

Original Article

The SRC-TOPK positive feedback loop promotes RB1 phosphorylation and drives the development of lung squamous cell carcinoma

Xiaofei Zeng^{1,2,3,†}, Longzhen Cui^{4,†}, Beibei Tang⁴, Fei Wang⁵, Hua He^{1,2}, Feng Zhu^{4,*}, Minjie Ma^{1,2,*}, and Chang Chen^{1,2,*}

¹The First School of Clinical Medicine, Lanzhou University, Lanzhou 730030, China, ²Department of Thoracic Surgery, the First Hospital of Lanzhou University, Lanzhou 730030, China, ³School of Clinical Medicine, Chengdu Medical College; Department of Cardiothoracic Surgery, the First Affiliated Hospital of Chengdu Medical College, Chengdu 610500, China, ⁴Translational Medicine Center, Huaihe Hospital of Henan University, Henan University, Kaifeng 475000, China, and ⁵Cancer Center, Renmin Hospital of Wuhan University, Wuhan 430060, China

[†]These authors contributed equally to this work.

*Correspondence address. E-mail: fengzhu@hust.edu.cn (F.Z.); lztsma@outlook.com (M.M.); chenthoracic@163.com (C.C.)

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Abstract

Lung squamous cell carcinoma (LUSC) is a common subtype of non-small cell lung cancer, with limited treatment options and poor patient prognosis. Currently, common driver mutations in lung adenocarcinoma rarely occur in LUSC; the mutated genes found in LUSCs lack corresponding targeted drugs. Therefore, it is necessary to discover new therapeutic targets for LUSC and provide patients with more treatment options. By analyzing different databases and tissue microarray immunohistochemistry staining, we firstly find that the expression of SRC/TOPK is elevated and positively correlated in LUSC and that patients with high SRC/TOPK expression have shorter survival time. Changing the expression levels of SRC/TOPK in LUSC cells can affect cell growth and colony formation, as there is a positive feedback loop between SRC and TOPK that regulates the transcription factor RB1, thereby altering the expressions of key factors in some growth-related signaling pathways. These inhibitors can synergistically promote apoptosis and have been validated *in vivo*. Therefore, the positive feedback loop between SRC and TOPK promotes tumorigenicity by inhibiting RB1 function, and has the potential to become a precise therapeutic target for LUSC, providing new possibilities for targeted therapy.

Key words lung squamous cell carcinoma, SRC, TOPK, targeted therapy

Introduction

Lung squamous cell carcinoma (LUSC) is a type of non-small cell lung cancer, which accounts for approximately 20%–30% of lung cancer cases [1]. At present, surgery, immunotherapy, radiotherapy, and chemotherapy are still the main treatments for LUSC patients. The treatment methods are relatively limited, and resistance to radiotherapy and chemotherapy often occurs; immunotherapy is also prone to adverse reactions or failure [2]. With the emergence of targeted drugs, the therapeutic effect on lung adenocarcinoma (LUAD) has improved, and the survival period of patients is continuously prolonged. However, unlike LUAD patients, LUSC patients have not yet benefited from targeted therapy. Common driver mutations in LUAD, such as those in

EGFR and KRAS, rarely occur in LUSC. The mutation sites discovered in LUSC, such as those in SOX2, FGFR1, and NSD3, have not yet been successfully identified as targeted drugs [3]. Therefore, new LUSC therapeutic targets still need to be identified.

SRC is a nonreceptor tyrosine kinase protein that is commonly expressed in normal tissues. SRC has been identified as an important oncoprotein that can promote cancer progression, invasion, metastasis and drug resistance in various cancers, including colorectal cancer, breast cancer, pancreatic cancer, and gastric cancer [4–6]. SRC has been studied in NSCLC, especially LUAD. Onodera *et al.* [7] demonstrated that the simultaneous inhibition of SRC and Rho/ROCK had a synergistic effect on the growth of NSCLC cells. SRC interacts with cell surface growth factor

receptors and intracellular signaling pathways to maintain cell survival in NSCLC [4]. However, there is still a lack of evidence to determine whether SRC is involved in the occurrence and development of LUSC.

T-LAK cell-derived protein kinase (TOPK), also known as PDZ binding kinase (PBK), is a serine threonine protein kinase belonging to the MAPK kinase family. TOPK is involved in inflammatory reactions and is also a procancer factor. Overexpression of TOPK is a poor prognostic factor in LUAD patients and is closely associated with P53 mutations [8,9]. KRAS^{G12C} mutation-induced TOPK overexpression promotes NSCLC tumor progression, mediating hypoxia-induced epithelial mesenchymal transition and NSCLC cell invasion through the HIF-1 α /snail axis [10,11]. In addition, our previous research revealed that TOPK promoted lung cancer resistance to EGFR inhibitors by phosphorylating c-Jun and that inhibiting the COX2/MET/TOPK signaling axis weakened NSCLC cell resistance to EGFR inhibitors [12,13].

RB1, a tumor suppressor gene, is involved mainly in cell cycle regulation, stable lineage plasticity, and the tumor microenvironment [14]. In prostate cancer cells, RB1 deficiency induces SOX2 and EZH2 activity, driving prostate cancer reprogramming into neuroendocrine lineage variants [15]. In addition, the inactivation or loss of RB1 is one of the key mechanisms for tumor-targeted therapy, immunotherapy, and acquired resistance to CDK4/6 inhibitors [16]. We previously reported that SRC can phosphorylate the Y74 site of TOPK *in vitro*, which has been validated in colorectal cancer cells, where it promoted cell proliferation [17]. However, there is currently a relative lack of in-depth research on SRC, TOPK, and RB1 in LUSC.

In this study, we revealed a positive feedback loop between SRC and TOPK, which inhibits the activity of RB1 by increasing its phosphorylation, thereby promoting the proliferation and colony formation ability of LUSC cells. Our study provides a new strategy for LUSC-targeted therapy.

Materials and Methods

Antibodies and reagents

Antibodies against TOPK (sc-293028, 1:1000), Caspase-7 (sc-81654, 1:1000) and β -actin (sc-47778, 1:1000) were purchased from Santa Cruz Technology (Dallas, USA). Antibodies against P-SRC (Tyr416) (#6943, 1:1000), SRC (#2190, 1:1000), AKT (#9272, 1:1000), P-AKT (#4060, 1:1000), ERK (#2695, 1:1000), P-ERK (#9101, 1:1000), RB (#9309, 1:1000), Cleaved-PARP (#5625, 1:500) and Cleaved Caspase-7 (#8438, 1:1000) were purchased from Cell Signaling Technology (Boston, USA). anti-HA tag (51064-2-AP, 1:2500), anti-PARP (66520-1-Ig, 1:1000), anti-His tag (66005-1-Ig, 1:1000), and anti-P-RB1 (30376-1-AP, 1:1000) antibodies, as well as HRP-labeled goat anti-mouse IgG (H+L) (SA00001-1, 1:5000), and goat anti-rabbit IgG (H+L) (SA00001-2, 1:5000) were purchased from Proteintech. (Wuhan, China). Anti-P-serine antibody (05-1000X, 1:1000) was purchased from Sigma-Aldrich (St Louis, USA). The anti-p-TOPK Y74 (1:1000) antibody was prepared by Abgent (Suzhou, China). All the antibodies were used according to the instructions of their respective manufacturers. EGF (HY-P7109), OTS964 (HY-12467), and eCF506 (HY-112096) were purchased from MedChemExpress (Shanghai, China).

Plasmids

The pcDNA3.1-HA-TOPK, pcDNA4-His-SRC, pMD2.G, and psPAX2 vectors were purchased from Addgene (Cambridge, USA). pCMV-

Flag-RB1 plasmid was purchased from QCHENG (Shanghai, China). Lentivirus plasmids shTOPK and shSRC were obtained from Sangon Biotech (Shanghai, China), and the sequences of shTOPKs and shSRCs are shown in Table 1. pLKO.1-puro Non-Target shRNA Control Plasmid DNA (SHC001) was purchased from Sigma-Aldrich.

Data collection and analysis

The expression data and corresponding clinical information of the LUSC patients in this study were obtained from the Cancer Genome Atlas (TCGA) (<https://portal.gdc.cancer.gov/>) Database and Gene Expression Omnibus (GEO) Database (<https://www.ncbi.nlm.nih.gov/geo/>). The GEO datasets included GSE30219 and GSE19188. The relevant results were analyzed via GraphPad Prism software.

Cell culture

HEK293FT, NCI-H1703, NCI-H2170, and NCI-H520 cells were purchased from the American Type Culture Collection (ATCC, Manassas, USA). The cells were cultivated according to the procedures provided by the ATCC and used within 6 months after resuscitation. The cells were cultured in RPMI-1640 medium (Corning, Shanghai, China) supplemented with 10% fetal bovine serum (Royacel Serum, Lanzhou, China) at 37°C and 5% CO₂.

Cell transfection

The transfection reagent Simple Fect was purchased from Signaling Dawn Biotech (Wuhan, China). According to the manufacturer's recommended protocol, when the cell fusion rate reached 50%–60%, Simple Fect was used to transfect plasmids into the cells.

Regarding lentivirus packaging, shRNA and packaging plasmids psPAX2 and pMD2.G were transfected together into HEK293FT cells in a 4:3:1 ratio. Culture media were collected for 48 h and 72 h, filtered through a 0.22- μ m filter membrane for sterilization, and stored at –80°C for future use. And 4 mL of lentivirus solution was added to a 10-cm cell culture dish. After 24 h of infection, the culture medium was replaced and 2 μ g/mL puromycin (Coolaber, Beijing, China) was added for screening and cultivation. Purimycin was removed in advance, and the cells were further cultured for 72 h before conducting other experiments.

The eukaryotic expression plasmid (8 μ g) was transfected into a 10-cm H1703 cell culture dish using Simple Fect, and corresponding antibiotics were added for screening for at least one week. G418 (600 μ g/mL; Sigma-Aldrich) corresponds to the pcDNA3.1-HA-TOPK plasmid, while Zeocin (50 μ g/mL; Sigma-Aldrich) corre-

Table 1. The sequences of shRNAs used in this study

Name	Sequence
shMOCK	5'-CCTAAGGTTAAGTCGCCCTCG-3'
shTOPK #1	5'-GGGAAGTAGGCCACCTATTAA-3'
shTOPK #2	5'-GAACTGTGGCTTGCCTAAATA-3'
shTOPK #3	5'-GTAATGATCATTATCGAAGTG-3'
shTOPK #4	5'-CCTTCAGAAGGTTGCTGAGTA-3'
shTOPK #5	5'-GATTCACACATTAATCTTTC-3'
shSRC #1	5'-GCTCGGCTCATTGAAGACAAT-3'
shSRC #2	5'-GACAGACCTGTCCTCAAGAA-3'
shSRC #3	5'-GTCATGAAGAAGCTGAGGCAT-3'
shSRC #4	5'-TCAGAGCGGTTACTGCTCAAT-3'
shSRC #5	5'-GCTGACAGTTTGTGGCATCTT-3'

sponds to the pcDNA4-His-SRC plasmid. Antibiotics were withdrawn at least 72 h in advance before conducting other cell experiments. Transfection of expression plasmids into HEK293FT cells did not require screening, and proteins were collected after 48 h of culture for subsequent experiments.

MTT assay

MTT (Beyotime, Shanghai, China) was dissolved in PBS at a concentration of 5 mg/mL, filtered through a 0.22- μ m membrane to remove bacteria, and stored at 4°C in the dark. The MTT solution was added to a 96-well plate at 20 μ L/well, incubated at 37°C for 4 h, and the supernatant was aspirated. Then, 150 μ L of dimethyl sulfoxide was added to each well and shaken at low speed on a shaker for 10 min to fully dissolve the crystals. The absorbance value of each well was measured at 490 nm using an enzyme-linked immunosorbent assay detector (Molecular Devices, San Jose, USA). The results were analyzed using GraphPad Prism software.

Drug toxicity experiments

Cells were inoculated into a 96-well plate at 8×10^3 cells/well and incubated for 24 h. Then, the cells were exposed to different concentrations of TOPK inhibitor (OTS964) or SRC inhibitor (eCF506) for 48 h. Subsequently, cell viability was tested by MTT assay, and the cell survival rate was analyzed via GraphPad Prism software to calculate the IC₅₀ values of drugs.

Analysis of the effectiveness of combination therapy

OTS964 and eCF506 were used at different concentrations separately or in combination. Cell proliferation was measured after 24 and 48 h of treatment via MTT assay, and the efficacy of drug combinations was evaluated via nonconstant ratios of each drug. CompuSyn (ComboSyn, Paramus, USA) was used to evaluate the interaction of combination drugs, and the Chou Talalay method was used to calculate the combination index (CI) value to evaluate the synergy/additivity/antagonism [18]. $CI < 1$, $CI = 1$, and $CI > 1$ indicate synergistic, additive, and antagonistic effects, respectively.

CCK-8 assay

The cells (2×10^3 cells/well) were inoculated into a 96-well plate, and cell proliferation was detected at 0 h, 24 h, 48 h, 72 h, 96 h, and 120 h. Specifically, 10 μ L of CCK-8 solution was added to each well, which were incubated at 37°C for 1 h, and the absorbance at 450 nm was detected via a microplate reader (SpectraMax i3x; Molecular Devices, San Jose, USA). GraphPad Prism software was used for analysis and plotting.

Colony formation assay

One milliliter of 0.33% Eagle basal medium (Sigma-Aldrich) agar containing 7×10^3 cells was added to each well of a 6-well plate, and the mixture was cultivated on 3 mL of 0.5% Eagle basal medium agar containing 10% FBS. The cells were maintained at 37°C in a 5% CO₂ incubator for 5–10 days, after which the colonies were counted and scored under a microscope.

Western blot analysis

The cells were cultured in a 6-cm diameter dish until 70%–80% confluence was achieved. One hundred microliters of RIPA buffer (Beyotime) was added to extract the protein, and Bradford staining solution (Thermo Fisher Scientific, Waltham, USA) was added to

measure the protein content. The above operations were carried out according to the manufacturer's recommended plan. The sample containing 5 $\times$ loading buffer was heated at 95°C for 5 min and then cooled on ice. Afterwards, the samples were separated via 8%–15% SDS-PAGE and then transferred to PVDF membranes (Millipore, Billerica, USA). After the PVDF membrane was blocked with 5% milk at room temperature for 1 h, the primary antibody prepared with 5% milk was added and incubated overnight at 4°C. Then, the PVDF membrane was washed with an appropriate amount of TBST for 10 min on a shaker, repeated 3 times, and incubated with the corresponding secondary antibody at room temperature for 1 h. Finally, the membrane was washed with TBST 3 times, each time for 10 min. The antibody-binding protein was detected using a chemiluminescence assay kit (Bio-Rad, Hercules, USA). Untreated cell samples were used as negative controls.

Pull-down analysis

Cells were lysed with RIPA buffer, and then centrifuged at 13,000 g at 4°C for 15 min. A small amount of the supernatant was retained as a positive control. The remaining supernatant was divided into two parts, and 20 μ L of NTA-His-Control beads and 20 μ L of prokaryotic expression NI-NTA-His-TOPK beads (Qiagen, Valencia, USA) were added. The mixture was incubated overnight at 4°C, the beads were washed 6–8 times with RIPA buffer, followed by western blot analysis.

Immunoprecipitation (IP) assay

Cells were harvested from a 10-cm cell culture plate at a confluence of ~80% and crushed with 500 μ L of IP buffer (Cell Signaling Technology). The cells were then centrifuged at 12,000 g for 10 min. The supernatant was collected and incubated overnight with 1.0 μ g of control IgG and 20 μ L of protein A/G-agarose (Santa Cruz) at 4°C, and then centrifuged at 3000 g for 3 min. The supernatant was transferred to a 1.5-mL EP tube, incubated overnight with mouse monoclonal anti-TOPK antibody and 20 μ L of protein A/G-agarose at 4°C, and then centrifuged at 3000 g for 3 min. The supernatant was carefully aspirated and discarded, and the immunoprecipitate was collected. Afterwards, the samples were separated via 8% SDS-PAGE and analyzed via western blotting.

In vitro kinase assay

Kinase buffer (10 $\times$) was purchased from Millipore. The inactive substrate (2 μ g) and active kinase (0.2 μ g in a 30 μ L reaction mixture) were incubated at 37°C for 1 h in 1 $\times$ kinase buffer containing 100 μ M unlabeled ATP. Then, 5 $\times$ SDS buffer was added to the sample, which was analyzed via SDS-PAGE and visualized via western blotting.

Phosphorylation omics analysis

The cell protein samples were prepared, and then liquid chromatography-mass spectrometry (LC-MS/MS) was performed via TMT technology at SpecAly Life Technology (Wuhan, China). Specifically, after the protein sample was digested with trypsin, the peptides were labeled using a TMT assay kit (Thermo Fisher Scientific). Next, the TMT-labeled peptides were graded via high-pH reversed-phase high-performance liquid chromatography (HPLC) via an Agilent 300Extend C18 column (5 μ m particles, 4.6 mm inner diameter, 250 mm length). Each component was subsequently enriched with phosphopeptides via immobilized metal affinity chromatography (IMAC)-TiQ2. Specific peptides were subsequently selected for dissolution digestion and LC-MS/MS, and the resulting

MS data were processed via the MaxQuant search engine (v.1.5.2.8). Finally, bioinformatics methods were used to analyze the MS data.

Immunohistochemistry (IHC) analysis

The LUSC tissue chip (HLugs180Su02) was purchased from Shanghai Outdo Biotech Co., Ltd. (Shanghai, China). The tissue chip was dewaxed, rehydrated, and treated with 3% hydrogen peroxide for 25 min. Antigen retrieval was performed in citrate buffer at pH 6.0 in a microwave oven for 8 min. After the glass slides were blocked with 3% bovine serum albumin in phosphate-buffered saline (PBS) for 30 min, the tissue sections were incubated overnight with the designated primary antibody at 4°C, and the corresponding secondary antibody was added for color development. The images were captured with a BX51 microscope (Olympus, Tokyo, Japan). The immunostaining area and intensity were graded according to the Remmele scoring method, and the product of the two was the final score.

Tumor xenografts

Five-week-old male NOD.Cg-Prkdc^{scid}Il2rg^{tm1Sug}/Jicrl mice (18–20 g, $n = 20$) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). After one week of adaptation, NCI-H520 cells (4×10^6 cells/0.1 mL RPMI-1640 medium) were inoculated subcutaneously into the right abdomen of each mouse and randomly divided into four groups ($n = 5$ in each group) for drug testing on the second day after inoculation. The dosage regimens used were as follows: eCF506 (30 mg/kg) was administered orally once a day; OTS964 (30 mg/kg) was administered via intraperitoneal injection every three days; or a combina-

tion of two treatments was administered. All the groups were treated continuously until the mouse tumor size reached 1000 mm³, and the experiment was terminated. This experiment adopted a double-blind method. During this period, the mice were weighed every 3 days, and the tumor volume was calculated via the ellipsoid formula (length $\times$ width $\times$ height $\times$ 0.52). Animal maintenance and experimental procedures were approved by the Animal Care Committee of Servicebio (Wuhan, China).

Statistical analysis

All the experiments were repeated three times. Data are presented as the mean $\pm$ SEM. Statistical analysis was conducted via Student's *t*-test, with $P < 0.05$ indicating significant difference.

Results

Increased expression of TOPK in LUSC tissue is associated with patient prognosis and tumor stage

To explore the role of TOPK in LUSC, we analyzed its mRNA expression in LUSC through the TCGA and GEO databases. Compared with that in normal tissues, the expression of TOPK mRNA was significantly increased in LUSC tissues (Figure 1A,B). We subsequently used a LUSC tissue chip to detect the protein expression of TOPK. The IHC results of the tissue chip revealed that in LUSC tissue, the area and intensity of TOPK staining were significantly greater than those in normal tissue, and the score was also significantly greater (Figure 1C), indicating a significant increase in the protein expression of TOPK in tumor tissue. To confirm whether SRC affects patient survival, we divided 90 patients into a TOPK low-expression group (0–5 points; $n = 45$)

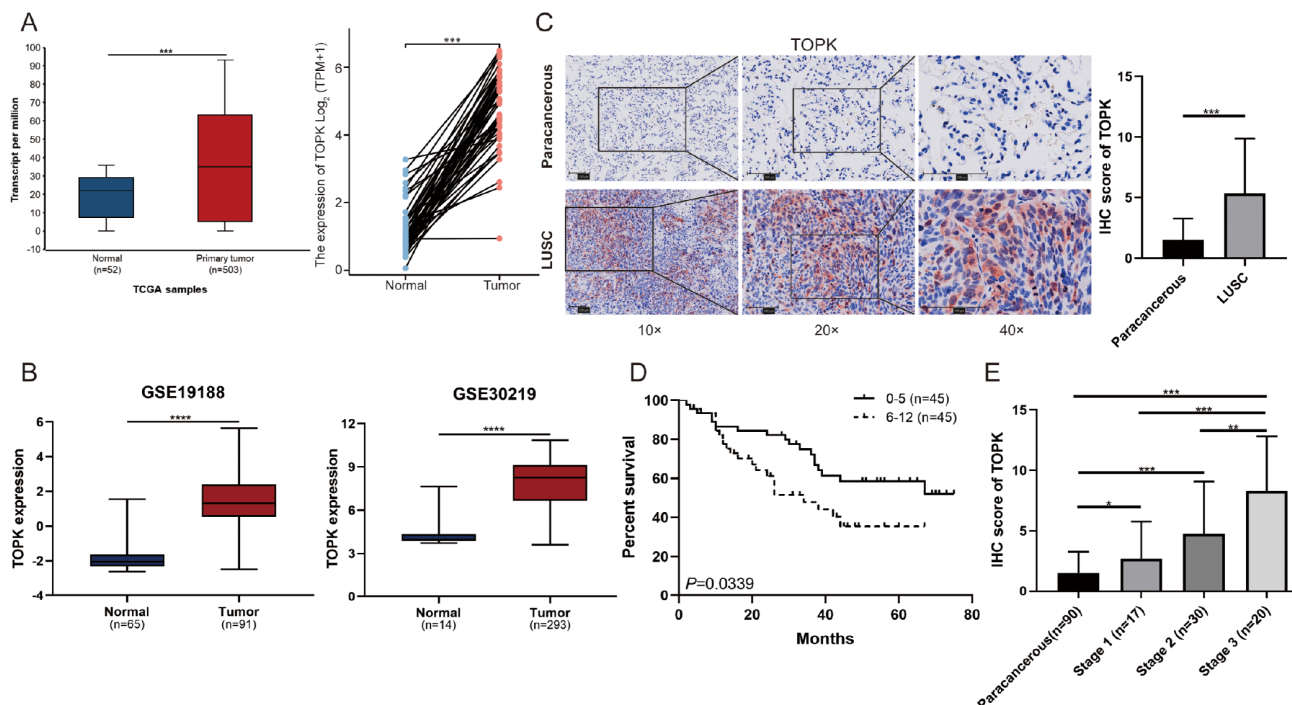


Figure 1. TOPK is abnormally highly expressed in LUSC (A) TCGA database analysis demonstrated that TOPK expression was increased at the mRNA level in tumor tissues. On the left are the overall analysis results of mRNA expression for the two groups; on the right is the result of paired analysis. (B) TOPK expression was elevated in LUSC tumor tissues in the GSE19188 and GSE30219 datasets. (C) The IHC score of TOPK was greater in LUSC tumor tissue. (D) LUSC patients with high IHC TOPK scores had shorter survival time. (E) The higher the stage of LUSC patients was, the higher the IHC score of TOPK was. LUSC: lung squamous cell carcinoma; IHC: immunohistochemistry. Data are presented as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

and a TOPK high-expression group (6–12 points; $n = 45$) on the basis of the scores in Figure 1C. Survival analysis revealed that the group with high TOPK expression had a shorter survival time (Figure 1D, $P = 0.0339$). Moreover, the higher the tumor stage of a patient was, the greater the expression of TOPK was (Figure 1E). These results indicate that TOPK is likely to promote the progression of LUSC and affect the survival of patients.

Increased expression of SRC in LUSC tissue is also associated with patient prognosis and tumor stage

Owing to our previous discovery that SRC can phosphorylate the TOPK Y74 site and activate it in CRC [17], we speculated that SRC

may also regulate tumor growth through TOPK in LUSC. To verify this hypothesis, we examined the expression of SRC in LUSC. TCGA database analysis revealed that the expression of SRC was abnormally elevated in LUSC tissues compared with normal tissues (Figure 2A). The same method as in Figure 1 was used for IHC staining of SRC on tissue chips with the same item number. The results indicated that the SRC score was greater in carcinoma tissues (Figure 2B). Patients were subsequently divided into two groups: SRC^{low} (0–5, $n = 35$) and SRC^{high} (6–12, $n = 55$). The SRC^{high} group had shorter survival time (Figure 2C). Moreover, the expression of SRC was significantly increased in Stages 2 and 3 patients (Figure 2D). Correlation analysis revealed a positive correlation between

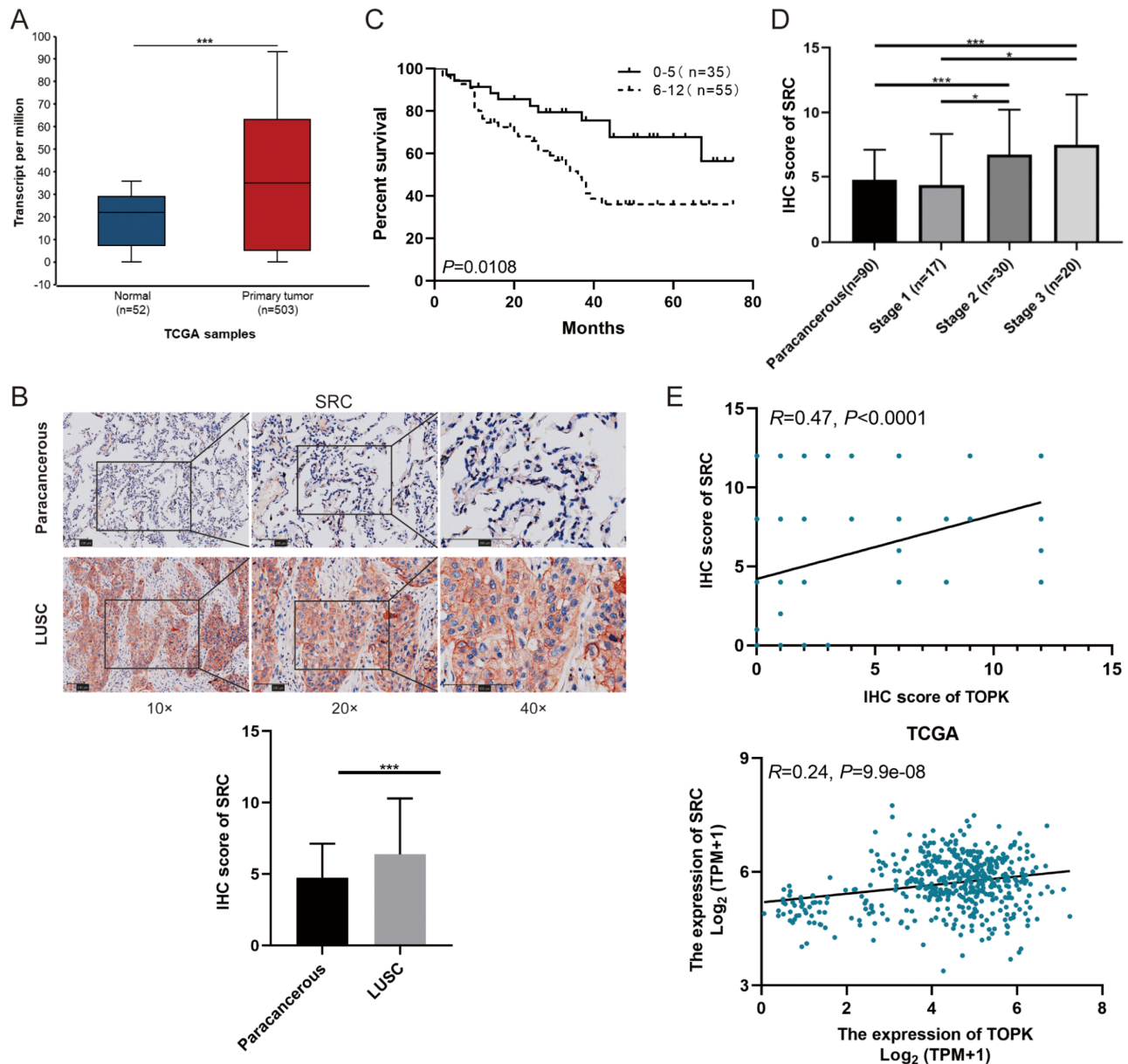


Figure 2. SRC is abnormally overexpressed in LUSC (A) SRC mRNA expression was increased in LUSC tissue. (B) The IHC results of the LUSC tissue chip indicated that SRC scores were higher in tumor tissues. (C) LUSC patients with high IHC SRC scores had shorter survival time. (D) The higher the stage of LUSC patients was, the higher the IHC score of SRC. (E) In LUSC, the expressions of SRC and TOPK were positively correlated. Up: the results of the analysis of the IHC staining scores for TOPK and SRC in the organizational chips; bottom: the results of the analysis of TOPK and SRC expressions in the TCGA database. LUSC: lung squamous cell carcinoma; IHC: immunohistochemistry. Data are presented as the mean $\pm$ SEM. * $P < 0.05$, *** $P < 0.001$.

SRC and TOPK expression in LUSC patients (Figure 2E). These results indicate that SRC is likely to interact with TOPK in LUSC, thereby regulating tumor growth.

SRC or TOPK overexpression promotes LUSC cell growth

To explore the mechanisms of SRC and TOPK in LUSC, we examined their expression in different LUSC cells and found that both were upregulated in NCI-H520 cells and downregulated in NCI-H1703 cells (Figure 3A). Subsequently, NCI-H1703 was

selected to construct cell lines overexpressing SRC or TOPK: NCI-H1703 His-SRC and NCI-H1703 HA-TOPK (WT) (Figure 3B,C), and cell growth curve experiments and soft agar assays were conducted. The results revealed that the overexpression of SRC or TOPK increased the cell growth rate (Figure 3D) and enhanced the colony formation ability (Figure 3E). To clarify the mechanism of occurrence, we detected the expressions of cell proliferation-related pathway proteins in overexpressing cell lines. After starvation of NCI-H1703 cells for 24 h and stimulation with EGF for 20 min, as expected, we found that overexpression of SRC led to an increase in

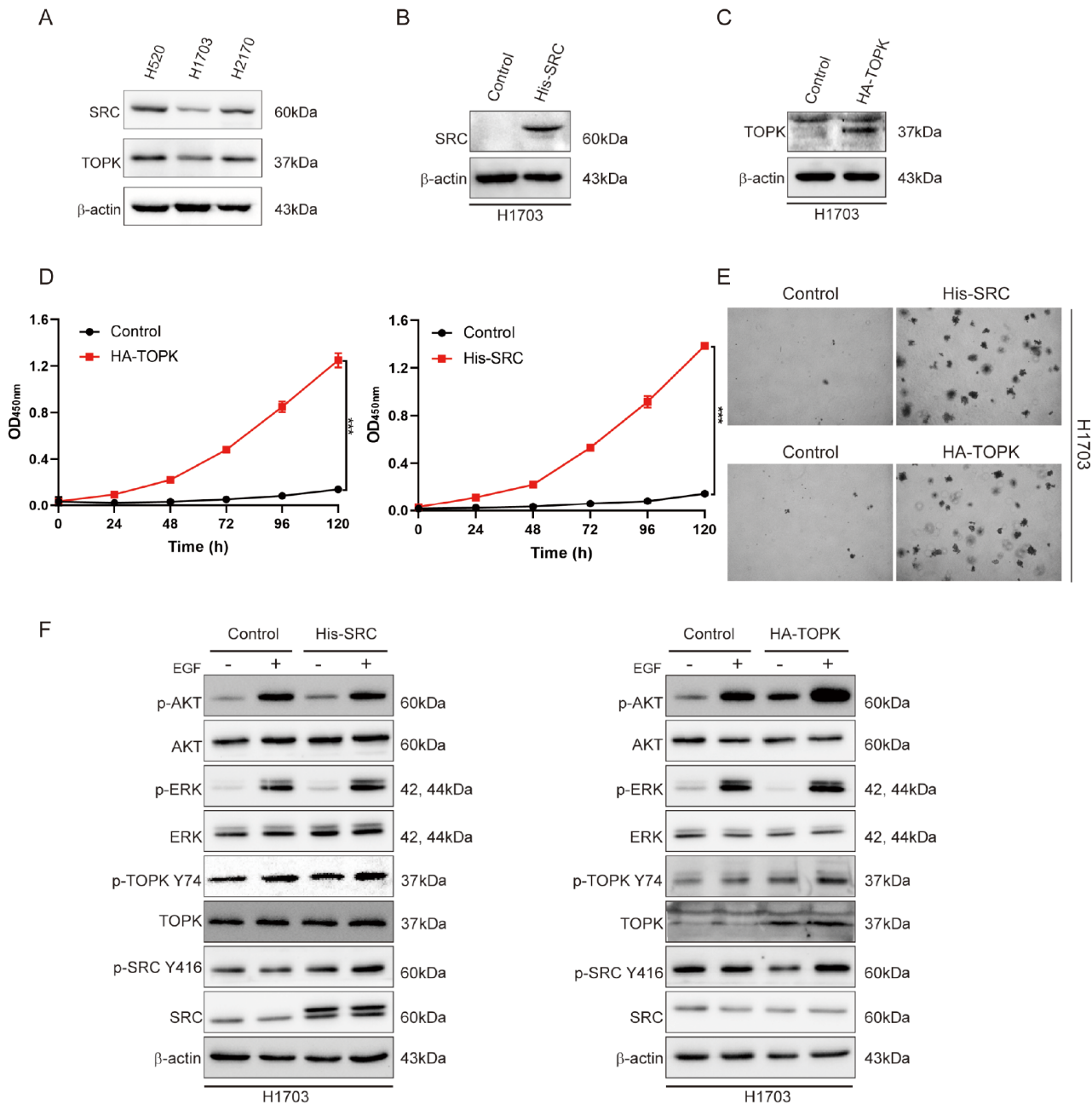


Figure 3. The overexpression of SRC or TOPK in LUSC cells promotes cell growth (A) The expression levels of SRC and TOPK in various LUSC cell lines. (B,C) Successfully constructed NCI-H1703 cell lines overexpressing SRC or TOPK. (D) Overexpression of SRC/TOPK increased the growth rate of NCI-H1703 cells. (E) Overexpression of SRC/TOPK enhanced the *in vitro* colony formation ability of NCI-H1703 cells. Magnification: 4x. (F) Overexpression of SRC/TOPK increased the expressions of p-AKT and p-ERK. LUSC: lung squamous cell carcinoma. Data are presented as the mean ± SEM. ****P* < 0.001.

TOPK expression; surprisingly, overexpression of TOPK also increased the activity of SRC; moreover, overexpression of SRC or TOPK significantly increased the expression levels of p-AKT and p-ERK, the main proteins associated with cell proliferation (Figure 3F). These data indicate that SRC or TOPK promotes NCI-H1703 cell growth by activating these signaling pathways.

SRC or TOPK knockdown inhibits LUSC cell growth

As shown in Figure 3A, a cell line with silenced SRC or TOPK was constructed from NCI-H520 cells. On the basis of the results in Figure 4A,B, we selected NCI-H520 shTOPK #2/3 and NCI-H520 shSRC #1/2, which had better silencing effects, for subsequent experiments. The cell survival curve and soft agar assay results indicated that knocking down TOPK or SRC inhibited cell proliferation and colony formation ability (Figure 4C,D). Similarly, we continued to investigate cell proliferation-related pathways and found that inhibiting the expression of SRC or TOPK resulted in a decrease in the expressions of p-AKT and p-ERK, which inhibited cell proliferation. In addition, consistent with the results in Figure 3F, downregulating the expression of SRC resulted in a decrease in TOPK expression; reducing the expression of TOPK led to a decrease in SRC activity, suggesting that TOPK may also positively regulate SRC in LUSC via feedback (Figure 4E).

SRC-TOPK promotes tumorigenicity through enhancing Rb1 phosphorylation in LUSC

To verify whether TOPK can activate SRC, we conducted *in vitro* kinase experiments. The results showed that active TOPK kinase increased the serine phosphorylation level and activity of SRC. Moreover, the activated SRC in the reaction system in turn activated TOPK, slowing down the migration rate of the TOPK protein and shifting the band position upward (Figure 5A). These findings prove that there is indeed a positive feedback loop between SRC and TOPK. Given the previous research on SRC regulation of TOPK, we focused on testing whether TOPK can activate SRC in 293FT cells. After the overexpression of TOPK, SRC activity increased; after the addition of the TOPK inhibitor OTS964, the changes weakened, indicating that TOPK indeed positively regulates the activity of SRC (Figure 5B).

Next, we investigated the transcription factors regulated by the SRC-TOPK feedback loop. First, H1703 cells overexpressing TOPK were subjected to phosphogenomic sequencing. The phosphorylation omics data revealed that as TOPK expression increased, the tumor suppressor RB1 was the transcription factor with the greatest increase in phosphorylation level (Figure 5C). We found that TOPK can phosphorylate RB1 *in vitro* (Figure 5D). IP and pull-down experiments revealed a direct interaction between TOPK and RB1 in H520 cells (Figure 5E). Moreover, overexpression of SRC/TOPK resulted in increased expression of p-RB1, indicating inactivation of RB1; downregulating SRC/TOPK expression resulted in the opposite effect (Figure 5F,G).

These results indicate that the SRC-TOPK positive feedback loop in LUSC promotes tumor cell proliferation by phosphorylating RB1 and inhibiting its activity.

TOPK inhibitors and SRC inhibitors can synergistically inhibit cell proliferation *in vitro* and *in vivo*

Drug resistance and a lack of effective targeted therapy are the main issues in the clinical treatment of LUSC, and finding new

therapeutic targets is crucial. Positive feedback regulation between SRC and TOPK affects LUSC cell proliferation, suggesting that these proteins may be therapeutic targets. According to the IHC results shown in Figures 1 and 2, patients with high expressions of SRC and TOPK in LUSC had a shorter survival period, which was not conducive to patient prognosis (Figure 6A). We treated NCI-H520 cells with the TOPK inhibitor OTS964 and the SRC inhibitor eCF506 and found that OTS964 inhibited the proliferation of NCI-520 cells, whereas eCF506 partially inhibited proliferation (Figure 6B,C). We conducted a combination therapy experiment on the basis of the IC₅₀ values of the two inhibitors in NCI-H520 cells. On the basis of the comprehensive results at 24 h and 48 h, the combination of 15 nM OTS964 and 0.5 μ M eCF506 had the most stable inhibition rate and CI value, indicating the best combined effect (Figure 6D). These findings indicated that the two inhibitors had a synergistic effect and that their combination effectively inhibited the proliferation of LUSC cells.

To explore the mechanism of the inhibitors, we detected the expressions of relevant proteins. After treatment with OTS964 and eCF506, the expressions of Caspase 7 and PARP decreased, whereas the expressions of Cleaved-Caspase 7 and Cleaved-PARP increased, indicating an increase in the degree of apoptosis (Figure 6E). The combination of the two drugs resulted in the most significant changes, indicating that the two inhibitors can synergistically promote the apoptosis of LUSC cells.

To clarify whether SRC and TOPK inhibitors still have synergistic effects *in vivo*, we conducted mouse xenograft experiments. The results showed that at the end of the experiment, the mice in the two-drug combination group presented the smallest tumor size and the lightest weight (Figure 6F,G). The tumor growth curve confirmed these results (Figure 6H). Although the weight of the mice in the drug combination group slightly decreased throughout the entire experiment, there was no statistically significant difference compared with that of the control group, indicating that drug toxicity can be ignored (Figure 6I). These data indicate that TOPK and SRC inhibitors can synergistically inhibit LUSC cell proliferation and have been validated in animal models, providing new possibilities for targeted therapy.

Discussion

In recent years, significant breakthroughs have been made in the treatment of cancer, but specific targeted therapies for LUSC are still lacking because of its unique location of onset and high mutation rate [19]. Most LUSC patients are diagnosed at a late stage, with limited systemic treatment options and high disease-related mortality rates. Even with the addition of PD-L1 inhibitors, the median OS of LUSC patients receiving first-line combination immunotherapy and chemotherapy was only 17.1 months [20]. The dysregulation of kinase activity plays an important role in the pathogenesis of many malignant tumors and is one of the most important drug targets in the 21st century. Multiple tyrosine kinase inhibitors (TKIs) have been approved by the FDA and have been widely used in LUAD. Unfortunately, efforts to find TKIs in LUSCs have not been successful. The reason may be that although abnormal signaling through the EGFR, FGFR, or DDR pathways contributes to tumor growth, LUSCs are unlikely to rely entirely on these receptor tyrosine kinases for survival [21]. More precise biomarkers or combination therapies to overcome bypass pathways may be better choices. Therefore, it is essential to search for new

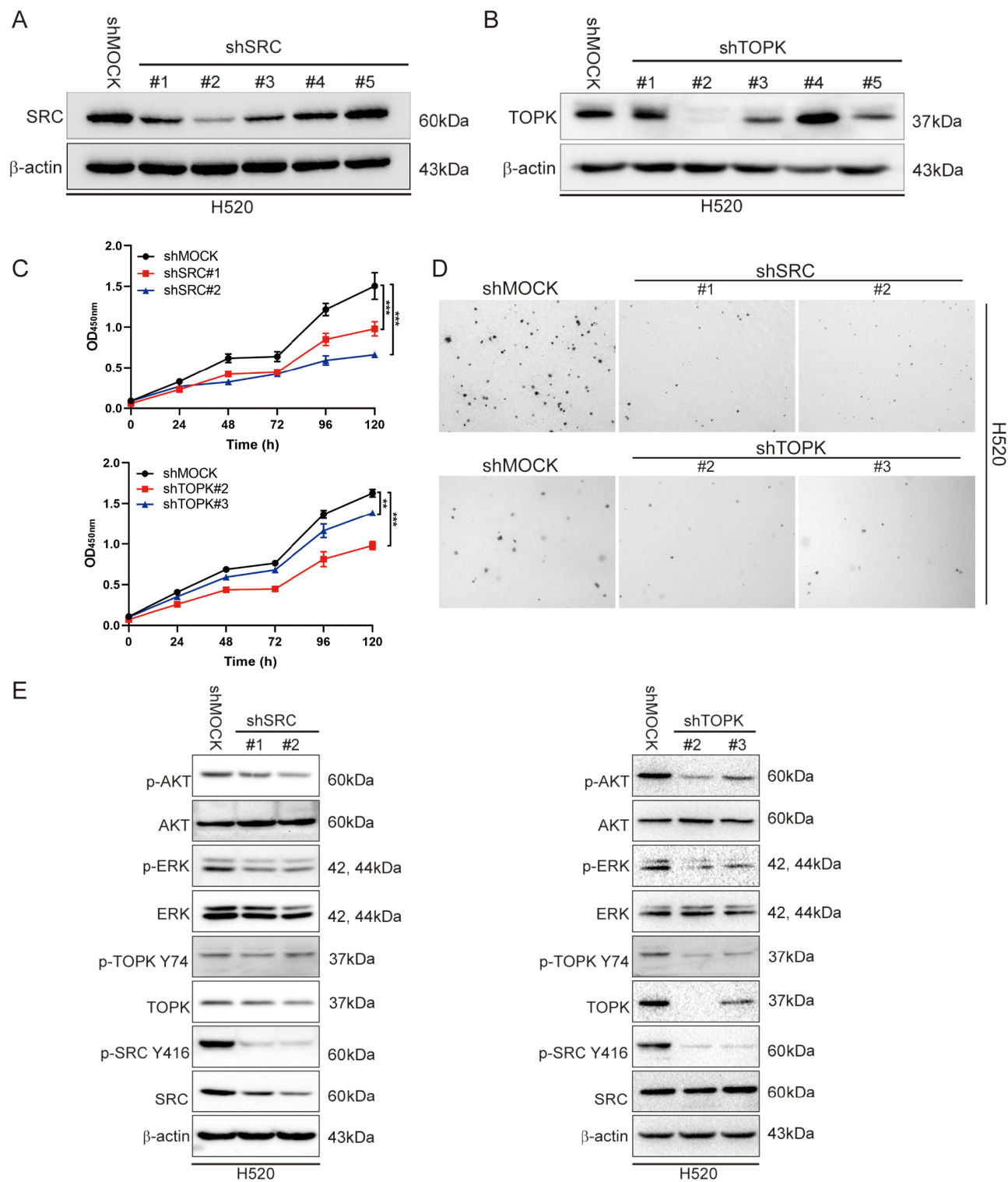


Figure 4. SRC/TOPK knockdown inhibits cell growth in LUSC cells (A,B) Construction of a stable SRC/TOPK-knockdown cell line in NCI-H520 cells. (C) SRC/TOPK knockdown inhibited the growth rate of NCI-H520 cells. (D) SRC/TOPK knockdown weakened the *in vitro* colony formation ability of NCI-H520 cells. Magnification: 4x. (E) SRC/TOPK knockdown resulted in a decrease in the expressions of p-AKT and p-ERK. LUSC: lung squamous cell carcinoma. Data are presented as the mean ± SEM. ** $P < 0.01$, *** $P < 0.001$.

therapeutic targets. In this study, we investigated the roles of the SRC-TOPK positive feedback loop in LUSC, providing new possibilities for treatment.

Both SRC and TOPK have been found to be associated with poor prognosis in LUAD patients [9,22,23]. Our study revealed that in LUSC, the expression of SRC or TOPK increased with tumor stage,

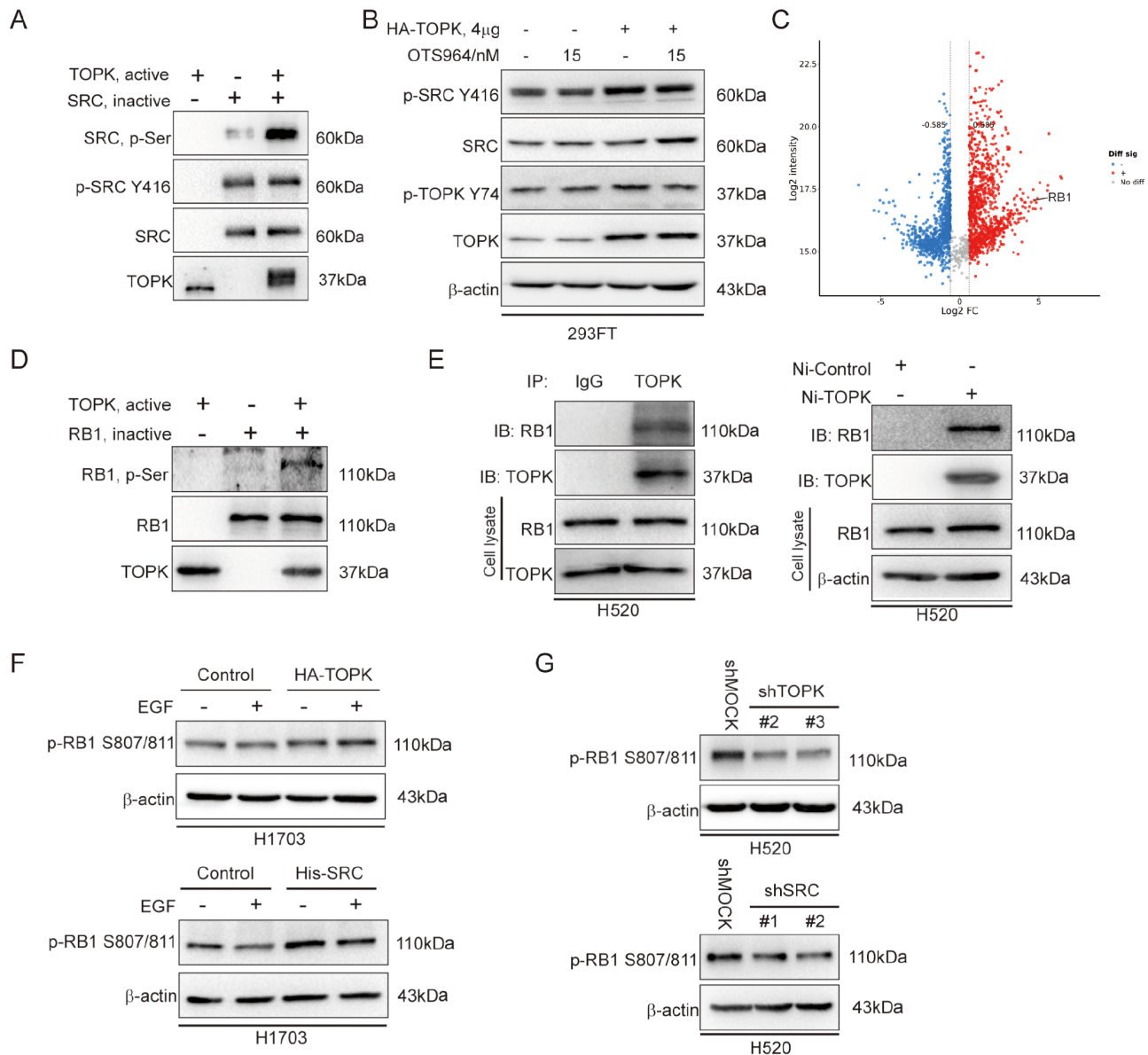


Figure 5. The SRC-TOPK positive feedback loop promotes tumorigenesis by regulating RB1 in LUSC (A) *In vitro* kinase experiments revealed a positive feedback loop between SRC and TOPK. (B) Exogenous TOPK increased the activity of SRC in 293FT cells. (C) Phosphoromics revealed that RB1 is the transcription factor with the greatest change in H1703 cells after overexpression of TOPK. (D) *In vitro* kinase experiments with the TOPK and RB1 proteins. (E) IP and pull-down experiments revealed a direct interaction between TOPK and RB1 in H520 cells. (F) Overexpression of SRC/TOPK increased RB1 phosphorylation in H1703 cells. (G) SRC/TOPK knockdown resulted in decreased phosphorylation of RB1. LUSC: lung squamous cell carcinoma.

and patients with high expression had shorter survival time, indicating that both promoted the malignant progression of tumors. Therefore, SRC or TOPK may serve as a clinical diagnostic marker to predict the prognosis of patients.

Our previous studies revealed that SRC can phosphorylate the TOPK Y74 site, which was confirmed in this study. In addition, we found that TOPK can phosphorylate SRC and increase its activity. With respect to the phosphorylation of SRC, the Y416 site is an autophosphorylation site, indicating increased activity [24]; there are few reports on serine phosphorylation sites, and some studies have shown that Ser17 phosphorylation may affect multiple signaling networks [25,26]. According to our experimental results, TOPK mainly increased the serine phosphorylation level of SRC,

which may involve two possibilities: (i) it directly enhances the activity of SRC; and (ii) it results in conformational changes in SRC, increases Y416 phosphorylation, and promotes SRC activation. In summary, the specific serine sites and roles of TOPK phosphorylation in SRC still need to be further explored.

We first discovered a direct interaction between TOPK and RB1 in LUSC cells. The SRC/TOPK positive feedback loop increased the protein expression of p-RB1, inhibiting the function of RB1. When RB1 is phosphorylated in S807/811, it loses its activity, leading to transcriptional activation of downstream genes, allowing the cell cycle to enter the S phase and subsequent expression of necessary downstream genes, thereby promoting tumor cell proliferation [14].

As mentioned earlier, the growth of LUSC cells may not be

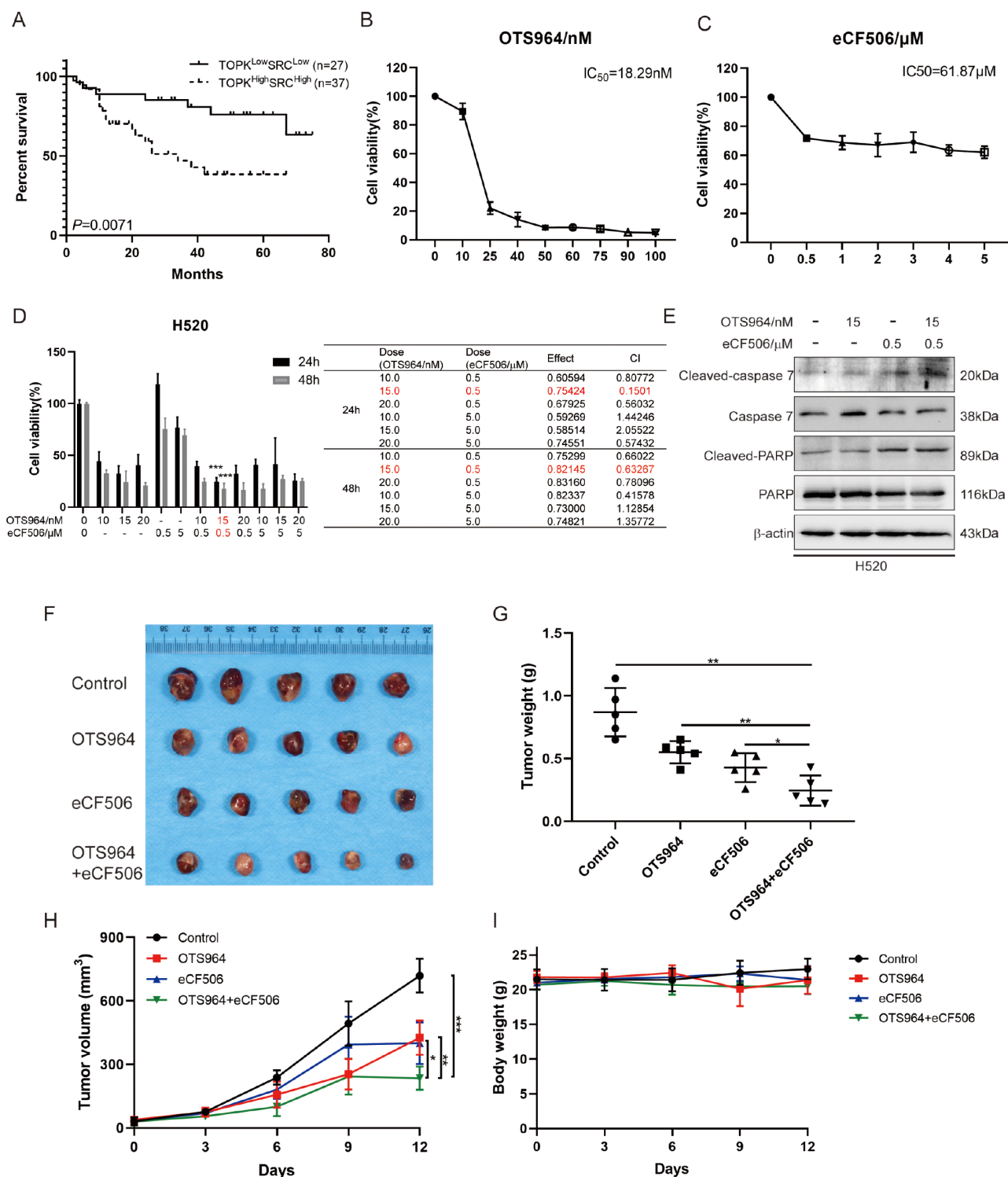


Figure 6. SRC and TOPK inhibitors synergistically inhibit LUSC proliferation *in vitro* and *in vivo* (A) LUSC patients with high IHC scores for both SRC and TOPK had shorter survival time. (B) TOPK inhibitor OTS964 inhibited the proliferation of NCI-H520 cells. (C) eCF506 inhibitor SRC partially inhibited the proliferation of NCI-H520 cells. (D) The combination of OTS964 and eCF506 had a synergistic effect in LUSC cells. (E) Two LUSC inhibitors promoted apoptosis. (F) Tumor sizes in the 4 groups of experimental mice. (G) Tumor weights in the 4 groups of experimental mice. (H) Tumor growth curves of the 4 experimental groups. (I) Body weight curves of the 4 experimental groups. The number of experimental mice in each group was $n = 5$. LUSC: lung squamous cell carcinoma; IHC: immunohistochemistry. Data are presented as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

entirely dependent on common RTKs, as indicated by the NCI-H520 cell line used in this study. It has an EGFR deletion mutation and cannot be activated by EGF stimulation when it amplifies cell growth signals, but its cell growth ability and malignancy are not weakened. However, our results showed that inhibitors of SRC and TOPK can synergistically inhibit the proliferation of NCI-H520 cells and promote apoptosis both *in vitro* and *in vivo*. There are currently approved SRC inhibitors on the market, and TOPK inhibitors are also undergoing clinical trials. The results of these trials have great potential to provide new therapeutic targets for clinical translation in the future.

In summary, we found that both SRC and TOPK are upregulated in LUSC and are unfavorable factors for patient survival. The positive feedback loop between SRC and TOPK promotes tumor growth and increases the expressions of p-AKT and p-ERK through enhancing Rb1 phosphorylation. SRC and TOPK inhibitors can synergistically inhibit cell proliferation by promoting the activation of the apoptosis-related proteins Cleaved-Caspase 7 and Cleaved-PARP. These genes may be potential therapeutic targets for LUSC.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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