1 expression in 56 pairs of tumors and adjacent normal tissues from patients with NSCLC. The results revealed that TPTEP1 was downregulated in most tumors (Fig. 1B). It was also indicated that TPTEP1 expression was lower in high-grade (stage III-IV) tumors compared with that in low-grade tumors (stage I-II; Fig. 1C). Low expression of TPTEP1 was associated with high-grade tumors (stage III-IV; $P = 0.006$), but no significant association was determined between TPTEP1 expression and sex, age and smoking history (Table I). The prognostic value of TPTEP1 in patients with NSCLC was then analyzed. For the dataset GSE30219, the Kaplan-Meier plot indicated that high expression of TPTEP1 was associated with a favorable overall survival for patients with NSCLC (log-rank $P = 0.001$; Fig. 1D). In addition, in the tumor tissues collected from patients with NSCLC, high expression of TPTEP1 was further confirmed to be associated with a relatively good prognosis (log-rank $P = 0.036$; Fig. 1E). Next, TPTEP1 expression was analyzed in a panel of NSCLC cell lines and lung epithelial cells. TPTEP1 was ~2-fold decreased in NSCLC cells (A549 and NCI-H1299) compared with that in lung epithelial cells (BEAS-2B) (Fig. 1F).

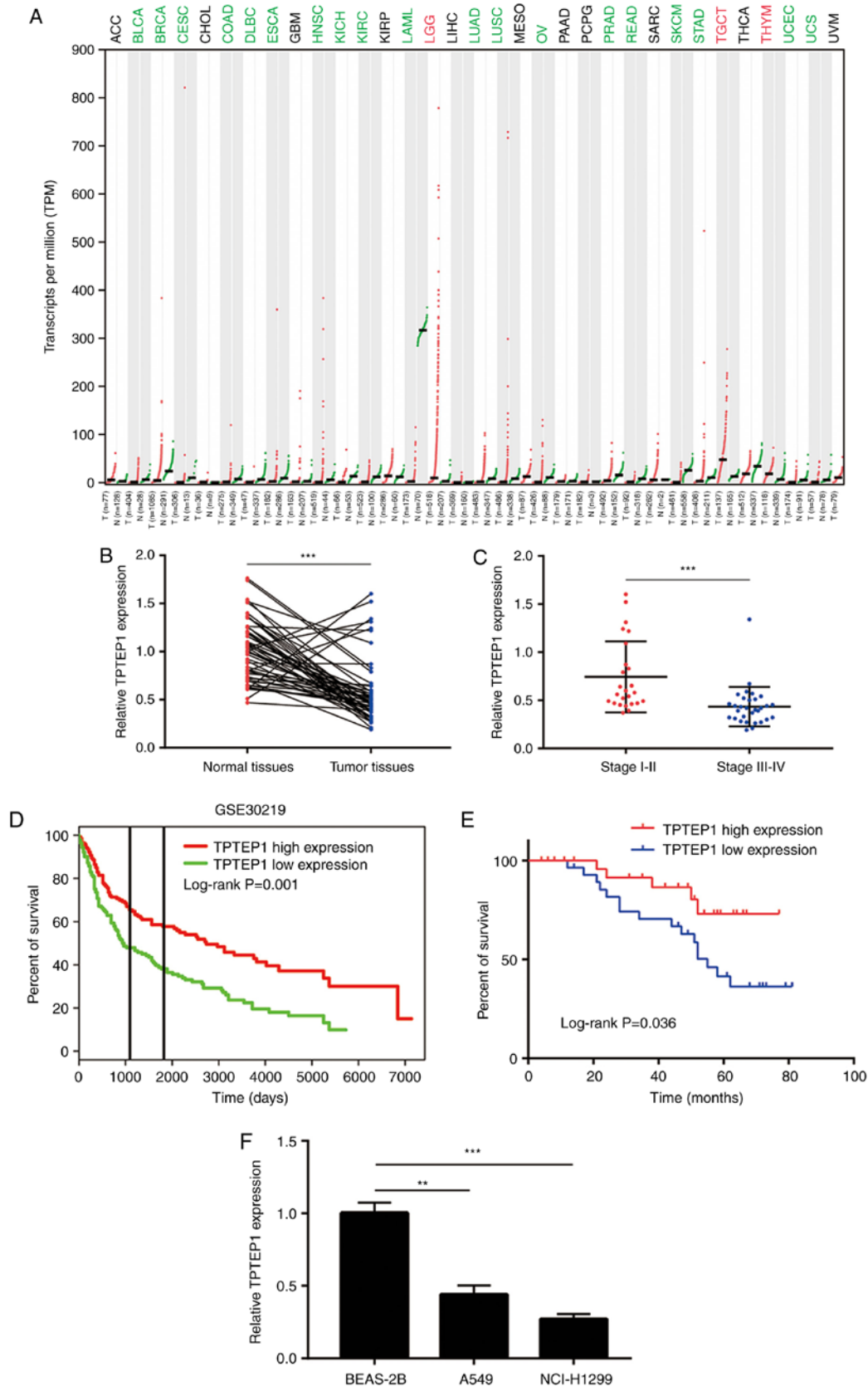


Figure 1. TPTEP1 is downregulated in NSCLC and predicts a favorable prognosis. (A) According to scatter diagram depiction of TPTEP1 expression in multiple cancers and normal tissues generated from GEPIA database, TPTEP1 was downregulated in several cancer types including LUAD and LUSC. (B) RT-qPCR was performed to detect TPTEP1 expression in 56 pairs of tumors and matched normal tissues from patients with NSCLC. TPTEP1 was significantly downregulated in NSCLC tumors. (C) Expression of TPTEP1 was decreased in high grade (grade III-IV) NSCLC tumors compared to those of low grade (grade I-II). (D) Using data from GSE30219 (n=307), the Kaplan-Meier analysis revealed that the TPTEP1 high-expression group exhibited a prolonged overall survival compared with those with low expression. (E) Kaplan-Meier analysis based on 56 patients with NSCLC revealed that patients with high expression of TPTEP1 exhibited a prolonged overall survival compared to those with low expression. (F) RT-qPCR revealed that TPTEP1 was downregulated in NSCLC cells (A549 and NCI-H1299) compared to lung epithelial cells (BEAS-2B). **P<0.01; ***P<0.001. TPTEP1, TPTE pseudogene 1; NSCLC, non-small cell lung cancer; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma.

Table I. Association between clinicopathological characteristics and TPTEP1 expression in patients with NSCLC.

Characteristics	Total cases	TPTEP1 expression		P-value
		High expression	Low expression	
Sex				0.227
Male	41	23	18	
Female	15	5	10	
Age				0.391
<60	18	7	11	
≥60	38	21	17	
Smoking history				0.503
No	11	7	4	
Yes	45	21	24	
TNM stage				0.006
I-II	23	17	6	
III-IV	33	11	22	

TPTEP1, TPTE pseudogene 1; NSCLC, non-small cell lung cancer.

Overexpression of TPTEP1 inhibits NSCLC cell proliferation and induces apoptosis. To study the role of TPTEP1 in NSCLC, pcDNA3.1-TPTEP1 with full-length TPTEP1 was constructed and transfected into NSCLC cells. Transfection of TPTEP1 led to a >4-fold increase of TPTEP1 in A549 and NCI-H1299 cells (Fig. 2A and B). In the cell proliferation assay, overexpression of TPTEP1 led to a significant decrease in the proliferation rate of A549 and NCI-H1299 cells (Fig. 2C and D). The strong cell growth-inhibitory effect of TPTEP1 suggested that cell apoptosis may be involved in this process. Flow cytometry was used to determine the ratio of apoptotic cells following overexpression of TPTEP1. The results indicated that overexpression of TPTEP1 significantly increased the apoptotic ratio of A549 cells (Fig. 2E). Similarly, a significant increase of cell apoptosis was observed in NCI-H1299 cells transfected with TPTEP1 (Fig. 2F). The results collectively indicated that TPTEP1 was pivotal for resistance to apoptosis of NSCLC cells.

TPTEP1 sponges miR-328-5p in NSCLC cells. lncRNAs may function as ceRNAs to sponge certain miRNAs and thereby regulate cancer progression (31). By using miRDB, 38 miRNAs containing a putative binding site for TPTEP1 were predicted. Among them, miR-328-5p was previously reported as an upregulated miRNA in NSCLC (32). As indicated in Fig. 3A, a putative binding site for miR-328-5p on TPTEP1 was predicted. In the 56 NSCLC tumors, a significant negative correlation between miR-328-5p levels and TPTEP1 expression was observed ($r=-0.416$, $P<0.01$; Fig. 3B). To explore the regulatory association between miR-328-5p and TPTEP1, miR-328-5p was knocked down in A549 and NCI-H1299 cells by transfection of miR-328-5p inhibitor (Fig. 3C and D). Downregulation of miR-328-5p led to an increase of TPTEP1 expression in A549 cells (Fig. 3E). A similar effect was also observed in NCI-H1299 cells (Fig. 3F). Conversely, overexpression of TPTEP1 decreased miR-328-5p levels in

A549 and NCI-H1299 cells (Fig. 3G and H). Transfection of miR-328-5p mimics was confirmed to increase miR-328-5p levels in A549 and NCI-H1299 cells (Fig. 3I and J). In the dual-luciferase reporter assay, overexpression of miR-328-5p was indicated to decrease the relative luciferase activity of pGL3-TPTEP1-wild-type (WT) plasmid but did not influence the relative luciferase activity of pGL3-TPTEP1-Mut in A549 cells (Fig. 3K). Similarly, miR-328-5p mimics also suppressed the relative luciferase activity of pGL3-TPTEP1-WT in NCI-H1299 cells (Fig. 3L). These results indicated that TPTEP1 directly interacts with miR-328-5p in NSCLC cells.

Downregulation of miR-328-5p inhibits NSCLC cell proliferation and induces cell apoptosis. Although it has been previously indicated that miR-328-5p was overexpressed in NSCLC and may serve as a diagnostic biomarker (32), the function of miR-328-5p in NSCLC has remained elusive. The present results indicated that in A549 and NCI-H1299 cells, downregulation of miR-328-5p inhibited cell proliferation (Fig. 4A and B). In addition, miR-328-5p inhibitor induced apoptosis in A549 cells (Fig. 4C), which was also observed in NCI-H1299 cells (Fig. 4D). Collectively, similar to the effect of overexpression of TPTEP1, downregulation of miR-328-5p inhibited cell proliferation and induced apoptosis in NSCLC cells.

TPTEP1 causes upregulation of SRCIN1 via sponging miR-328-5p. Using TargetScan, several target genes of miR-328-5p were predicted, and among them, SRCIN1 is a well-characterized tumor suppressor (33) which was indicated to share a putative binding site for miR-328-5p (Fig. 5A). Overexpression of TPTEP1 increased SRCIN1 mRNA levels, and transfection of miR-328-5p mimics attenuated the upregulation of SRCIN1 mRNA in A549 and NCI-H1299 cells (Fig. 5B and C). Previous studies reported that SRCIN1 directly inactivated Src signaling and indirectly inactivated STAT3 signaling in cancer cells (34,35). In the present study, western blot analysis revealed that TPTEP1 over-

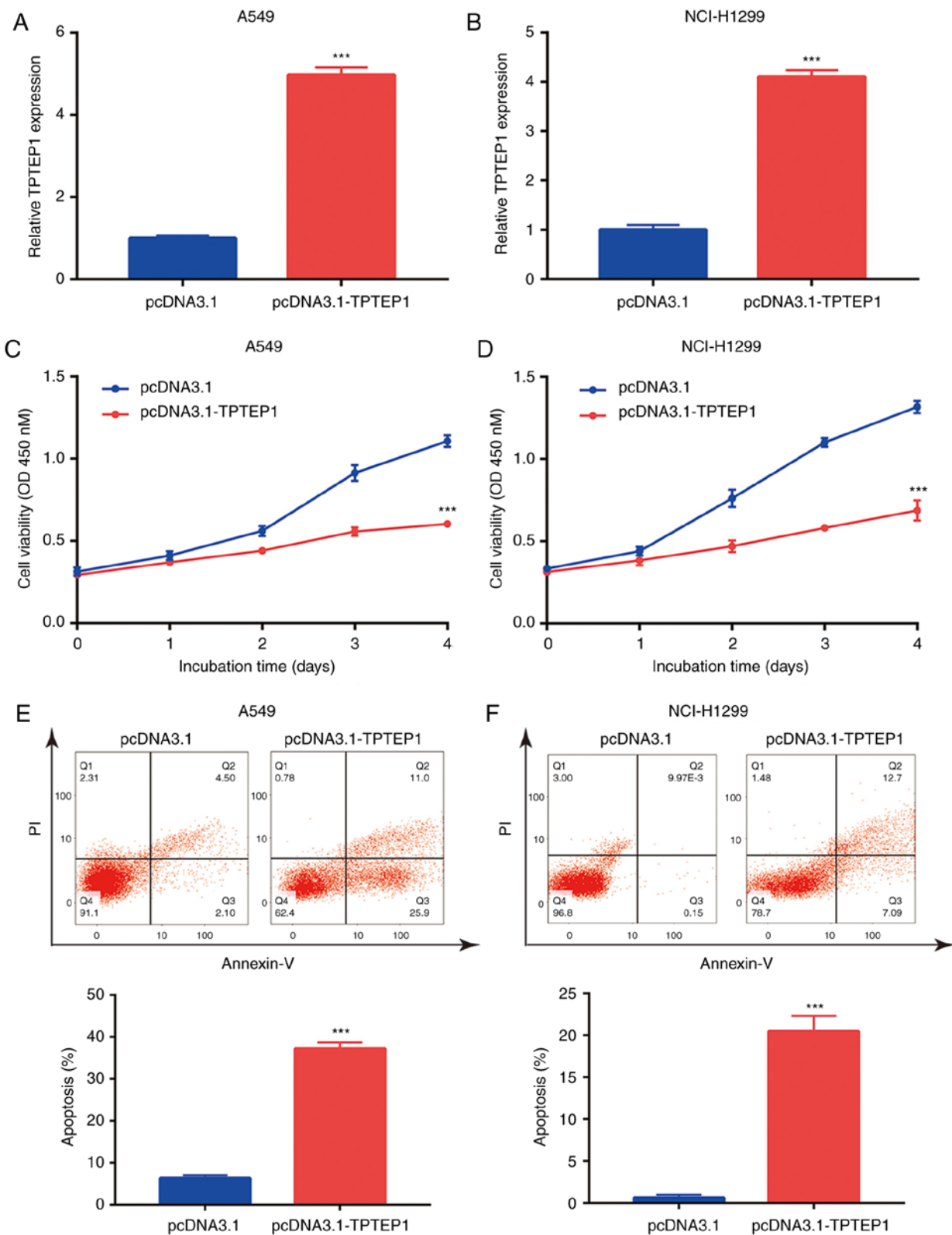


Figure 2. TPTEP1 overexpression inhibits cell proliferation and induces cell apoptosis in NSCLC cells. (A) Transfection of pcDNA3.1-TPTEP1 increased TPTEP1 expression in A549 cells. (B) Transfection of pcDNA3.1-TPTEP1 increased TPTEP1 expression in NCI-H1299 cells. (C) Overexpression of TPTEP1 inhibited cell proliferation in A549 cells. (D) Overexpression of TPTEP1 inhibited cell proliferation in NCI-H1299 cells. (E) Overexpression of TPTEP1 induced cell apoptosis in A549 cells. (F) Overexpression of TPTEP1 induced cell apoptosis in NCI-H1299 cells. *** $P < 0.001$. TPTEP1, TPTE pseudogene 1; NSCLC, non-small cell lung cancer.

expression increased SRCIN1 protein expression and decreased p-Src and p-STAT3 protein levels in A549 cells (Fig. 5D). The decrease of the p-SRC/t-SRC and p-STAT3/t-STAT3 ratios

indicated inactivation of SRC and STAT3 signaling in response to TPTEP1 overexpression in A549 cells. Similar to the effect in A549 cells, TPTEP1 overexpression increased SRCIN1 protein

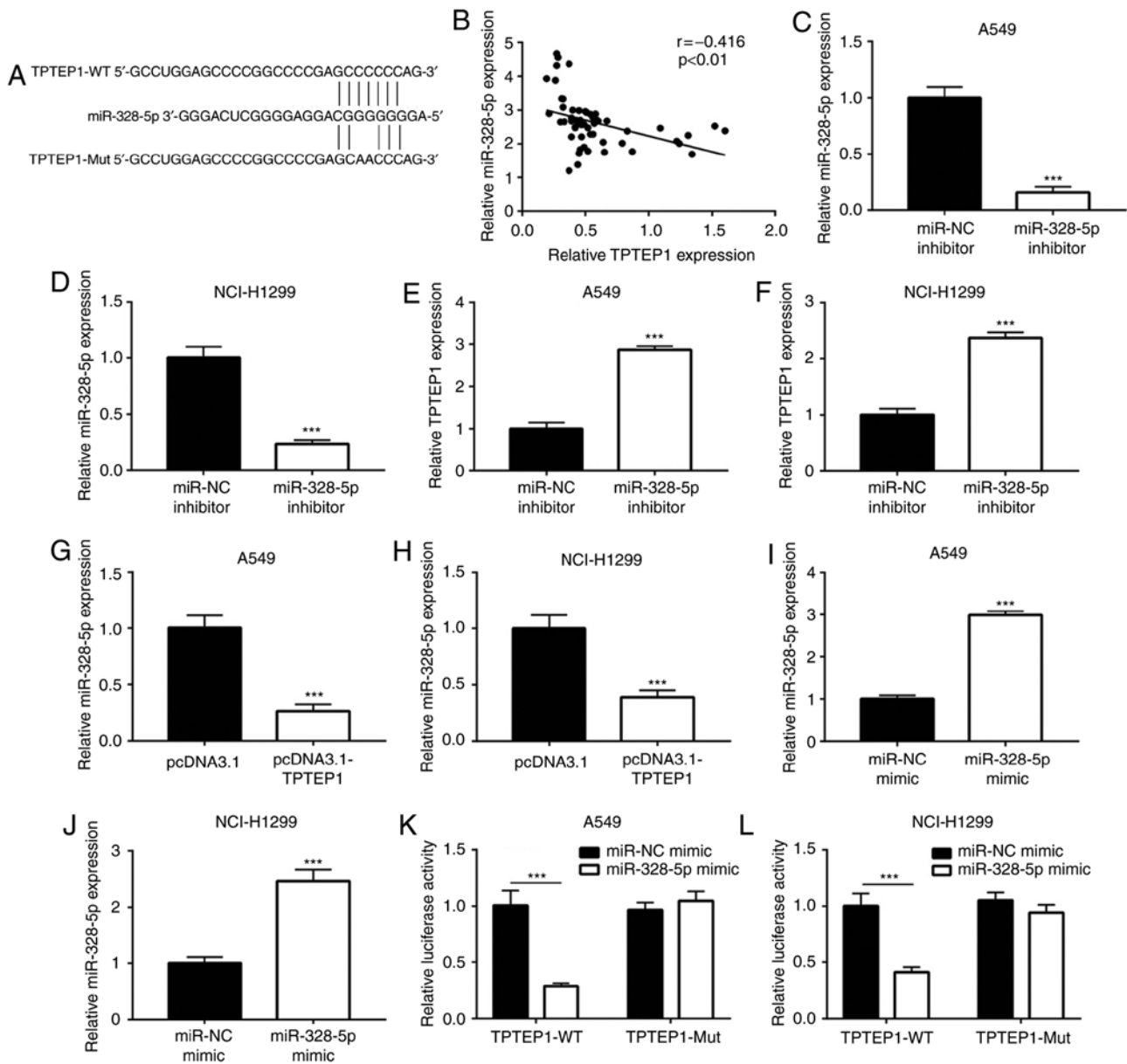


Figure 3. TPTEP1 sponges miR-328-5p in NSCLC cells. (A) Sequence alignment of TPTEP1 to miR-328-5p is revealed. Two site mutations were introduced into putative binding sites of miR-328-5p in TPTEP1 sequence. (B) Pearson correlation analysis revealed a negative correlation between TPTEP1 and miR-328-5p levels in NSCLC tumors. (C) Transfection of miR-328-5p inhibitor decreased miR-328-5p levels in A549 cells. (D) Transfection of miR-328-5p inhibitor decreased miR-328-5p levels in NCI-H1299 cells. (E) Transfection of miR-328-5p inhibitor increased TPTEP1 levels in A549 cells. (F) Transfection of miR-328-5p inhibitor increased TPTEP1 levels in NCI-H1299 cells. (G) Overexpression of TPTEP1 decreased miR-328-5p expression in A549 cells. (H) Overexpression of TPTEP1 decreased miR-328-5p expression in NCI-H1299 cells. (I) Transfection of miR-328-5p mimic increased miR-328-5p levels in A549 cells. (J) Transfection of miR-328-5p mimic increased miR-328-5p levels in NCI-H1299 cells. (K) Transfection of miR-328-5p mimic decreased the relative luciferase activity of pGL3-TPTEP1-WT not pGL3-TPTEP1-Mut in A549 cells. (L) Transfection of miR-328-5p mimic decreased the relative luciferase activity of pGL3-TPTEP1-WT not pGL3-TPTEP1-Mut in NCI-H1299 cells. *** $P < 0.001$. TPTEP1, TPTE pseudogene 1; NSCLC, non-small cell lung cancer.

expression and decreased p-SRC and p-STAT3 protein levels in NCI-H1299 cells (Fig. 5E). SRC and STAT3 signaling became inactivated in NCI-H1299 after TPTEP1 overexpression. In the dual-luciferase reporter assay, miR-328-5p mimics suppressed the relative luciferase activity of the plasmid driven by the SRCIN1 3'UTR in A549 and NCI-H1299 cells (Fig. 5F and G), revealing that miR-328-5p directly binds to the 3'UTR of SRCIN1 mRNA. Hyperactivation of Src and STAT3 signaling are pivotal for cancer cell survival (36,37). The present results indicated that TPTEP1 sponged miR-328-5p to upregulate SRCIN1 and inactivated Src and STAT3 signaling to promote NSCLC cell apoptosis.

TPTEP1 inhibits cell proliferation and induces apoptosis via upregulation of SRCIN1. To investigate whether SRCIN1 is involved in TPTEP1-mediated cell growth arrest and cell apoptosis, SRCIN1 siRNA was transfected into A549 and NCI-H1299 cells to knock down SRCIN1 expression. Western blot analysis confirmed that transfection of SRCIN1 siRNA decreased SRCIN1 protein expression in A549 and NCI-H1299 cells (Fig. 6A). The cell proliferation assay indicated that silencing of SRCIN1 abrogated the inhibitory effect of TPTEP1 overexpression on the proliferation of A549 and NCI-H1299 cells (Fig. 6B and C). Furthermore, flow cytometric analysis revealed that silencing of SRCIN1 inhibited

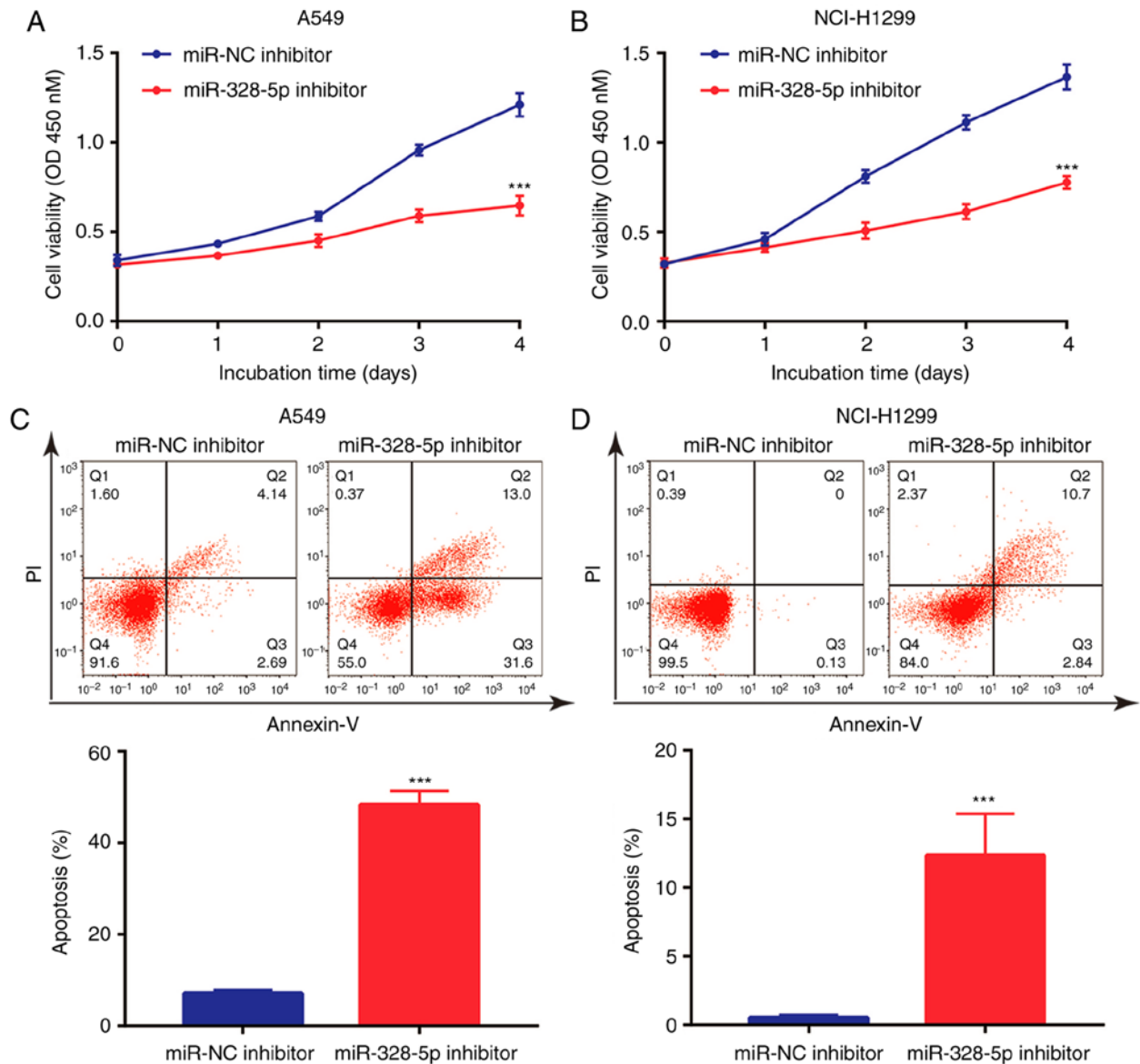


Figure 4. miR-328-5p promotes cell proliferation and survival of NSCLC cells. (A) Transfection of miR-328-5p inhibitor inhibited cell proliferation of A549 cells. (B) Transfection of miR-328-5p inhibitor inhibited cell proliferation of NCI-H1299 cells. (C) Transfection of miR-328-5p inhibitor induced cell apoptosis of A549 cells. (D) Transfection of miR-328-5p inhibitor induced cell apoptosis of NCI-H1299 cells. *** $P < 0.001$. TPTEP1, TPTE pseudogene 1; NSCLC, non-small cell lung cancer.

the effect of TPTEP1 overexpression to induce apoptosis in A549 cells (Fig. 6D), which was also observed in NCI-H1299 cells (Fig. 6E).

TPTEP1 expression is positively correlated with SRCIN1 mRNA levels in NSCLC tumors. To examine the association between SRCIN1 and TPTEP1 in clinical samples, RT-qPCR was performed to detect SRCIN1 mRNA levels in 56 pairs of NSCLC tumors and adjacent normal tissues. Downregulation of SRCIN1 was observed in NSCLC tumors ($P < 0.001$; Fig. 7A), which was consistent with the results of a previous study (33). In addition, SRCIN1 protein expression was detected in 10 pairs of NSCLC tumors and normal tissues by western blot analysis. Consistently, SRCIN1 protein expression was significantly downregulated in tumors ($P < 0.001$; Fig. 7B). Pearson correlation analysis revealed a strong positive correlation between TPTEP1 and SRCIN1 mRNA expression ($r = 0.591$,

$P < 0.001$; Fig. 7C) and a negative correlation between SRCIN1 mRNA and miR-328-5p levels ($r = -0.436$, $P < 0.001$; Fig. 7D) in tumors.

Discussion

Due to the development of RNA sequencing technology, numerous aberrantly expressed lncRNAs have been identified in NSCLC (38). Several lncRNAs were experimentally identified to function as tumor suppressors or oncogenes during NSCLC (38-40). DiGeorge syndrome critical region gene 5, an upregulated lncRNA in lung adenocarcinoma identified through integrated analysis of TCGA data (20), was indicated to be pivotal for proliferation and metastasis of lung cancer cells via sponging miR-1180 and miR-873-5p (41,42). The present study focused on TPTEP1, another differentially expressed lncRNA in lung adenocarcinoma, and indicated for the first time to the

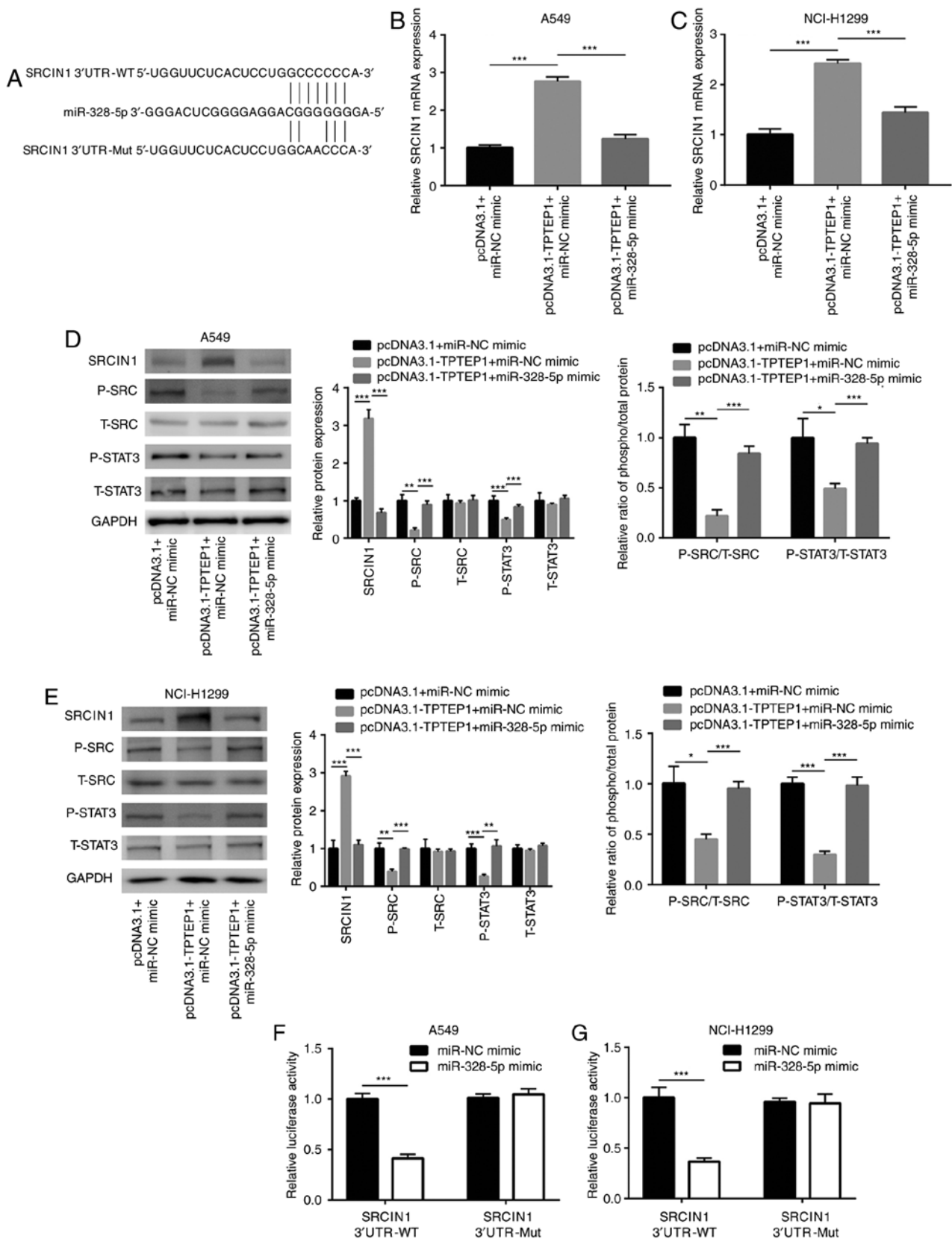


Figure 5. TPTEP1 promoted SRCIN1 expression via sponging miR-328-5p. (A) There was a putative binding site for miR-328-5p on SRCIN1 3'UTR. Two sites mutations were introduced into SRCIN1 3'UTR on the putative binding site. (B) SRCIN1 mRNA was upregulated after TPTEP1 overexpression which was reversed after miR-328-5p overexpression in A549 cells. (C) SRCIN1 mRNA was upregulated after TPTEP1 overexpression which was reversed after miR-328-5p overexpression in NCI-H1299 cells. (D) Western blotting revealed that TPTEP1 overexpression increased SRCIN1 and decreased p-SRC, p-STAT3 protein expression which was reversed after miR-328-5p overexpression in A549 cells. (E) Western blotting revealed that TPTEP1 overexpression increased SRCIN1 and decreased p-SRC, p-STAT3 protein expression which was reversed after miR-328-5p overexpression in NCI-H1299 cells. (F) The dual luciferase reporter assay revealed that miR-328-5p mimic reduced the relative luciferase activity of SRCIN1 3'UTR-WT not SRCIN1 3'UTR-Mut in A549 cells. (G) The dual luciferase reporter assay revealed that miR-328-5p mimic reduced the relative luciferase activity of SRCIN1 3'UTR-WT not SRCIN1 3'UTR-Mut in NCI-H1299 cells. *P<0.05; **P<0.01; ***P<0.001. TPTEP1, TPTE pseudogene 1; SRCIN1, Src kinase signaling inhibitor 1.

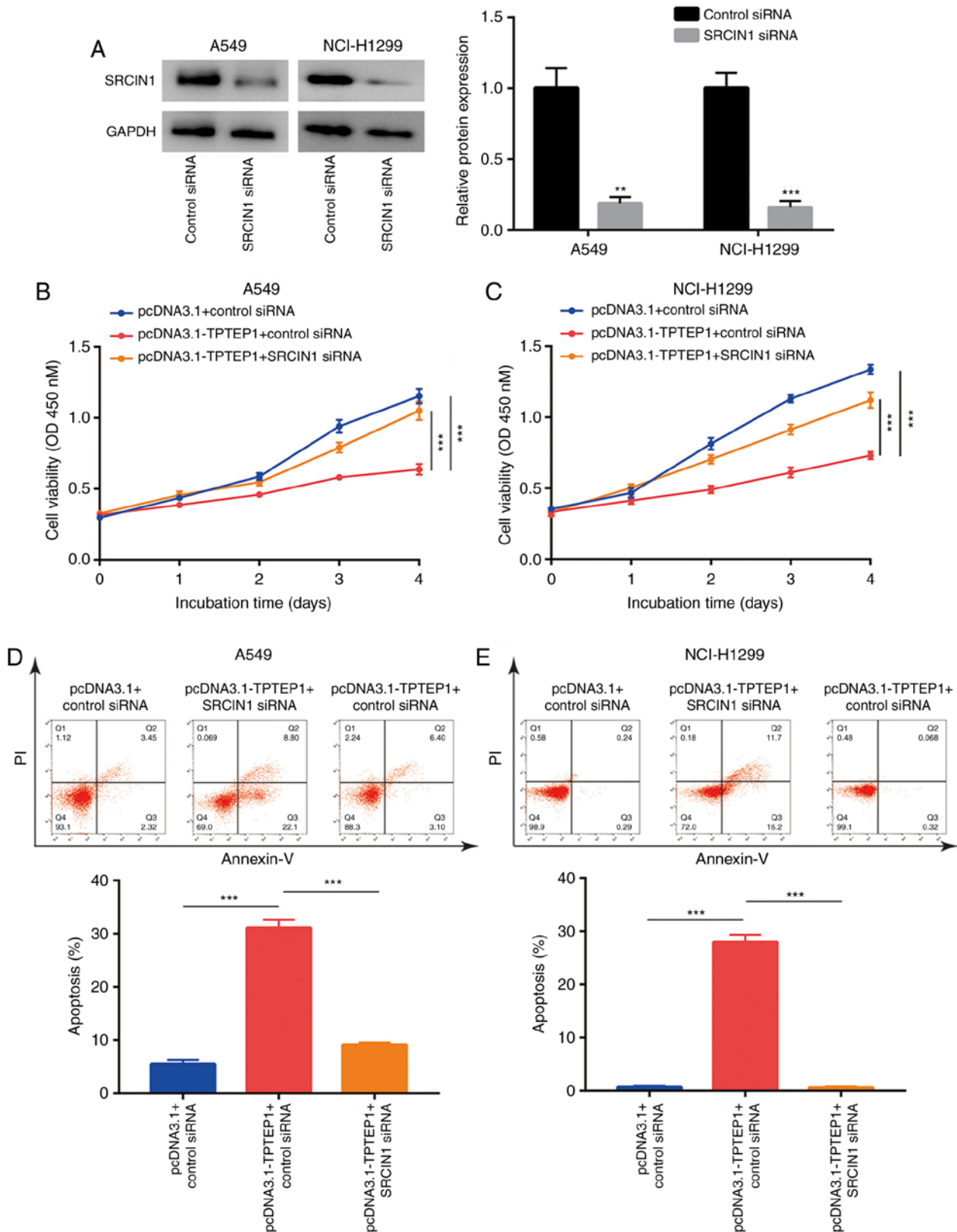


Figure 6. TPTEP1 negatively regulates cell proliferation and induces cell apoptosis via upregulation of SRCIN1 in NSCLC cells. (A) Transfection of SRCIN1 siRNA decreased SRCIN1 protein expression in A549 and NCI-H1299 cells. (B) Overexpression of TPTEP1 decreased cell proliferation which was reversed after SRCIN1 silencing in A549 cells. (C) Overexpression of TPTEP1 decreased cell proliferation which was reversed after SRCIN1 silencing in NCI-H1299 cells. (D) Overexpression of TPTEP1 induced cell apoptosis which was reversed after SRCIN1 silencing in A549 cells. (E) Overexpression of TPTEP1 induced cell apoptosis which was reversed after SRCIN1 silencing in NCI-H1299 cells. ** $P < 0.01$; *** $P < 0.001$. TPTEP1, TPTE pseudogene 1; SRCIN1, Src kinase signaling inhibitor 1.

best of our knowledge, that TPTEP1 regulated cell proliferation and apoptosis in NSCLC.

Downregulation of TPTEP1 was first reported in kidney, liver, lung and stomach cancers as a result of DNA methyl-

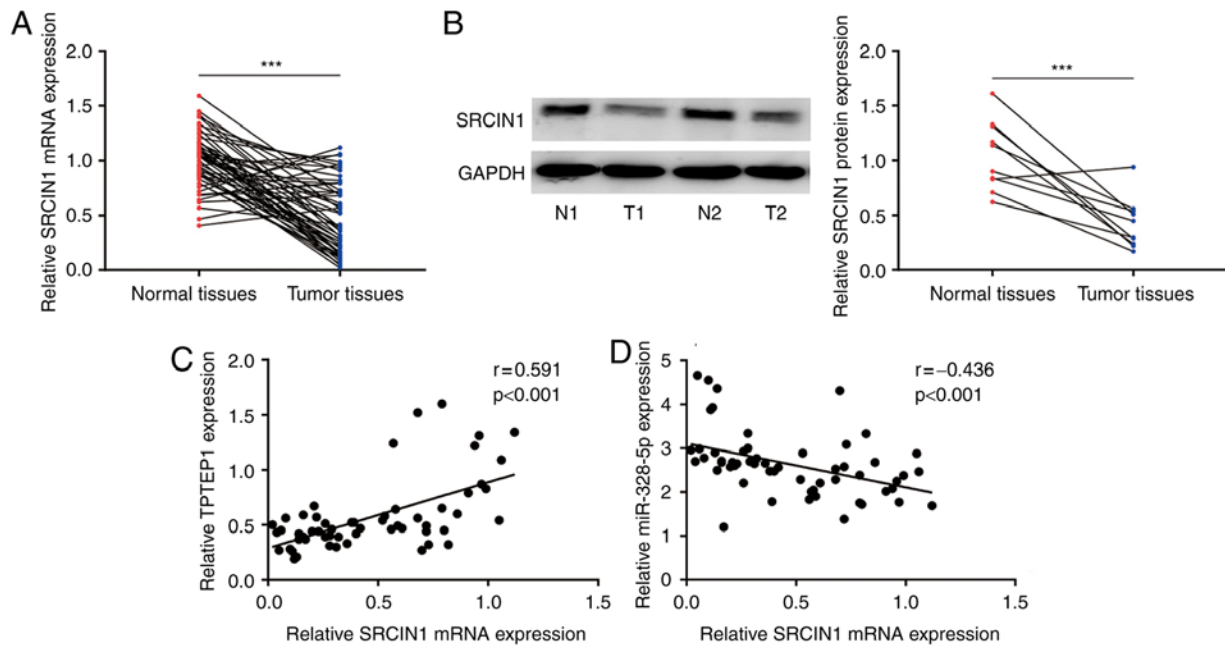


Figure 7. Expression of TPTEP1 is negatively correlated with SRCIN1 mRNA levels in NSCLC tumors. (A) RT-qPCR revealed that SRCIN1 mRNA was decreased in NSCLC tumors compared to matched normal tissues from 56 patients with NSCLC. (B) Western blotting revealed that SRCIN1 protein was decreased in 10 NSCLC tumors compared to matched normal tissues. The blots of two representative tumors and normal tissues are presented. (C) Pearson Correlation analysis revealed that TPTEP1 expression was positively correlated with SRCIN1 mRNA levels in 56 NSCLC tumors. (D) Pearson Correlation analysis revealed that SRCIN1 mRNA levels were negatively correlated with miR-328-5p levels in 56 NSCLC tumors. *** $P<0.001$. TPTEP1, TPTE pseudo-gene 1; SRCIN1, Src kinase signaling inhibitor 1; NSCLC, non-small cell lung cancer.

tion (43). In the present study, the expression pattern of TPTEP1 was analyzed in several cancer types and normal tissues according to TCGA datasets, indicating that TPTEP1 was down-regulated in a majority of cancers, including kidney renal clear cell carcinoma, LUAD, LUSC and stomach adenocarcinoma. The present RT-qPCR data further confirmed the downregulation of TPTEP1 in NSCLC tumors. In addition, based on an analysis of previously published data (30), it was indicated that the high expression of TPTEP1 was able to predict a prolonged overall survival of patients with lung cancer. The result was verified in the samples collected from 56 patients with NSCLC. Certain lncRNAs are crucial for cancer cell proliferation and survival (44,45). In hepatocellular carcinoma, overexpression of TPTEP1 suppressed cell proliferation and metastasis but did not affect cell apoptosis (46). The present results indicated that overexpression of TPTEP1 significantly inhibited cell proliferation and induced apoptosis in NSCLC cells. These results indicated that TPTEP1 may have a pivotal role by inducing cell death to inhibit overgrowth of NSCLC cells.

The role of miR-328-5p in cancers is controversial. miR-328-5p levels were high in the serum of patients with NSCLC and may serve as an excellent biomarker for early prediction of NSCLC (32). In breast cancer, loss of miR-328-5p expression was observed in tumors and miR-328-5p exerted an anti-proliferative effect on breast cancer cells by targeting RAGE (47). In the present study, miR-328-5p was determined to be overexpressed in NSCLC tumors. This is consistent with the RT-qPCR data from a previous study of NSCLC tumors (48). Similar to the effect in NSCLC cells transfected with TPTEP1, knockdown of miR-328-5p inhibited cell proliferation and induced cell apoptosis in NSCLC cells. The dual-luciferase assay confirmed the direct binding of TPTEP1 and miR-328-5p

in NSCLC cells. Aberrant expression of miR-328-5p has been reported in several cancer types, and was negatively regulated by circRNA (circRNA-5692) and lncRNA (LINC00210) (47,49,50). Our data implied that TPTEP1 downregulation may contribute to the increase of miR-328-5p in NSCLC, indicating TPTEP1 as a novel regulator of miR-328-5p.

As a non-receptor kinase, Src drives NSCLC cell proliferation, drug resistance and cell survival by activating pathways including STAT3 signaling (51,52). SRCIN1 suppresses Src activity and according to a previous study, its expression was relatively low in tumors (53). Upregulation of miR-873 and miR-346 were indicated to be responsible for the down-regulation of SRCIN1 in various tumor types (33,54). In the present study, a bioinformatics analysis indicated that SRCIN1 is a potential target gene of miR-328-5p. In addition, down-regulation of miR-328-5p increased SRCIN1 expression and decreased the phosphorylation levels of Src and STAT3, indicating inactivation of Src and STAT3 signaling. Furthermore, SRCIN1 was confirmed as a target gene of miR-328-5p in the dual-luciferase reporter assay. miR-328-5p targeted mRNA of several genes such as RAGE, PLCE1 and PTEN to exert its role during cancer progression (47,55,56). To the best of our knowledge, the present study was the first to indicate that miR-328-5p promotes NSCLC cell proliferation and sustains cell survival at least partially via targeting of SRCIN1 to activate the Src and STAT3 pathways.

In conclusion, the present results revealed that TPTEP1 was downregulated in NSCLC and predicted a good prognosis for patients with NSCLC. Mechanistically, TPTEP1 inactivated Src and STAT3 signaling via sponging miR-328-5p to upregulate SRCIN1. These results may provide novel insights into the molecular basis of NSCLC.

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Availability of materials and methods

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

FC, ZW, YF, HZ and MY carried out the experimental studies and analyzed the data. XW, HZ and SZ designed the study, and performed the literature research. XW and SZ also carried out the experimental studies and prepared the manuscript preparation. All 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

The present study was approved by the Ethics Committee of Shangqiu First People's Hospital and the First Affiliated Hospital of Henan University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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