SP4 Facilitates Esophageal Squamous Cell Carcinoma Progression by Activating PHF14 Transcription and Wnt/B-Catenin Signaling

Li Wei[1], Chaowei Deng[2], Bo Zhang[3], Guanghui Wang[4], Yan Meng[3], and Hao Qin[3]

ABSTRACT

Specificity protein 4 transcription factor (SP4), a member of the Sp/Krüppel-like family (KLF), could bind to GT and GC box promoters, and plays an essential role in transcriptional activating. Despite *SP4* having been detected to be highly expressed in a variety of human tumors, its biological effect and underlying molecular mechanism in esophageal squamous cell carcinoma (ESCC) remains unclear. Our research discovered that high SP4 expression is detected in primary ESCC specimens and cell lines and is strongly associated with the ESCC tumor grade and poor prognosis. *In vitro*, knockdown of SP4 suppressed cell proliferation and cell-cycle progression and promoted apoptosis, whereas overexpression of SP4 did the opposite. *In vivo*, inhibiting SP4 expression in ESCC cells suppresses tumor growth. Subsequently, we demonstrated that SP4 acts as the transcriptional upstream of *PHF14*, which binds to *PHF14* promoter region, thus promoting *PHF14* transcription. PHF14 was also significantly expressed in patient tissues and various ESCC cell lines and its expression promoted cell proliferation and inhibited apoptosis. Moreover, knockdown of SP4 inhibited the Wnt/β-catenin signaling pathway, whereas overexpression of PHF14 eliminated the effects of SP4 knockdown in ESCC cells. These results demonstrate that SP4 activates the Wnt/β-catenin signaling pathway by driving *PHF14* transcription, thereby promoting ESCC progression, which indicates that SP4 might act as a prospective prognostic indicator or therapeutic target for patients with ESCC.

Implications: This study identified *SP4/PH14* axis as a new mechanism to promote the progression of ESCC, which may serve as a novel therapeutic target for patients with ESCC.

Introduction

Esophageal squamous cell carcinoma (ESCC) is the main histologic subtype of esophageal cancer which is a malignant tumor that occurs in the esophageal epithelium, accounting for more than 90% of esophageal cancer cases (1–4). Its incidence rate in East Asia ranks first in the world (5). In recent years, the incidence rate of ESCC has decreased significantly due to the reduction of the poor population, but the postoperative prognosis of patients is still very poor, resulting in more than 500,000 deaths every year (2, 6–9). The reason for this on one hand is due to the fact that most patients with ESCC are usually diagnosed at advanced stage (9). On the other hand, because the most predominant neoadjuvant chemoradiotherapy for ESCC has not achieved satisfactory therapeutic effect, that is, half of the patients relapse before and after surgery, and the most common is distant metastasis (9–12). Therefore, the clinical treatment of ESCC has a long way to go, and new biomarkers and drug targets should urgently be found (13–15).

Specificity protein 4 transcription factor (SP4) is a member of the SP/KLF family, which includes 25 transcription factors (16, 17). As a transcription factor, SP4 could bind to GC-rich DNA motifs and play multiple roles in maintaining cellular homeostasis (16–18). SP4 has been reported to be overexpressed in a variety of cancers, including colon cancer (19), pancreatic cancer (20), breast cancer (21), thyroid cancer (22), prostate cancer (23), bladder cancer (24), and esophageal adenocarcinoma (25). In addition, SP4 plays an oncogenic role in SKBR3 and MDA-MB-231, A549, SW480, 786-o, PANC1, l3.6pl, miapaca2, Rh30 cell lines (26, 27) and also contributes to cancer cell proliferation, survival, and angiogenesis (19, 20, 23, 24, 28–30). However, the focus on SP1 has left the functional role of SP4 in cancer cells uninvestigated in depth (26, 31). Although SP4 shares a similar modular structure with SP1 and both bind GC-rich promoter sequences, they also exhibit different numbers of isoforms and unique DNA-binding characteristics (31–33). Therefore, an in-depth study of the biological function of SP4 in tumors and its molecular mechanism is warranted.

The plant homeodomain (PHD) finger protein 14 (PHF14) is a member of the plant homeodomain finger protein family, some members of which have been reported to be cancer associated (34). Whereas the biological functions of PHF14 in tumors vary according to different cancer types (35). PHF14 inhibits DNA damage in colorectal cancer, thus inhibiting apoptosis (34) and it is also reported that PHF14 depletion could inhibit cell proliferation in lung cancer cells and bladder cancer cells (36). In addition, PHF14 inhibits apoptosis and promotes proliferation and invasion of glioblastoma cells through the Wnt signaling pathway (35). PHF14 promotes gastric cancer cell proliferation and migration through AKT and ERK1/2 pathways (37). Broadly speaking, PHF14 tends to have an active role in tumorigenesis and progression.

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Although a certain handful of researches have investigated the relationship between SP4 and human cancers, its functions in ESCC are yet to be explored. In this study, we are the first to evaluate the role of SP4 in ESCC. Mechanistically, we first found that PHF14 is transcriptionally downstream of SP4 and transcriptionally regulated by SP4. In view of this, we were also the first to evaluate the expression of PHF14 in ESCC, and further explored the role of SP4 transcriptionally regulating PHF14 as well as its molecular mechanism in ESCC, which provided a new idea for the underlying mechanism of ESCC development, and a theoretical reference for SP4 to be a promising prognostic indicator or chemotherapy target.

Materials and Methods

Clinical specimens

With patients' written informed consent, the 73 ESCC patients' clinical tissue samples and adjacent nontumor tissues and corresponding clinicopathologic features such as age, gender, and initial stage were obtained from the Department of Oncology Surgery, the First Affiliated Hospital of Medical College in Xi'an Jiaotong University, P.R. China. Chemotherapy or radiotherapy had not been implemented on these patients before surgery according to the patients' pathology records. The study was approved by the Ethical Committee of Xi'an Jiaotong University Health Science Center. Every individual patient who participated in the research signed written informed consent. Ethical approval was given by the Ethics Committee of The First Affiliated Hospital of Xi'an Jiaotong University. The whole study was performed in accordance with ethical standards just as in the 1964 Declaration of Helsinki and its later amendments.

Reagents and antibodies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; M5655) and dimethyl sulfoxide (DMSO; D5879), were purchased from Sigma-Aldrich. RIPA lysis buffer, phenylmethylsulfonylfluoride (PMSF), and transfection reagent Lipofectamine 2000 were obtained from Thermo Fisher Scientific (New York, NY). Annexin V-APC (2005128) and propidium iodide (PI; 2048964) were obtained from Invitrogen. Super ECL prime (catalog no. S6008–100 mL) was purchased from US Everbright Inc. Anti-SP4 (sc-390124) antibody was purchased from Santa Cruz Biotechnology. CDK4 (#12790), CDK6 (#13331), Cyclin D1 (#2922), active Caspase-3 (#9661), and Wnt3a (#2721) were purchased from Cell Signaling Technology, Inc. The GAPDH (60004-1-Ig), β-catenin (51067-2-AP), PHF14 (ProteinTech, catalog no. 24787–1-AP, RRID: AB_2879724), and mouse IgG (ProteinTech, catalog no. B900620, RRID: AB_2883054) antibodies were purchased from ProteinTech Group.

Cell culture

EC109 (RRID: CVCL_6898), TE-1 (RRID: CVCL_1759), SEG-1, and Het-1A (RRID: CVCL_3702) cell lines were used in this study and obtained from the Procell Life Science & Technology Co. Ltd. Short tandem repeat analysis for cell lines used in the study has been conducted by Shanghai Biowing Applied Biotechnology Co. Ltd. on March 3, 2022. All the cell lines were free of *Mycoplasma* contamination as tested by vendors using a MycoAlert kit from Lonza annually, and were grown in RPMI1640 medium with 1% penicillin–streptomycin Solution and 10% FBS at standard conditions (5% CO_2, 37°C). Throughout the entire study, the cell lines were passaged approximately 10–15 times between thawing and use.

Quantitative and reverse transcriptional PCR

According to previous research methods (38), total RNA was isolated from collected cells and human tissues using TRizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was reverse-transcribed to cDNA using a PrimeScript RT reagent Kit (TaKaRa Biotechnology Co., Ltd.). qRT-PCR was conducted using the iCycler IQ Multicolor qRT-PCR Detection System (Bio-Rad). The results were normalized to *GAPDH* gene expression. Each reaction was repeated three times. All primers were shown as follows:

SP4-F: 5′-TCCACCAAGCAGATTGTCTTTCTTC-3′
SP4-R: 5′-TCATCTGCCTGTTGTGCATTTCT-3′
PHF14-F: 5′-GCAACTTGCAAGGGAACTGG-3′
PHF14-R: 5′-AAGAGGTTTCCGGGATTGCC-3′
GAPDH-F: 5′-GCCGTATCGCTCAGACAC-3′
GAPDH-R: 5′-GCCTAATACGACCAAATCC-3′

Transfection, infection experiments, and plasmids

Lentiviruses for human SP4 were obtained from Gene Pharma Co. Ltd. Small-interfering siRNA for human SP4 and PHF14 knockdown as well as negative control were obtained from Gene Pharma Co. Ltd., and showed as follows:

Negative siRNA (NC-siRNA) sense: 5′-UUCUCCGAACGUGUCACGUTT-3′
Negative siRNA (NC-siRNA) antisense: 5′- ACGUGACACGUUCGGAGAATT-3′
SP4 siRNA-1 sense: 5′-GUGGAUUACAGAGUAGGAATT-3′
SP4 siRNA-1 antisense: 5′-UUCCUACUCUGUAAUCCACTT-3′
SP4 siRNA-2 sense: 5′-GGCCAGCAGAAAAUUCUCUTT-3′
SP4 siRNA-2 antisense: 5′-AGAGAAUUUUCUGCUGGCCTT-3′
PHF14 siRNA-1 sense: 5′-GUCAGCGAGCCAAAAAAAUTT-3′
PHF14 siRNA-1 antisense: 5′-AUUUUUUUGGCUCGCUGACTT-3′
PHF14 siRNA-2 sense: 5′-CCAUGAUCAGCAUCUUCUUTT-3′
PHF14 siRNA-2 antisense: 5′-AAGAAGAUGCUGAUCAUGGTT-3′
sh-control: 5′-AAAAGAGGCTTGCACAGTGCATTCAAGACGTGCACTGTGCAAGCCTCTTTT-3′
sh-SP4: 5′-TGGAGAAACTGATGTGGATTTTCTCGAGAGAGAATTTTCTGCTGGCCTTTTTTC-3′

The recombinant plasmid containing the human SP4 as well as PHF14 full-length cDNA cloned into the PCDH-CMV-MCS-EF1-Hygro vector was purchased from Youbao Company.

For transfection, EC109 as well as TE-1 cells in the logarithmic phase were inoculated into 6-well plates (2×10^5 cells in 2 mL medium per well) and cultured overnight in a 37°C incubator. Lipofectamine 2000 (6 μL/well) was used to optimize siRNA and overexpress plasmid (2 μg/well) transfection for 72 hours.

For infection, lentiviruses were used to infect EC109 cells twice at 40% confluency, 12 hours per infection. The infected cells were screened by treatment for 36 hours with puromycin (Life Technologies), and the surviving cells were frozen and stored in liquid nitrogen for subsequent experiments.

Cell viability detection

Cells were seeded at 3,000 cells in 100 mL RPMI1640 per well in 96-well culture plates, respectively, treated with siRNA or overexpression plasmid for 24, 48, and 72 hours. At these indicated time points, 10 μL of 5 mg/mL MTT was added to each well and incubated at 37°C for 4 hours. Next, suspension liquid was thrown away and 150 μL DMSO was used to dissolve formazan crystals. The absorbance values

(OD 492 nm) were measured using a multi-microplate test system (POLARstar OPTIMA, BMG Lab Technologies).

Live-cell imaging system

ESCC cells were seeded at 3,000 cells in 100 mL RPMI1640 per well in 96-well culture plates, respectively, treated with siRNA or overexpression plasmid. Cells were imaged on 96-well culture plates using LeicaAndor and Nikon-Andor spinning disc confocal imaging systems with an EM-CCD camera. The number of cells was recorded at 24, 48, and 72 hours, respectively.

Cell-cycle analysis

Treated ESCC cells were placed in 6-well plates with a confluence of 50% 24 hours before analysis, then were collected by trypsin digestion, and resuspended in PBS. Next, ice-cold ethanol (70%) was applied to immobilize these treated cells at 4°C overnight. The collected cells were stained with 50 μg/mL PI containing 50 μg/mL RNase A (DNase free) for 20 minutes at room temperature. Samples were subjected to flow cytometry for cell-cycle analysis by FACS (FACSCalibur, BD Biosciences).

Apoptosis analysis

Treated ESCC cells were placed in 6-well plates with a confluence of 50% 24 hours before analysis, then were collected by trypsin digestion and resuspended in PBS. The harvested cells were stained with Annexin-V FITC Apoptosis Detection Kit (Invitrogen) according to the manufacturer's instructions. Samples were subjected to flow cytometry for apoptosis analysis by using a flow cytometer (FACSCalibur, BD Biosciences), and the apoptosis populations were determined by ModFit software.

Western blot analysis

ESCC clinical tissues, normal esophageal squamous tissues, and ESCC cells were lysed with RIPA lysis buffer supplemented with PMSF and protease inhibitors (Roche) to obtain protein. Equal amounts of protein lysates were separated with 10% SDS polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was blocked with 5% BSA at room temperature for 2 hours. Next, the primary antibodies were used to incubate the membranes overnight at 4°C. Then, the PVDF membrane was incubated with the secondary antibody (peroxidase-labeled anti-mouse and anti-rabbit antibodies) at room temperature for 2 hours. The ECL reagent (Amersham) was used to incubate the membranes. Then, the luminescent signal was scanned, recorded, and quantified with Syngene GBox (Syngene).

Subcutaneous tumor xenografts

All operations were conducted orderly following to the Guidelines for Animal Health and Use (Ministry of Science and Technology, China, 2006). Ten 5-week-old female nude mice purchased from Huafukang Biotechnology Co., Ltd., were randomly divided into two groups to feed, and observed in the SPF room for a week to adapt to the new environment. By previous research methods (39), EC109 cells were stably transfected with shCtrl and shSP4. These transfected cells (1×10^6 cells per mouse) were suspended in 0.1 mL serum-free RPMI1640 and subcutaneously inoculated on the left upper back of two groups of mice respectively on a sterile workbench of an SPF room. Prior to this, mice were subjected to nasal anesthesia with isoflurane. Before and after subcutaneous injection, mouse epidermis was disinfected with 75% medical alcohol. After 1 week of injection, tumor volume [tumor volume = (length × width^2)/2] was measured every 3 days under strict and standardized feeding conditions. Before tumor collection, mice were given nasal anesthesia (isoflurane) to alleviate their pain. Then, these mice were sacrificed, and tumors were harvested and weighed. The bodies were frozen at −20°C before incineration. Finally, these tumors were photographed and recorded.

IHC staining

Tumors embedded in paraffin were cut into 5 mm–thick sections, dewaxed, hydrated, and then, these paraffin slices were placed in citrate buffer (pH 6.0) and heated in a microwave oven to 95°C for 20 minutes to facilitate antigen retrieval. After that, endogenous peroxidase activity was quenched, then using normal goat serum blocked this process. The anti-SP4 antibody was diluted with 5% normal goat serum (1:50), and added to the paraffin sections and incubated overnight at 4°C. The second antibody linked to horseradish peroxidase was added and incubated with these slices, followed by the addition of DBA reagent. Before counterstaining with hematoxylin, the results were observed under a microscope.

Chromatin immunoprecipitation-qRT-PCR assay

In accordance with previous research methods (40), the chromatin immunoprecipitation (ChIP) assay was fulfilled by using a ChIP Assay Kit (Millipore) according to the manufacturer's instructions. Formaldehyde (1%) was used to crosslink for 15 minutes in EC109 and TE-1 cells and glycine was applied to quench. DNA was sheared into 200–800 bp fragments by using sonication. These DNA fragments were incubated using SP4 or IgG antibodies for 12 hours at 4°C, respectively. Then, Dynabeads Protein A (Thermo Fisher Scientific) was applied to obtain DNA–protein complexes and TE buffer was used to elute the complexes at 65°C. Next, these complexes were treated for reversing crosslinking 8 hours at 65°C. These DNA products were isolated by using the QIA Quick PCR Purification Kit (QIAGEN) for qRT-PCR. The correlative gene-specific primers were shown as follows:

PHF14 (ChIP)-Primer 1-F: 5′-CTGATTGCGCGTTACTTCATTTACA-3′
PHF14 (ChIP)-Primer 1-R: 5′-CGACCTGAGCAGCGGCTCAAGGGCC-3′
PHF14 (ChIP)-Primer 2-F: 5′-AGCCGCTGCTCAGGTCGCTTCCGTA-3′
PHF14 (ChIP)-Primer 2-R: 5′-GTTGTCCAGGTTCTTGGCGTTTTTA-3′
PHF14 (ChIP)-Primer 3-F: 5′-GAACCTGGACAACTTGCAGTCACGA-3′
PHF14 (ChIP)-Primer 3-R: 5′-TTTTGTTTCTGATCTGTTTTTTAAG-3′
PHF14 (ChIP)-Primer 4-F: 5′-GATCAGAAACAAAAACTGGCCTCCC-3′
PHF14 (ChIP)-Primer 4-R: 5′-GGAGCAGAGATCCCCGCCAAACCCT-3′
PHF14 (ChIP)-Primer 5-F: 5′-CTCTGCTCCGCCTTGGCACTACTCG-3′
PHF14 (ChIP)-Primer 5-R: 5′-TGAGAGCAGCAGGAATTGAGGGCGC-3′
PHF14 (ChIP)-Primer 6-F: 5′-CTCAGGGCTGCGCTCAGCCTCGTGT-3′
PHF14 (ChIP)-Primer 6-R: 5′-ACCAGAAGCAACGAAAGACCAGAAA-3′
PHF14 (ChIP)-Primer 7-F: 5′-TTTCTGGTCTTTCGTTGCTTCTGGT-3′
PHF14 (ChIP)-Primer 7-R: 5′-CAGCGCAGTCAACCCGGCGGCCCCG-3′

PHF14 (ChIP)-Primer 8-F: 5′-CTTTAATTTTTTTTCTTCTAGTTTT-3′
PHF14 (ChIP)-Primer 8-R: 5′-CTCGAGCCCCTTCCCACAGCGCTCC-3′
GAPDH (ChIP)-F: 5′-GTGGCAAAGTGGAGATTGTT-3′
GAPDH (ChIP)-R: 5′-CTCGCTCCTGGAAGATGG-3′

Luciferase reporter assay

Dual-Luciferase expression plasmids pGL3-luc, as well as pGL3-PHF14-Luc, were constructed by AuGCT DNA-SYN Biotechnology. ESCC cells were transfected with pGL3-Luc or pGL3-GIT1-luc to verify these plasmids. SP4 siRNA and NC-siRNA were cotransfected into ESCC cells that have been transferred with pGL3-PHF14-luc, so are overexpression SP4 and vector. The ESCC cells were collected 48 hours after transfection. Next, we examined and analyzed the luciferase activity by applying the Dual-Luciferase Reporter System (Promega).

Statistical analysis

All triplicate results were quantitative results of inter-independent experiments. The data were presented as mean ± SEM. Statistical analysis was performed with SPSS (RRID: SCR_002865) 19.0 software (Abbott Laboratories). Quantitative data were shown as mean ± SD and analyzed by Student *t* test, two-way ANOVA, and Pearson χ^2 test according to the data characteristics. $P < 0.05$ indicates statistical significance.

Data availability

The datasets analyzed during this study are available at https://xenabrowser.net/. All other data supporting the funding of this study are available in the article, as well as from the corresponding authors upon reasonable request.

Results

High expression of SP4 in ESCC is relevant to clinicopathologic characteristics

Aiming to describe the profile of SP4 in ESCC progression, we first investigated the correlation between SP4 expression and ESCC clinicopathologic features. University of California Santa Cruz (UCSC, Santa Cruz, CA) Xena online data illustrated that, compared with normal esophageal squamous tissue, SP4 had higher expression in ESCC tissues ($P < 0.05$) and was correlated with poor phase G, TNM ($P < 0.05$; **Fig. 1A–C**). Moreover, Kaplan–Meier analysis from UCSC Xena showed that high expression of SP4 was negatively associated with disease-specific survival (DSS) and progression-free interval (FPI; $P < 0.01$; **Fig. 1D** and **E**). We next analyzed its expression levels in 73 ESCC tissues and matched adjacent nontumor tissues by qRT-PCR to further confirm the SP4 expression in ESCC tissues. The results showed that mRNA expression of SP4 was upregulated in ESCC tissues compared with normal tissues (**Fig. 1F**; $P < 0.01$). Western blot analysis showed that SP4 was additionally expressed in ESCC tissues than in normal esophageal squamous tissues (**Fig. 1G**). Furthermore, the relevance between SP4 mRNA levels with the patient clinicopathologic feature of 73 ESCC sufferers was recorded in Supplementary Table S1, which illustrated that high *SP4* mRNA expression was correlated with histologic grade [G1: 61.9% (13/21); G2: 82.4% (28/34); G3: 94.4% (17/18)] ($P < 0.01$), T stage [T1/T2: 64.7% (11/17); T3/T4: 83.9% (47/56)] ($P < 0.01$), and TNM stage [I/II: 56.7% (17/30); III/IV: 95.3% (41/43)] ($P < 0.01$), and not associated with age and gender.

To facilitate subsequent *in vitro* experiments, we validated the above results in ESCC cell lines (EC109, TE-1, SEG-1) by Western blot and qRT-PCR analyses, which demonstrated that SP4 expression in ESCC cell lines was distinctly higher than that in a normal human esophageal epithelial cell line (Het-1A; **Fig. 1H** and **I**). In brief, these data deliver that SP4 might be a potential effective biomarker and involved in human ESCC progression.

SP4 promotes ESCC cell proliferation and inhibits apoptosis *in vitro* and *in vivo*

Considering the clinical significance of SP4 in ESCC, we next explored the effects of SP4 gain and knockdown on the malignant growth potential of ESCC cells to further identify the role of SP4 in ESCC. EC109 and TE-1 were used as the *in vitro* experimental material, which both express higher SP4 level. Western blot and qRT-PCR results showed that *SP4* siRNAs remarkably decreased SP4 expression in EC109 and TE-1 cells (**Fig. 2I** and **K**; $P < 0.01$), while SP4 overexpression vector availably enhanced SP4 expression (**Fig. 2J** and **L**; $P < 0.01$). MTT assays revealed reduced viability of ESCC cells with SP4 knockdown (**Fig. 2A**; $P < 0.01$), whereas increased viability of ESCC cells with SP4 overexpression (**Fig. 2B**; $P < 0.01$). Analogical results with the same trend could also be observed in the live-cell imaging system (**Fig. 2C** and **D**; $P < 0.01$). Understandably, flow cytometry analysis showed that SP4 knockdown resulted in cell-cycle arrest at the G_0–G_1 phase (**Fig. 2E**; $P < 0.01$) and increased percentage of apoptotic cells (**Fig. 2G**; $P < 0.01$), whereas overexpression of SP4 did the opposite expectantly (**Fig. 2F** and **H**; $P < 0.01$). After expectation, Western blot analysis demonstrated that SP4 knockdown downregulated CDK4, CDK6, and Cyclin D1 protein expression and upregulated active-caspase 3 protein expression in ESCC cells (**Fig. 2K**), whereas SP4 overexpression increased CDK4, CDK6, and Cyclin D1 protein expression and decreased active-caspase 3 expressions (**Fig. 2L**).

To further evaluate whether overexpression of SP4 could restore apoptosis of ESCC cells with SP4 knocked down, qRT-PCR, Western blot, and flow cytometry assays were performed, and the results indicated that the apoptosis of ESCC cells was significantly rescued (Supplementary Fig. S1A–S1C). Besides, we have conducted more detailed experiments, using 5-fluorouracil (5-FU) to simulate the influence of chemotherapy, demonstrating that overexpression of SP4 could rescue the apoptosis of ESCC cells caused by 5-FU, which demonstrating that SP4 are closely associated with cancer cell apoptosis (Supplementary Fig. S1D).

We next generated a stable SP4 knockdown EC109 cell line by using a shRNA lentivirus transfection system to more accurately address the importance of SP4 in regulating the growth of ESCC cells. Western blot and qRT-PCR results showed that *SP4* shRNAs remarkably suppressed SP4 expression in EC109 with decreased CDK4, CDK6, and Cyclin D1 protein expression and increased active-caspase 3 protein expression (**Fig. 3C** and **D**; $P < 0.01$), which inhibited ESCC cell growth as shown in MTT assay and live cell imaging system (**Fig. 3A**; $P < 0.01$). As the same results as *SP4* siRNA, flow cytometry analysis showed that SP4 shRNA resulted in cell-cycle arrest at the G_0–G_1 phase and increased percentage of apoptotic cells (**Fig. 3B**; $P < 0.01$).

Then the EC109 cells with *SP4* shRNA and sh-Control were subcutaneously injected into 5-week-old female nude mice, respectively. Cell-derived xenograft experiments indicated that the growth rate of tumors and the volume and weight of tumors with *SP4* shRNA were significantly decreased compared with those in the

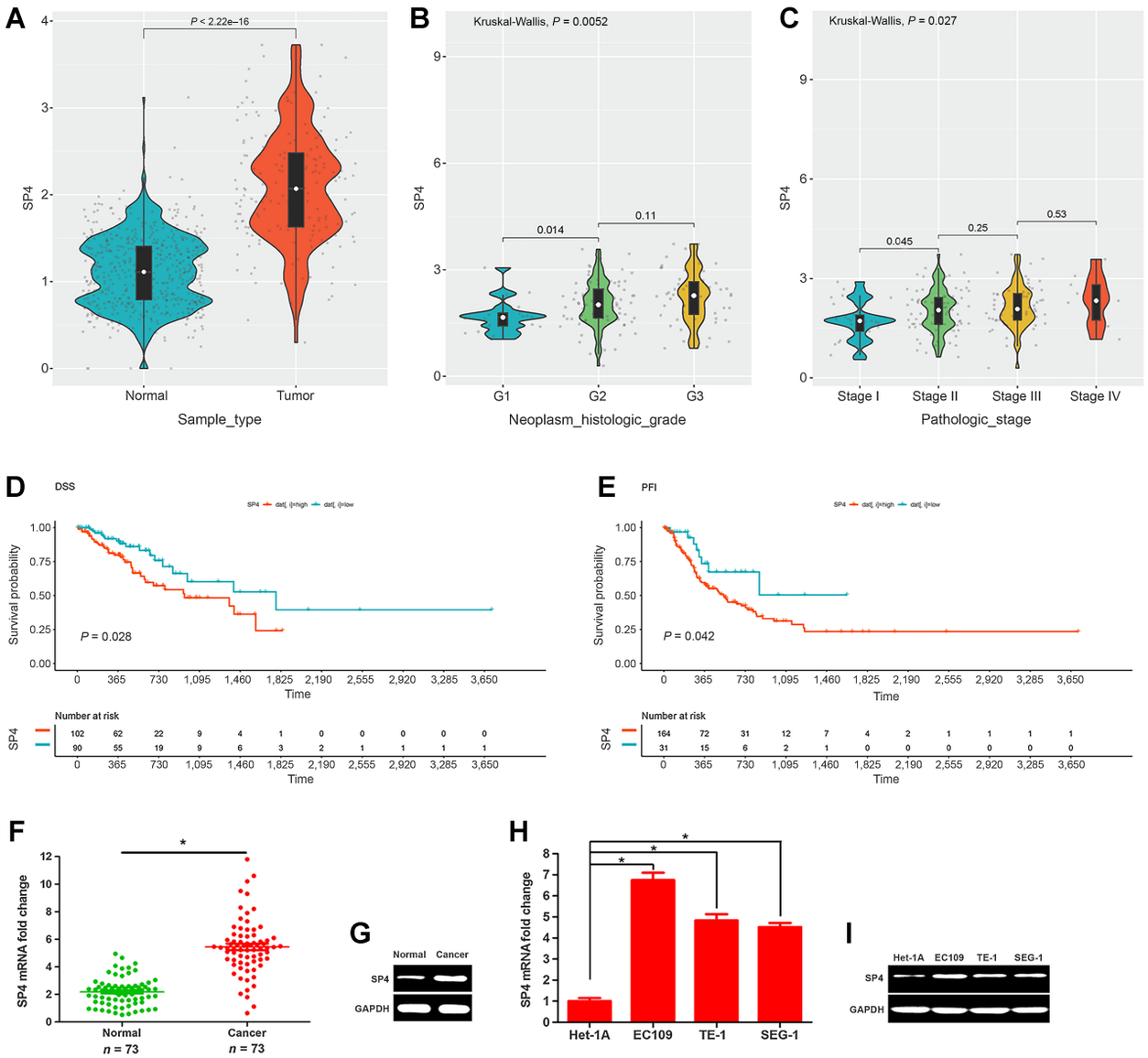

Figure 1.

High expression of SP4 in ESCC is relevant to clinicopathologic characteristics. **A,** SP4 expression in patients with ESCC. **B,** Correlation between SP4 expression and G stage in patients with ESCC. **C,** Correlation between SP4 expression and TNM stage in patients with ESCC. **D,** Patients' disease-specific survival (DSS) estimation datasets of SP4 high expression correlation group compared with low expression correlation group. **E,** Patients' progression-free interval estimation datasets of SP4 high expression correlation group compared with low expression correlation group. **F,** SP4 mRNA expression in ESCC tissues versus normal esophageal squamous tissues ($n = 73$; $P < 0.01$). **G,** SP4 protein expression in ESCC tissues versus normal esophageal squamous tissues. GAPDH was used as an internal reference. **H,** SP4 mRNA expression in ESCC cells (EC109, TE-1, SEG-1) versus esophageal squamous cells (Het-1A). GAPDH was used as an internal reference. **I,** SP4 protein expression in ESCC cells (EC109, TE-1, SEG-1) versus normal esophageal squamous cells (Het-1A). GAPDH was used as an internal reference. Each experiment was performed in triplicate. According to the data characteristics, quantitative data of (**A**–**C**, **F**, **H**) were analyzed by Student *t* test, quantitative data of **D** and **E** were analyzed by Pearson χ^2 test, *, $P < 0.05$.

control groups (**Fig. 3E-G**; $P < 0.01$). qRT-PCR, IHC staining, and Western blot analysis have been conducted to verify the downregulation of SP4 in tumors derived from SP4 shRNA–transduced EC109 cells (**Fig. 3H–J**; $P < 0.01$). Consistently, downregulated CDK4, CDK6, and Cyclin D1 protein expression and upregulated active-caspase 3 protein expression in these tumors were observed using Western blot analysis (**Fig. 3J**). Taken together, these data stated that SP4 promoted malignancy and progression of ESCC cells.

SP4 binds the promoter of PHF14 to mediate its transcription

Given the transcription factor role of SP4, bioinformatics analysis (UCSC Genome Browser) was used to hunt the SP4 target gene to further analyze the molecular mechanism of SP4 regulating ESCC

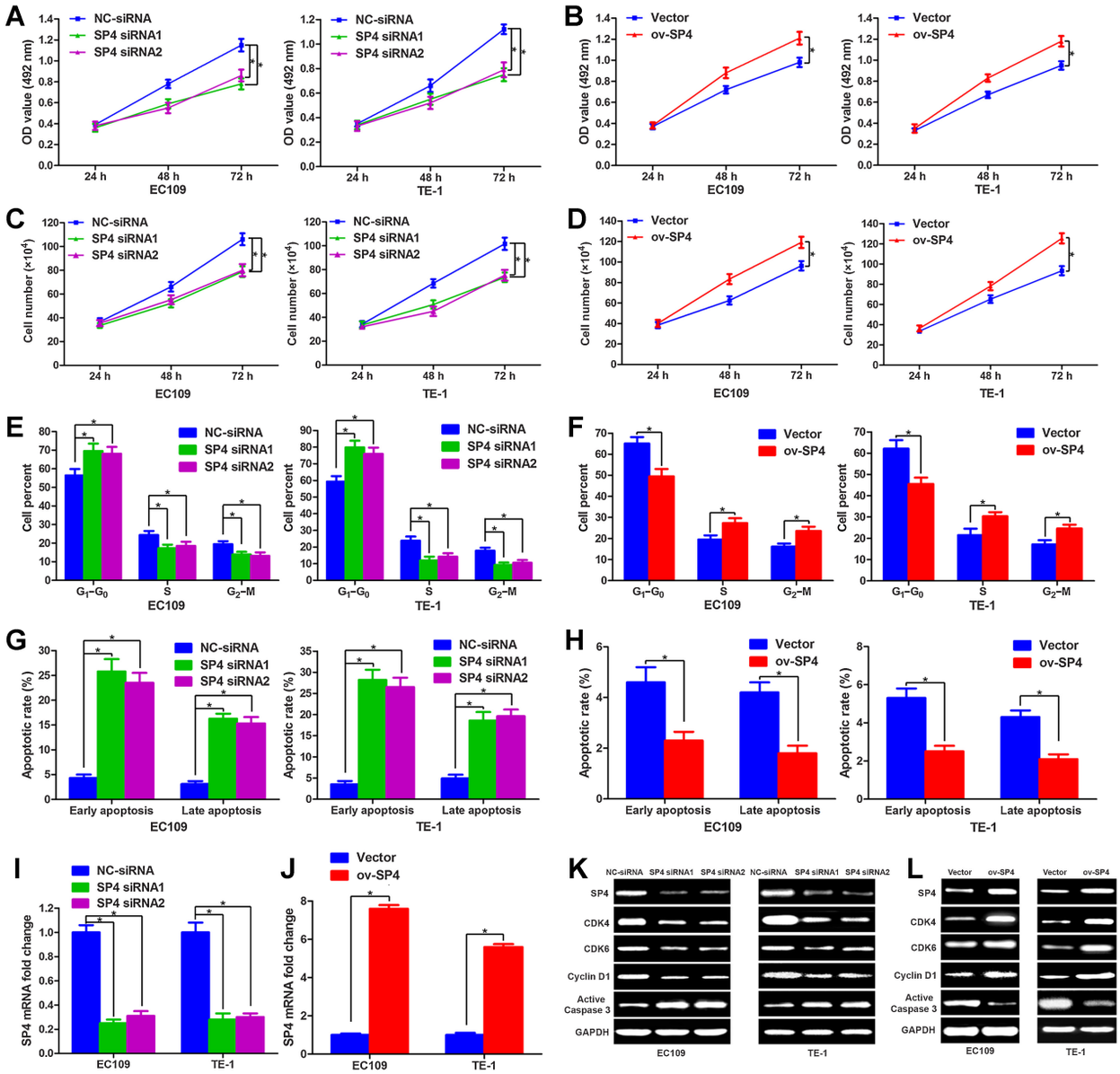

Figure 2.

SP4 promotes ESCC cell proliferation and inhibits apoptosis *in vitro*. **A,** Viability of ESCC cells after transfection with SP4 siRNA. **B,** Viability of ESCC cells after transfection with SP4 overexpression plasmid. **C,** Cell number of ESCC cells after transfection with SP4 siRNA. **D,** Cell number of ESCC cells after transfection with SP4 overexpression plasmid. **E,** The cell cycle of ESCC cells after transfection with SP4 siRNA was analyzed by flow cytometry. **F,** The cell cycle of ESCC cells after transfection with SP4 overexpression plasmid was analyzed by flow cytometry. **G,** Apoptosis of ESCC cells after transfection with SP4 siRNA was examined by flow cytometry. **H,** Apoptosis of ESCC cells after transfection with SP4 overexpression plasmid was examined by flow cytometry. **I,** SP4 mRNA expression after transfection with SP4 siRNA in ESCC cells. GAPDH was used as an internal reference. **J,** SP4 mRNA expression after transfection with SP4 overexpression plasmid in ESCC cells. GAPDH was used as an internal reference. **K,** SP4, CDK4, CDK6, Cyclin D1, and active caspase 3 expressions were measured after transfection with SP4 siRNA in ESCC cells. GAPDH was used as an internal reference. **L,** SP4, CDK4, CDK6, Cyclin D1, and active caspase 3 expressions were measured after transfection with SP4-overexpression plasmid in ESCC cells. GAPDH was used as an internal reference. Each experiment was performed in triplicate. According to the data characteristics, quantitative data of **A**–**D** were analyzed by Pearson χ^2 test, quantitative data of **E**–**J** were analyzed by Student *t* test, *, $P < 0.05$.

progression. According to the results, we found that SP4 could bind to the promoter region of PHF14 (**Fig. 4A**). The Cancer Genome Atlas (TCGA) online data showed that SP4 expression was positively related to PHF14 expression in human ESCC tissues (**Fig. 4B**; $P < 0.01$). The qRT-PCR results from 73 ESCC tissues and matched adjacent nontumor tissues also showed that SP4 mRNA expression was remarkably positively correlated with PHF14 mRNA expression in ESCC (**Fig. 4C**; $P < 0.01$). Most importantly, we verified that SP4 bound to the promoter of PHF14 in EC109 and TE-1 cells by ChIP-qRT-PCR assay (**Fig. 4D**; $P < 0.01$). A dual-luciferase reporter system

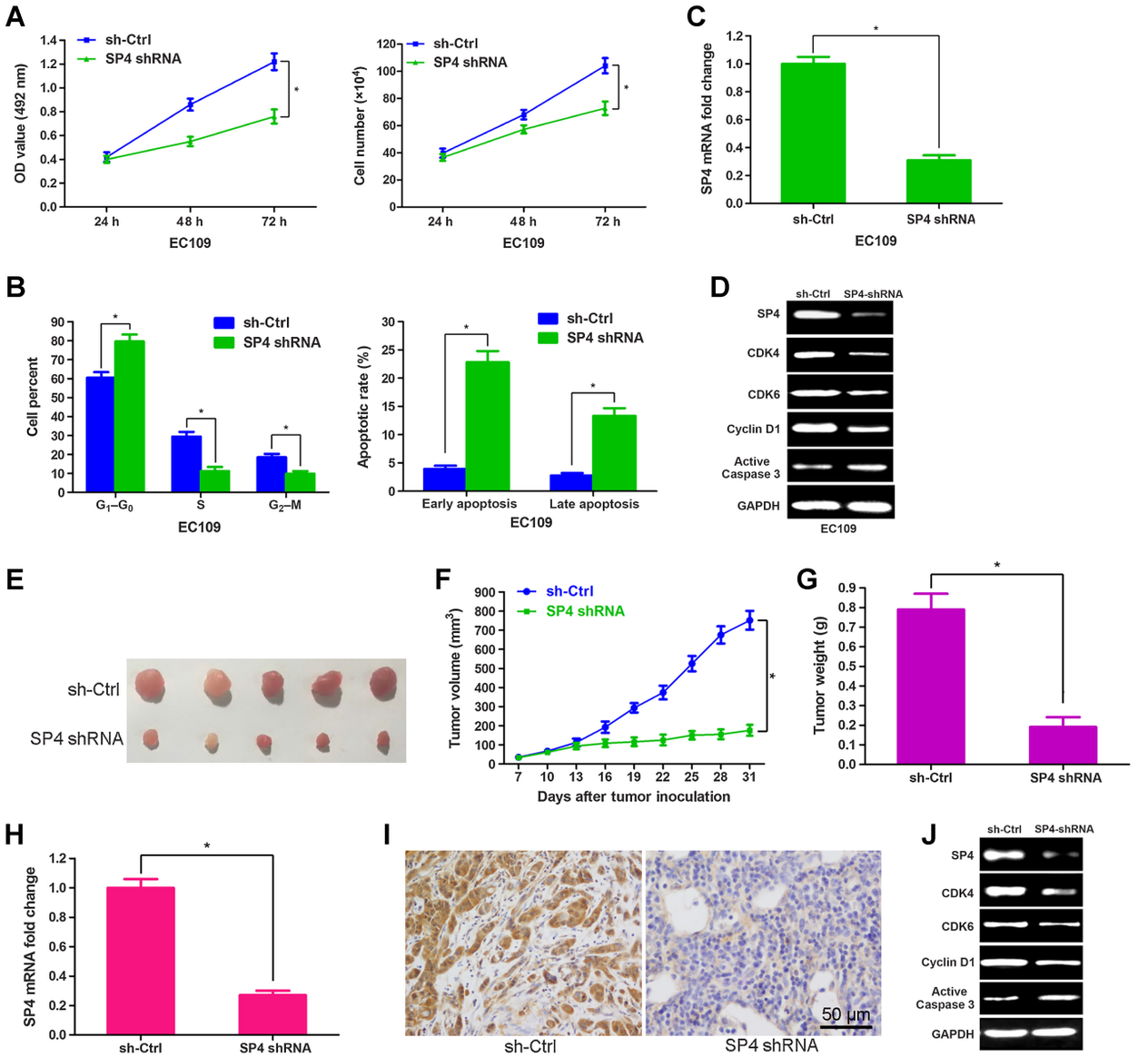

Figure 3.

SP4 shRNA inhibits ESCC cell proliferation and promotes apoptosis *in vitro* and *in vivo*. **A,** Viability and cell number of ESCC cells after SP4-knockdown. **B,** The cell cycle and apoptosis of ESCC cells after SP4-knockdown were analyzed by flow cytometry. **C,** SP4 mRNA expression after SP4 knockdown in ESCC cells. GAPDH was used as an internal reference. **D,** SP4, CDK4, CDK6, Cyclin D1, and active caspase 3 expressions were measured after SP4 knockdown in ESCC cells. GAPDH was used as an internal reference. **E,** Morphology of isolated tumors from nude mice. **F,** Growth curvatures of tumor volume were obtained from 7 to 31 days. **G,** Tumor weight of indicated mice at 31st day. **H,** SP4 mRNA expression in xenograft tumor. **I,** IHC staining was performed to detect the expression of SP4. **J,** SP4, CDK4, CDK6, Cyclin D1, active caspase 3 expressions in xenograft tumor. GAPDH was used as an internal reference. Each experiment was performed in triplicate. According to the data characteristics, quantitative data of **A** and **F** were analyzed by Pearson χ^2 test, quantitative data of **B**, **C**, **G**, and **H** were analyzed by Student *t* test. *, $P < 0.05$.

was implemented to verify this result. The target sequence of the promoter of *PHF14* was inserted into the luciferase gene in the pGL3 reporter plasmid, which was transferred into EC109 and TE-1 cells. After 48 hours, luciferase activity was recorded. The results showed that, compared with in pGL3-luc group, luciferase activity remarkably increased in the pGL3-*PHF14*-luc group (**Fig. 4E**; $P < 0.01$). Subsequently, *SP4* siRNAs and SP4 overexpression vector were, respectively, cotransfected with pGL3-*PHF14*-luc into EC109 and TE-1 cells. Remarkably reduced luciferase activity was observed in *SP4* siRNA groups compared with the NC-siRNA group (**Fig. 4F**; $P < 0.01$), whereas significantly enhanced luciferase activity was observed in the SP4 overexpression vector group compared with the control vector group (**Fig. 4G**; $P < 0.01$). To be essential, the effect of SP4 on PHF14 expression was analyzed through qRT-PCR and Western blot analysis. We found that *SP4* siRNAs observably suppressed PHF14 expression in EC109 and TE-1 cells (**Fig. 4H** and **J**; $P < 0.01$), while

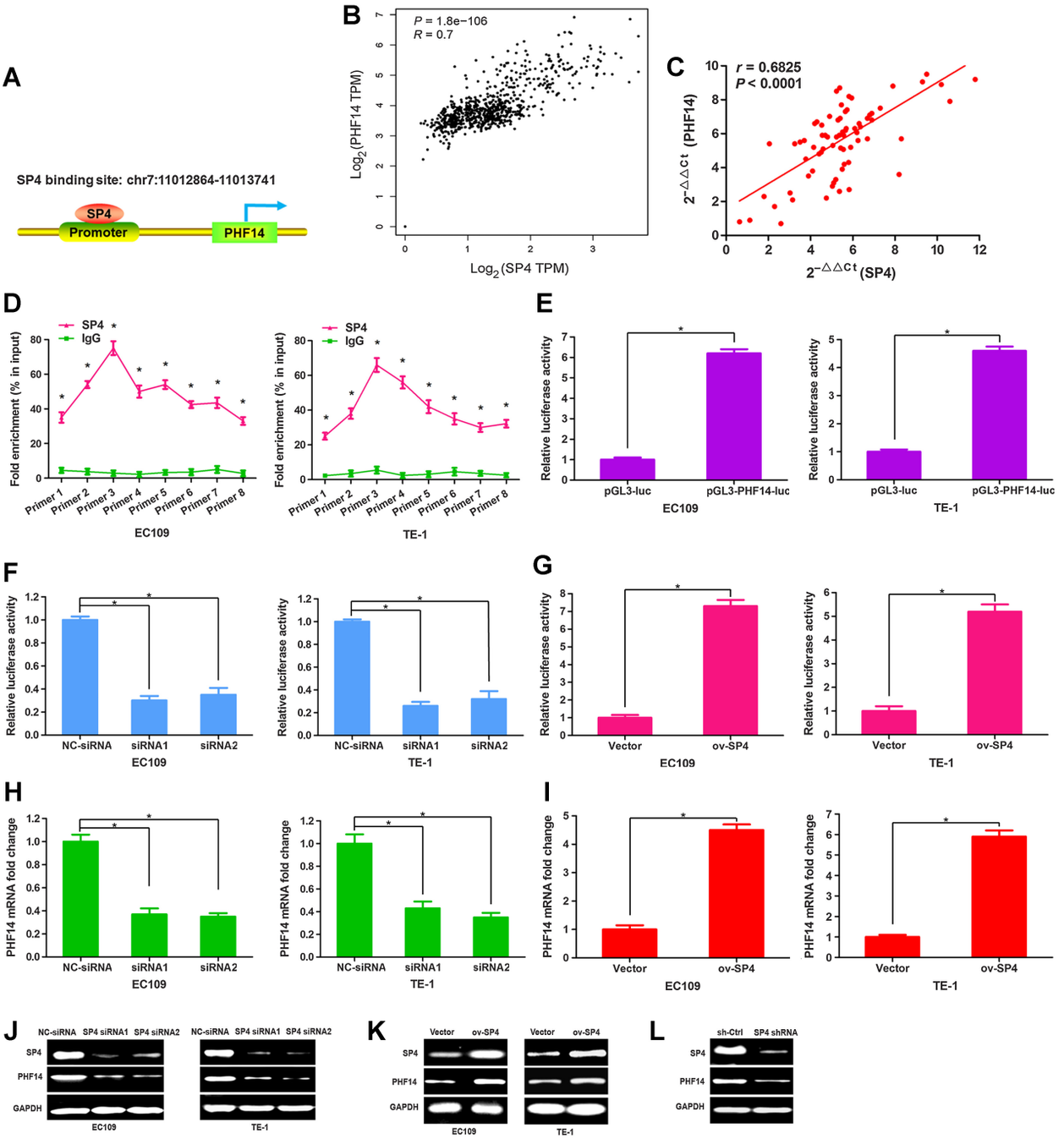

Figure 4.

SP4 binds the promoter of PHF14 to mediate its transcription. **A,** Bioinformatics analysis (UCSC Genome Browser) predicted the SP4 binding site in the promoter of PHF14. **B,** Correlation analysis between SP4 and PHF14 using TCGA data. **C,** mRNA correlation between SP4 and PHF14 in ESCC tissues. **D,** ChIP-qRT-PCR analysis showed that SP4 binds the promoter of PHF14. **E,** ESCC cells were transfected with pGL3-PHF14-luc (target sequences of SP4), and the luciferase activity was examined at 48 h after transfection. Renilla luciferase was known as the internal control. **F,** ESCC cells were co-transfected with pGL3-PHF14-luc and SP4 siRNAs; the luciferase activity was determined. *Renilla* luciferase was known as the internal control. **G,** ESCC cells were cotransfected with pGL3-PHF14-luc and SP4 siRNAs; the luciferase activity was determined. *Renilla* luciferase was known as the internal control. **H,** PHF14 mRNA expression after transfection with SP4 siRNA in ESCC cells. GAPDH was used as an internal reference. **I,** PHF14 mRNA expression after transfection with SP4 overexpression plasmid in ESCC cells. GAPDH was used as an internal reference. **J,** SP4 and PHF14 expressions were measured after transfection with SP4 siRNA in ESCC cells. GAPDH was used as an internal reference. **K,** SP4 and PHF14 expressions were measured after transfection with SP4 overexpression plasmid in ESCC cells. GAPDH was used as an internal reference. **L,** SP4 and PHF14 expressions were measured after SP4 knockdown in ESCC cells. GAPDH was used as an internal reference. Each experiment was performed in triplicate. According to the data characteristics, quantitative data of **B** and **C** were analyzed by two-way ANOVA, quantitative data of **D**–**I** were analyzed by Student *t* test. *, $P < 0.05$.

SP4 overexpression vector significantly enhanced PHF14 expression (**Fig. 4I** and **K**; $P < 0.01$). With the same results as *SP4* siRNA, *SP4* shRNA also decreased PHF14 expression in tumors derived from SP4 shRNA–transduced EC109 cells (**Fig. 4L**).

High expression of PHF14 occurred in ESCC and is associated with clinicopathologic feature

Previous studies have shown that PHF14 exerted carcinogenesis as well as altered tumor physiology in a variety of cancers, such as gastric cancer (37), biliary tract cancer (41), lung cancer (42), glioblastoma (35), and bladder cancer (36). However, the profile of PHF14 expression in patients with ESCC remains unknown. Therefore, we first analyzed the expression of PHF14 in ESCC tissues to explore the role of PHF14 transcriptionally regulated by SP4 in ESCC progression. Data from UCSC Xena revealed that PHF14 expression was significantly upregulated in ESCC tissues compared with normal esophageal squamous tissues (Supplementary Fig. S2A; $P < 0.01$) and correlated with poor phase G, TNM, and T (Supplementary Fig. S2B–S2D; $P < 0.05$). The high PHF14 expression was correlated to progression-free interval of patients with ESCC (Supplementary Fig. S2E; $P < 0.05$). The qRT-PCR results from 73 ESCC tissues and matched adjacent nontumor tissues showed that mRNA expression of PHF14 was upregulated in ESCC tissues compared with normal tissues (Supplementary Fig. S2F; $P < 0.01$). The same trend shown by Western blot analysis revealed that the PHF14 protein expression was observably increased in ESCC tissues (Supplementary Fig. S2H). Likewise, the PHF14 expression was also greatly upregulated in ESCC cell lines (Supplementary Fig. S2G and S2I). Furthermore, investigating the clinicopathologic feature of patients with ESCC, we found that high PHF14 mRNA expression was correlated with histologic grade [G1: 61.9% (13/21); G2: 76.5% (26/34); G3: 88.9% (16/18)] ($P < 0.01$), T stage [T1/T2: 47.1% (8/17); T3/T4: 83.9% (47/56)] ($P < 0.01$) and TNM stage [I/II: 50.0% (15/30); III/IV: 93.0% (40/43)] ($P < 0.01$), and not associated with age and gender (Supplementary Table S2).

PHF14 promotes ESCC cell proliferation and inhibits apoptosis *in vitro*

To further explore the role of PHF14 in ESCC, *PHF14* siRNAs and PHF14 overexpression vector were transfected into EC109 and TE-1 cells independently. Western blot and qRT-PCR results showed that *PHF14* siRNAs significantly decreased PHF14 expression in EC109 and TE-1 cells (**Fig. 5I** and **K**; $P < 0.01$), while PHF14 overexpression vector availably enhanced PHF14 expression (**Fig. 5J** and **L**; $P < 0.01$). MTT assays revealed reduced viability of ESCC cells with PHF14 knockdown (**Fig. 5A**; $P < 0.01$), whereas increased viability of ESCC cells with PHF14 overexpression (**Fig. 5B**; $P < 0.01$). Live-cell imaging system showed that the number of cells was reduced after knocking down the expression of the *PHF14* gene, whereas increased the number of cells in the PHF14 overexpression vector group (**Fig. 5C** and **D**; $P < 0.01$). Flow cytometry analysis showed that PHF149 knockdown also resulted in cell-cycle arrest at the G_0–G_1 phase (**Fig. 5E**; $P < 0.01$) and enhanced percentage of apoptotic cells (**Fig. 5G**; $P < 0.01$), whereas overexpression of PHF14 did the opposite (**Fig. 5F** and **H**; $P < 0.01$). In addition, using 5-FU to simulate the influence of chemotherapy, demonstrating that overexpression of PHF14 could rescue the apoptosis of ESCC cells caused by 5-FU, showed that PHF14 is closely associated with cancer cell apoptosis (Supplementary Fig. S1D). Meanwhile, the expression of cell cycle and apoptosis-related proteins was detected by Western blot analysis, which found that PHF14 knockdown also reduced CDK4, CDK6, and Cyclin D1 protein expression and upregulated active caspase 3 protein expression in ESCC cells (**Fig. 5K**), whereas PHF14 overexpression increased CDK4, CDK6, and Cyclin D1 protein expression and decreased active caspase 3 expressions (**Fig. 5L**). Besides, we have analyzed correlation of *WNT3A, CTNNB1*, and *SP4/PFH14* expression using qRT-PCR results from 73 ESCC tissues and matched adjacent nontumor tissues. The result showed that *WNT3A* and *CTNNB1* were both positive correlated with *SP4/PFH14* (Supplementary Fig. S3A–S3D), which was consistent with the previous report that PHF14 promotes proliferation and invasion and alleviates apoptosis through the Wnt signaling pathway (35).Considering the relevance between the Wnt pathway and SP4/PHF14, qRT-PCR assays were implemented to show that *SP4* and *PHF14* siRNAs significantly decreased Wnt3a and β-Catenin mRNA expression in EC109 and TE-1 cells (Supplementary Fig. S3E–S3H). Accordingly, Western blot analysis showed that PHF14 knockdown decreased Wnt3a, β-catenin protein expressions, but overexpressing PHF14 upregulated their protein levels (**Fig. 5K** and **L**). Collectively, as transcriptional downstream of SP4, PHF14 plays a carcinogenic role in ESCC and regulates cell cycle and apoptosis.

SP4 facilitates ESCC progression via modulating Wnt/β-catenin signaling pathway by promoting PHF14 transcription

We then tried to illustrate that SP4 exhibited oncogene function by regulating PHF14 transcription. Western blot and qRT-PCR results stated that *SP4* siRNA resulted in the downregulation of PHF14 expression, whereas overexpressing PHF14 reversed the effect of SP4 siRNA on PHF14 expression (**Fig. 6E** and **F**; $P < 0.01$). Cell viability detection by MTT assay showed that overexpressing PHF14 rescued the suppression effect of *SP4* siRNA on cell growth (**Fig. 6A**; $P < 0.01$). Analogical results with the same trend were also exhibited in the live cell imaging system (**Fig. 6B**; $P < 0.01$). Besides, flow cytometry analysis showed that overexpressing PHF14 rescued cell-cycle arrest at G_0–G_1 phase as well as apoptosis, both caused by SP4 knockdown (**Fig. 6C** and **D**; $P < 0.01$). Consistent with the results of flow cytometry analysis, Western blot analysis showed that overexpression of PHF14 rescued the downregulation of CDK4, CDK6, and Cyclin D1 caused by SP4 knockdown, meanwhile restored the upregulation of active caspase 3 caused by SP4 knockdown. Moreover, we examined key molecules in the signaling pathway using Western blot and qRT-PCR analyses discovering that SP4 knockdown also downregulated Wnt3a and β-Catenin protein expressions (**Fig. 6F**; Supplementary Fig. S3I), as SP4 is a transcriptional upstream protein of PHF14. As expected, Western blot and qRT-PCR analyses stated that co-knockdown of both SP4 and PHF14 also results in more decreased mRNA and protein levels of the Wnt3a and β-Catenin (Supplementary Fig. S4), and overexpressing PHF14 successfully rescued the downregulation of Wnt3a and β-Catenin caused by SP4 knockdown (**Fig. 6F**; Supplementary Fig. S3I). To further demonstrate whether the Wnt/β-Catenin signaling pathway is downstream of SP4/PHF14, we used Wnt inhibitor LGK974 treating ESCC cells after overexpression of SP4, and Western blot results showed that the LGK974 IC_{50}: 0.1 nmol/L indeed restored the upregulated Wnt3a and β-catenin levels after overexpression of SP4 (**Fig. 7E**). MTT assay and live-cell imaging system showed that LGK974 could restore the excessive cell vitality and proliferation after overexpression of SP4 (**Fig. 7A** and **B**). Flow cytometry analysis showed that LGK974 could restore cell-cycle G_0–G_1 to S-phase transition as well as apoptosis, both caused by overexpression of SP4 (**Fig. 7C** and **D**). In addition, Western blot assays showed that overexpression of Wnt3a could restore the

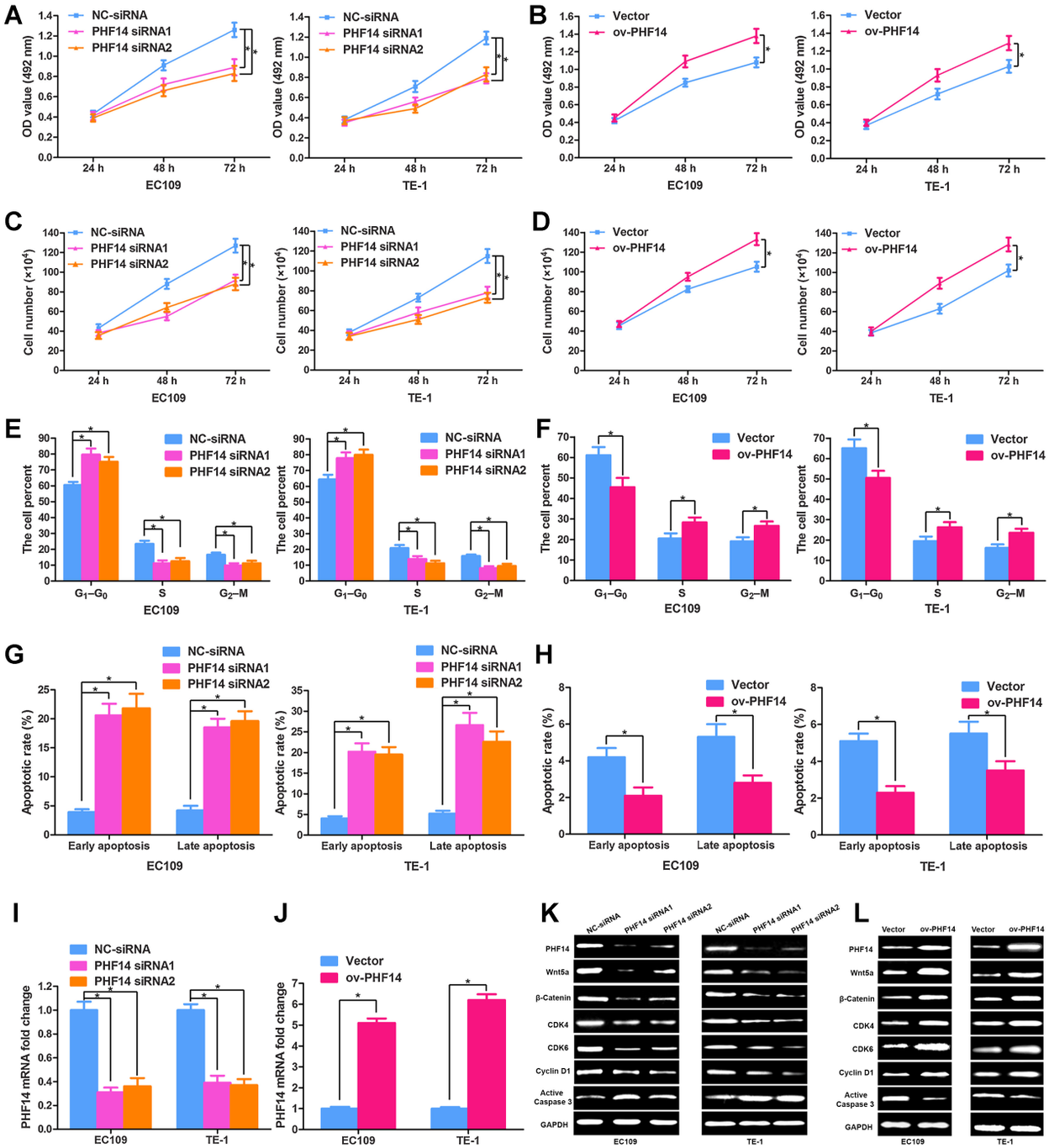

Figure 5.

PHF14 promotes ESCC cell proliferation and inhibits apoptosis *in vitro*. **A,** Viability of ESCC cells after transfection with PHF14 siRNA. **B,** Viability of ESCC cells after transfection with PHF14 overexpression plasmid. **C,** Cell number of ESCC cells after transfection with PHF14 siRNA. **D,** Cell number of ESCC cells after transfection with PHF14 overexpression plasmid. **E,** The cell cycle of ESCC cells after transfection with PHF14 siRNA was analyzed by flow cytometry. **F,** The cell cycle of ESCC cells after transfection with PHF14 overexpression plasmid was analyzed by flow cytometry. **G,** Apoptosis of ESCC cells after transfection with PHF14 siRNA was examined by flow cytometry. **H,** Apoptosis of ESCC cells after transfection with PHF14 overexpression plasmid was examined by flow cytometry. **I,** PHF14 mRNA expression after transfection with PHF14 siRNA in ESCC cells. **J,** PHF14 mRNA expression after transfection with PHF14 overexpression plasmid in ESCC cells. **K,** PHF14, Wnt3a, β-catenin, CDK4, CDK6, Cyclin D1, and active caspase 3 expressions were measured after transfection with PHF14 siRNA in ESCC cells. GAPDH was used as an internal reference. **L,** PHF14, Wnt3a, β-catenin, CDK4, CDK6, Cyclin D1, and active caspase 3 expressions were measured after transfection with PHF14 overexpression plasmid in ESCC cells. GAPDH was used as an internal reference. Each experiment was performed in triplicate. According to the data characteristics, quantitative data of **E**–**J** were analyzed by Student *t* test, quantitative data of **A**–**D** were analyzed by Pearson χ^2 test. *, $P < 0.05$.

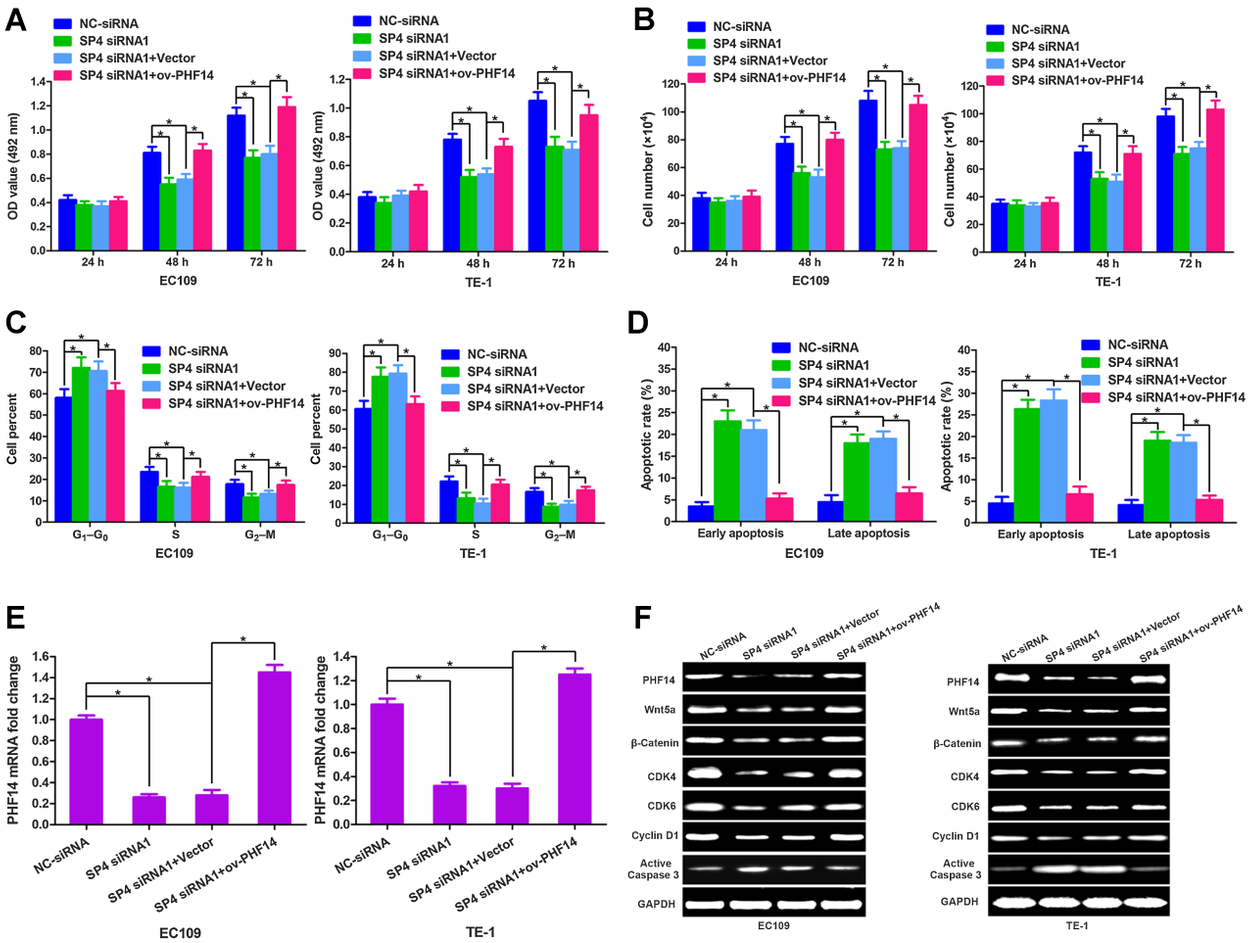

Figure 6.

SP4 facilitates ESCC progression via modulating the Wnt/β-catenin signaling pathway by promoting PHF14 transcription. **A,** Viability of ESCC cells after cotransfection with SP4 siRNA-1 and PHF14 overexpression plasmid. **B,** Cell number of ESCC cells after cotransfection with SP4 siRNA-1 and PHF14 overexpression plasmid. **C,** The cell cycle of ESCC cells after cotransfection with SP4 siRNA-1 and PHF14 overexpression plasmid was analyzed by flow cytometry. **D,** Apoptosis of ESCC cells after cotransfection with SP4 siRNA-1 and PHF14-overexpression plasmid was analyzed by flow cytometry. **E,** PHF14 mRNA level was measured in ESCC cells after cotransfection with SP4 siRNA-1 and PHF14 overexpression vector. **F,** PHF14, Wnt3a, β-Catenin, CDK4, CDK6, Cyclin D1, and active caspase 3 expressions were measured after cotransfection with SP4 siRNA-1 and PHF14 overexpression vector. GAPDH was used as an internal reference. Each experiment was performed in triplicate. According to the data characteristics, quantitative data of **A**–**E** were analyzed by Student *t* test. *, $P < 0.05$.

downregulation of Wnt3a, β-catenin, CDK4, CDK6, Cyclin D1, as well as the upregulation of active caspase3 caused by *PHF14* siRNA (**Fig. 7F**). Altogether, these findings suggested that SP4 activates Wnt/β-catenin signaling pathway by promoting the PHF14 transcription, thereby facilitates ESCC progression.

Discussion

Specificity protein (SP) transcription factors (TF) are essential for embryonic growth and early development, and their expression decreases with age (16, 43–45). Research on SP TF structure and function has focused on SP1, showing that SP1 and its modifications (phosphorylation, acetylation, and glycosylation) could play critical roles in the basal transcriptional machinery (16, 46). Meanwhile, high SP1 expression is also a poor prognostic factor in patients with pancreatic, glioma, colon, gastric, head and neck, prostate, lung, and breast cancers (47–55). In contrast, although SP4 has also been characterized as highly expressed in several cancers (56), there has been little in-depth investigation of its function as well as mechanism. Our data were the first to reveal that SP4 was highly expressed in ESCC tissues and cells and that the overexpression of SP4 was significantly correlated with clinicopathologic characteristics for patients with ESCC (**Fig. 1**; Supplementary Table S1). Subsequently, by knockdown and overexpression of SP4 in ESCC cells, we found that SP4 could promote the proliferation, G_0–G_1 to S-phase transition, and inhibit apoptosis of ESCC cells (**Fig. 2**; Supplementary Figs. S1 and S3). Correspondingly, cell-derived xenograft subcutaneous tumorigenesis experiments showed that SP4 significantly promoted the growth of tumors *in vivo* (**Fig. 3E–J**). In summary, these results suggest that SP4 promotes ESCC cell growth, proliferation, and inhibits cell apoptosis, playing a vital role in ESCC progression.

To further clarify the potential molecular mechanism of SP4 facilitating ESCC, we combined bioinformatics prediction, ChIP-qRT PCR, and dual luciferase reporting system to determine that

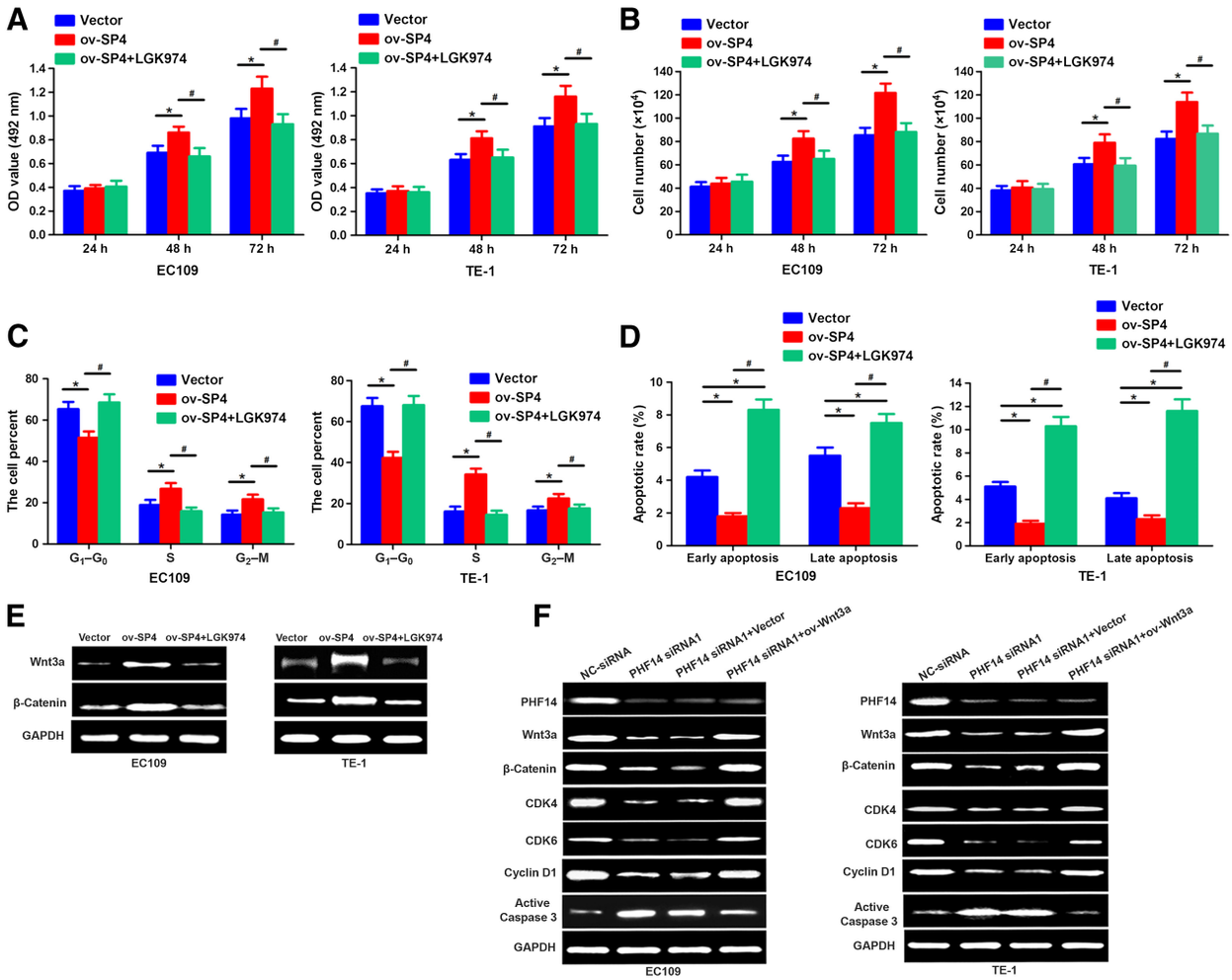

Figure 7.

Wnt/β-Catenin signaling pathway is downstream of SP4/PHF14. **A,** Viability of ESCC cells after treatment with Vector, ov-SP4, ov-SP4+LGK974. **B,** Cell number of ESCC cells after treatment with Vector, ov-SP4, ov-SP4+LGK974. **C,** The cell cycle of ESCC cells after treatment with vector, ov-SP4, ov-SP4+LGK974. **D,** Apoptosis of ESCC cells after treatment with vector, ov-SP4, ov-SP4+LGK974. **E,** Wnt3a, β-catenin expressions were measured after treatment with Vector, ov-SP4, ov-SP4+LGK974. GAPDH was used as an internal reference. **F,** PHF14, Wnt3a, β-catenin, CDK4, CDK6, Cyclin D1, and active caspase-3 expressions were measured after treatment with Vector, ov-SP4, ov-SP4+LGK974. GAPDH was used as an internal reference. Each experiment was performed in triplicate. According to the data characteristics, quantitative data of **A–D** were analyzed by Student *t* test. *, $P < 0.05$.

PHF14 is the transcriptional downstream of an SP4 transcription factor (**Fig. 4**). PHF14 is a chromatin-binding protein that interacts with histones via its PHD1 and PHD3 domains (57), indicative of its function in epigenetic modification and regulation. Therefore, PHF14 has gradually gained attention in the field of cancer research in recent years. PHF14 was reported to inhibit the growth of mesenchymal cells in biliary tract cancer (41). It also induces epithelial–mesenchymal transition (EMT) in tumor cells and enhances proliferation and invasiveness in bladder cancer (36). Furthermore, PHF14 promotes cell proliferation and migration in gastric cancer through the AKT and ERK1/2 pathways (37). In this study, we found that PHF14 expression was upregulated in ESCC, which was significantly correlated with clinicopathologic characteristics for patients with ESCC (Supplementary Fig. S2; Supplementary Table S2). Our data demonstrated that PHF14 promoted ESCC cell proliferation and suppressed apoptosis *in vitro* (**Fig 5A–J**).

PHF14 has been reported that could be involved in the Wnt/β-catenin signaling pathway which was closely related to tumorigenesis and progression and regulated many important biological processes such as tumor growth, apoptosis, and metastasis (35, 58). Given the effect of PHF14 on cell cycle and apoptosis, we speculate that PHF14 could also regulate the Wnt3a/β-catenin signaling pathway in ESCC cells. As expected, the results showed that *WNT3A* and *CTNNB1* were both positively correlated with *SP4/PFH14* (Supplementary Fig. S3A–S3D). PHF14 knockdown decreased Wnt3a and β-Catenin expressions, whereas overexpressing PHF14 upregulated their levels (**Fig. 5K** and **L**; Supplementary Fig. S3E–S3I). SP4 knockdown also downregulated Wnt3a and β-catenin expressions (**Fig. 6F**; Supplementary

Fig. S3I), and coknockdown of both SP4 and PHF14 also results in more decreased expression of the Wnt3a and β-catenin (Supplementary Fig. S4). Subsequently, the rescue experiments for SP4 knockdown using PHF14 overexpression vectors showed that PHF14 could rescue cell proliferation inhibition, apoptosis, and related protein changes caused by SP4 knockdown(**Fig. 6**). Overexpressing Wnt3a could rescue changes in proliferation and apoptosis-related proteins caused by PHF14 knockdown(**Fig. 7F**). Further combination of inhibitors LGK974 and SP4 overexpression revealed that LGK974 can restore proliferation promotion, apoptosis inhibition, and related protein changes induced by SP4 overexpression (**Fig. 7A–E**). These data illustrated that SP4 could activate the Wnt/β-catenin signaling pathway by facilitating PHF14 transcription, thereby facilitating ESCC progression (**Fig. 7**). It is a pity that this conclusion still needs to be supported by *in vivo* experimental data to be more robust. Not only that, the specific mechanism by which SP4 exerts transcription, the transcription regulatory factors it binds to, the DNA sites it binds to, and the structural domains that exert transcription still need further research. Furthermore, considering that Wnt/β-catenin signaling pathway participates in a variety of biological processes, we will also explore the role of SP4 in cancer metastasis and inflammation in the future to further enrich the mechanism of SP4 regulating cancer progression.

There is a strong statement that the *SP4* is a nononcogene addiction (NOA) gene in cancer (26). NOA suggests a situation in which some cellular stress pathways fail to drive tumorigenesis on their own, but are hyperactivated upon malignant transformation, manifested as being necessary for disease progression (59, 60), such as secretion of VEGFA as part of cellular response to hypoxia orchestrated by hypoxia-inducible factor 1α (61), and amplification of CDK4 and CDK6 or their partner Cyclin D1 observed in various human tumors (62). Mutations in the NOA genes cannot directly promote tumor formation because, although they are required for their pathways, as non-rate-limiting genes, they cannot increase the overall activity of their pathways. However, NOA genes could be reduced in activity to limit the rate of their associated pathways and NOA genes did not play as crucial a role in normal cells as they do in cancer cells, indicative of their value as potential drug targets (63, 64). For the past few years, an increasing number of chemical drugs targeting the NOA gene have been discovered, such as bevacizumab targeting VEGF to inhibit endothelial cell recruitment and tumor vasculature (65), AZD2281 targeting PARP1 to inhibit base excision repair in homologous recombination repair–deficient cancer cells (66), methotrexate targeting DHFR to inhibit thymidine biosynthesis and induces replicative stress (67). Our data suggested that SP4, which is highly expressed in ESCC, is consistent with its identity as an NOA gene and has the potential to be a clinical drug target. Fortunately, some drugs with cancer chemopreventive activity, such as aspirin, sulindac, and its metabolites, the antidiabetic drug metformin, curcumin, phenethyl isothiocyanate, triterpenoid natural products, celastrol, betulinic acid and bardoxolone, resveratrol, and several polyphenolics have been reported to induce SP4 downregulation in cancer (68–74). Our studies on SP4 in cancer progression enrich the anticancer mechanism of these drugs.

Collectively, in this study, we were the first to reveal that the SP4 expression was upregulated in ESCC and promoted ESCC progression via modulating the Wnt/β-catenin signaling pathway by promoting PHF14 transcription (Supplementary Fig. S5). These findings provided a theoretical reference for SP4 to be a promising prognostic indicator or chemotherapy target.

Authors' Disclosures

H. Qin reports grants from Shaanxi Province Natural Science Foundation during the conduct of the study. No disclosures were reported by the other authors.

Authors' Contributions

L. Wei: Conceptualization, data curation, software, methodology. **C. Deng:** Data curation, visualization, methodology, writing–original draft. **B. Zhang:** Validation, investigation. **G. Wang:** Methodology, project administration. **Y. Meng:** Resources, supervision. **H. Qin:** Resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, project administration.

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Note

Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018;68:394–424.
2. Wu LL, Zhong JD, Zhu JL, Kang L, Huang YY, Lin P, et al. Postoperative survival effect of the number of examined lymph nodes on esophageal squamous cell carcinoma with pathological stage T1–3N0M0. BMC Cancer 2022;22:118.
3. Abnet CC, Arnold M, Wei WQ. Epidemiology of esophageal squamous cell carcinoma. Gastroenterology 2018;154:360–73.
4. Arnold M, Soerjomataram I, Ferlay J, Forman D. Global incidence of oesophageal cancer by histological subtype in 2012. Gut 2015;64:381–7.
5. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021;71:209–49.
6. Akhtar S, Sheikh AA, Qureshi HU. Chewing areca nut, betel quid, oral snuff, cigarette smoking and the risk of oesophageal squamous-cell carcinoma in South Asians: a multicentre case-control study. Eur J Cancer 2012;48:655–61.
7. Herskovic A, Russell W, Liptay M, Fidler MJ, Al-Sarraf M. Esophageal carcinoma advances in treatment results for locally advanced disease: review. Ann Oncol 2012;23:1095–103.
8. Iacob R, Mandea M, Iacob S, Pietrosanu C, Paul D, Hainarosie R, et al. Liquid biopsy in squamous cell carcinoma of the esophagus and of the head and neck. Front Med (Lausanne) 2022;9:827297.
9. Huang R, Dai Q, Yang R, Duan Y, Zhao Q, Haybaeck J, et al. A review: PI3K/AKT/mTOR signaling pathway and its regulated eukaryotic translation initiation factors may be a potential therapeutic target in esophageal squamous cell carcinoma. Front Oncol 2022;12:817916.
10. Watanabe M, Mine S, Nishida K, Yamada K, Shigaki H, Matsumoto A, et al. Salvage esophagectomy after definitive chemoradiotherapy for patients with esophageal squamous cell carcinoma: who really benefits from this high-risk surgery? Ann Surg Oncol 2015;22:4438–44.
11. Shapiro J, van Lanschot JJB, Hulshof MCCM, van Hagen P, van Berge Henegouwen MI, Wijnhoven BPL, et al. A Neoadjuvant chemoradiotherapy plus surgery versus surgery alone for oesophageal or junctional cancer

(CROSS): long-term results of a randomised controlled trial. Lancet Oncol 2015;16:1090–8.
12. Yang Y, Zhu L, Cheng Y, Liu Z, Cai X, Shao J, et al. Three-arm phase II trial comparing camrelizumab plus chemotherapy versus camrelizumab plus chemoradiation versus chemoradiation as preoperative treatment for locally advanced esophageal squamous cell carcinoma (NICE-2 Study). BMC Cancer 2022;22:506.
13. Reichenbach ZW, Murray MG, Saxena R, Farkas D, Karassik EG, Klochkova A, et al. Clinical and translational advances in esophageal squamous cell carcinoma. Adv Cancer Res 2019;144:95–135.
14. Yang Y, Zhang Y, Lin Z, Wu K, He Z, Zhu D, et al. Silencing of histone deacetylase 3 suppresses the development of esophageal squamous cell carcinoma through regulation of miR-494-mediated TGIF1. Cancer Cell Int 2022;22:191.
15. Zhang Y, Han N, Zeng X, Sun C, Sun S, Ma X, et al. Impact of platelets to lymphocytes ratio and lymphocytes during radical concurrent radiotherapy and chemotherapy on patients with nonmetastatic esophageal squamous cell carcinoma. J Oncol 2022;2022:3412349.
16. Suske G, Bruford E, Philipsen S. Mammalian SP/KLF transcription factors: bring in the family. Genomics 2005;85:551–6.
17. Safe S, Abbruzzese J, Abdelrahim M, Hedrick E. Specificity protein transcription factors and cancer: opportunities for drug development. Cancer Prev Res 2018; 11:371–82.
18. Suske G. The Sp-family of transcription factors. Gene 1999;238:291–300.
19. Abdelrahim M, Safe S. Cyclooxygenase-2 inhibitors decrease vascular endothelial growth factor expression in colon cancer cells by enhanced degradation of Sp1 and Sp4 proteins. Mol Pharmacol 2005;68:317–29.
20. Abdelrahim M, Baker CH, Abbruzzese JL, Safe S. Tolfenamic acid and pancreatic cancer growth, angiogenesis, and Sp protein degradation. J Natl Cancer Inst 2006;98:855–68.
21. Mertens-Talcott SU, Chintharlapalli S, Li X, Safe S. The oncogenic microRNA-27a targets genes that regulate specificity protein transcription factors and the G2-M checkpoint in MDA-MB-231 breast cancer cells. Cancer Res 2007;67: 11001–11.
22. Chintharlapalli S, Papineni S, Lee SO, Lei P, Jin UH, Sherman SI, et al. Inhibition of pituitary tumor-transforming gene-1 in thyroid cancer cells by drugs that decrease specificity proteins. Mol Carcinog 2011;50:655–67.
23. Chintharlapalli S, Papineni S, Ramaiah SK, Safe S. Betulinic acid inhibits prostate cancer growth through inhibition of specificity protein transcription factors. Cancer Res 2007;67:2816–23.
24. Chadalapaka G, Jutooru I, Chintharlapalli S, Papineni S, Smith R III, Li X, et al. Curcumin decreases specificity protein expression in bladder cancer cells. Cancer Res 2008;68:5345–54.
25. Papineni S, Chintharlapalli S, Abdelrahim M, Lee SO, Burghardt R, Abudayyeh A, et al. Tolfenamic acid inhibits esophageal cancer through repression of specificity proteins and c-Met. Carcinogenesis 2009;30:1193–201.
26. Hedrick E, Cheng Y, Jin UH, Kim K, Safe S. Specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 are non-oncogene addiction genes in cancer cells. Oncotarget 2016;7:22245–56.
27. Hedrick E, Crose L, Linardic CM, Safe S. Histone deacetylase inhibitors inhibit rhabdomyosarcoma by reactive oxygen species-dependent targeting of specificity protein transcription factors. Mol Cancer Ther 2015;14:2143–53.
28. Abdelrahim M, Smith R III, Burghardt R, Safe S. Role of Sp proteins in regulation of vascular endothelial growth factor expression and proliferation of pancreatic cancer cells. Cancer Res 2004;64:6740–9.
29. Higgins KJ, Abdelrahim M, Liu S, Yoon K, Safe S. Regulation of vascular endothelial growth factor receptor-2 expression in pancreatic cancer cells by Sp proteins. Biochem Biophys Res Commun 2006;345:292–301.
30. Abdelrahim M, Baker CH, Abbruzzese JL, Sheikh-Hamad D, Liu S, Cho SD, et al. Regulation of vascular endothelial growth factor receptor-1 expression by specificity proteins 1, 3, and 4 in pancreatic cancer cells. Cancer Res 2007;67: 3286–94.
31. Beishline K, Azizkhan-Clifford J. Sp1 and the 'hallmarks of cancer'. FEBS J 2015; 282:224–58.
32. Kennett SB, Udvadia AJ, Horowitz JM. Sp3 encodes multiple proteins that differ in their capacity to stimulate or repress transcription. Nucleic Acids Res 1997;25: 3110–7.
33. Li L, Davie JR. The role of Sp1 and Sp3 in normal and cancer cell biology. Ann Anat 2010;192:275–83.
34. Pan G, Zhang K, Geng S, Lan C, Hu X, Li C, et al. PHF14 knockdown causes apoptosis by inducing DNA damage and impairing the activity of the damage response complex in colorectal cancer. Cancer Lett 2022;531: 109–23.
35. Wu S, Luo C, Li F, Hameed NUF, Jin Q, Zhang J. Silencing expression of PHF14 in glioblastoma promotes apoptosis, mitigates proliferation and invasiveness via Wnt signal pathway. Cancer Cell Int 2019;19:314.
36. Miao L, Liu HY, Zhou C, He X. LINC00612 enhances the proliferation and invasion ability of bladder cancer cells as ceRNA by sponging miR-590 to elevate expression of PHF14. J Exp Clin Cancer Res 2019;38:143.
37. Zhao Y, He J, Li Y, Xu M, Peng X, Mao J, et al. PHF14 promotes cell proliferation and migration through the AKT and ERK1/2 pathways in gastric cancer cells. Biomed Res Int 2020;2020:6507510.
38. Deng C, Zhang L, Ma X, Cai S, Jia Y, Zhao L. RFTN1 facilitates gastric cancer progression by modulating AKT/p38 signaling pathways. Pathol Res Pract 2022; 234:153902.
39. Li C, Deng C, Pan G, Wang X, Zhang K, Dong Z, et al. Lycorine hydrochloride inhibits cell proliferation and induces apoptosis through promoting FBXW7-MCL1 axis in gastric cancer. J Exp Clin Cancer Res 2020;39:230.
40. Zhang J, Zhang J, Liu W, Ge R, Gao T, Tian Q, et al. UBTF facilitates melanoma progression via modulating MEK1/2-ERK1/2 signalling pathways by promoting GIT1 transcription. Cancer Cell Int 2021;21:543.
41. Akazawa T, Yasui K, Gen Y, Yamada N, Tomie A, Dohi O, et al. Aberrant expression of the PHF14 gene in biliary tract cancer cells. Oncol Lett 2013;5: 1849–53.
42. Zhang L, Huang Q, Lou J, Zou L, Wang Y, Zhang P, et al. A novel PHD-finger protein 14/KIF4A complex overexpressed in lung cancer is involved in cell mitosis regulation and tumorigenesis. Oncotarget 2017;8:19684–98.
43. Oh JE, Han JA, Hwang ES. Downregulation of transcription factor, Sp1, during cellular senescence. Biochem Biophys Res Commun 2007;353:86–91.
44. Ammendola R, Mesuraca M, Russo T, Cimino F. Sp1 DNA binding efficiency is highly reduced in nuclear extracts from aged rat tissues. J Biol Chem 1992;267: 17944–8.
45. Adrian GS, Seto E, Fischbach KS, Rivera EV, Adrian EK, Herbert DC, et al. YY1 and Sp1 transcription factors bind the human transferrin gene in an age-related manner. J Gerontol A Biol Sci Med Sci 1996;51:66–75.
46. Vizcaino C, Mansilla S, Portugal J. Sp1 transcription factor: A long-standing target in cancer chemotherapy. Pharmacol Ther 2015;152:111–24.
47. Jiang NY, Woda BA, Banner BF, Whalen GF, Dresser KA, Lu D. Sp1, a new biomarker that identifies a subset of aggressive pancreatic ductal adenocarcinoma. Cancer Epidemiol Biomarkers Prev 2008;17:1648–52.
48. Guan H, Cai J, Zhang N, Wu J, Yuan J, Li J, et al. Sp1 is upregulated in human glioma, promotes MMP-2-mediated cell invasion and predicts poor clinical outcome. Int J Cancer 2012;130:593–601.
49. Maurer GD, Leupold JH, Schewe DM, Biller T, Kates RE, Hornung HM, et al. Analysis of specific transcriptional regulators as early predictors of independent prognostic relevance in resected colorectal cancer. Clin Cancer Res 2007;13: 1123–32.
50. Wang F, Ma YL, Zhang P, Shen TY, Shi CZ, Yang YZ, et al. SP1 mediates the link between methylation of the tumour suppressor miR-149 and outcome in colorectal cancer. J Pathol 2013;229:12–24.
51. Wang L, Wei D, Huang S, Peng Z, Le X, Wu TT, et al. Transcription factor Sp1 expression is a significant predictor of survival in human gastric cancer. Clin Cancer Res 2003;9:6371–80.
52. Essafi-Benkhadir K, Grosso S, Puissant A, Robert G, Essafi M, Deckert M, et al. Dual role of Sp3 transcription factor as an inducer of apoptosis and a marker of tumour aggressiveness. PLoS One 2009;4:4478.
53. Bedolla RG, Gong J, Prihoda TJ, Yeh IT, Thompson IM, Ghosh R, et al. Predictive value of Sp1/Sp3/FLIP signature for prostate cancer recurrence. PLoS One 2012; 7:44917.
54. Kong LM, Liao CG, Fei F, Guo X, Xing JL, Chen ZN. Transcription factor Sp1 regulates expression of cancer-associated molecule CD147 in human lung cancer. Cancer Sci 2010;101:1463–70.
55. Wang XB, Peng WQ, Yi ZJ, Zhu SL, Gan QH. [Expression and prognostic value of transcriptional factor sp1 in breast cancer]. Ai Zheng 2007;26:996–1000.
56. Safe S, Imanirad P, Sreevalsan S, Nair V, Jutooru I. Transcription factor Sp1, also known as specificity protein 1 as a therapeutic target. Expert Opin Ther Targets 2014;18:759–69.
57. Huang Q, Zhang L, Wang Y, Zhang C, Zhou S, Yang G, et al. Depletion of PHF14, a novel histone-binding protein gene, causes neonatal lethality in mice due to respiratory failure. Acta Biochim Biophys Sin 2013;45:622–33.

58. Brunt L, Scholpp S. The function of endocytosis in Wnt signaling. Cell Mol Life Sci 2018;75:785–95.
59. Nagel R, Semenova EA, Berns A. Drugging the addict: non-oncogene addiction as a target for cancer therapy. EMBO Rep 2016;17:1516–31.
60. Eifert C, Powers RS. From cancer genomes to oncogenic drivers, tumour dependencies and therapeutic targets. Nat Rev Cancer 2012;12:572–8.
61. Apte RS, Chen DS, Ferrara N. VEGF in signaling and disease: beyond discovery and development. Cell 2019;176:1248–64.
62. Kent LN, Leone G. The broken cycle: E2F dysfunction in cancer. Nat Rev Cancer 2019;19:326–38.
63. Solimini NL, Luo J, Elledge SJ. Non-oncogene addiction and the stress phenotype of cancer cells. Cell 2007;130:986–8.
64. Luo J, Solimini NL, Elledge SJ. Principles of cancer therapy: oncogene and non-oncogene addiction. Cell 2009;136:823–37.
65. Folkman J. Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov 2007;6:273–86.
66. Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature 2005;434:913–7.
67. McGuire JJ. Anticancer antifolates: current status and future directions. Curr Pharm Des 2003;9:2593–613.
68. Costa C, Tsatsakis A, Mamoulakis C, Teodoro M, Briguglio G, Caruso E, et al. Current evidence on the effect of dietary polyphenols intake on chronic diseases. Food Chem Toxicol 2017;110:286–99.
69. Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 2003;3:768–80.
70. Gupta SC, Kim JH, Prasad S, Aggarwal BB. Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals. Cancer Metastasis Rev 2010;29:405–34.
71. Park W, Amin AR, Chen ZG, Shin DM. New perspectives of curcumin in cancer prevention. Cancer Prev Res 2013;6:387–400.
72. Rauf A, Imran M, Suleria HAR, Ahmad B, Peters DG, Mubarak MS. A comprehensive review of the health perspectives of resveratrol. Food Funct 2017;8:4284–305.
73. Romero R, Erez O, Huttemann M, Maymon E, Panaitescu B, Conde-Agudelo A, et al. Metformin, the aspirin of the 21st century: its role in gestational diabetes mellitus, prevention of preeclampsia and cancer, and the promotion of longevity. Am J Obstet Gynecol 2017;217:282–302.
74. Elwood PC, Gallagher AM, Duthie GG, Mur LA, Morgan G. Aspirin, salicylates, and cancer. Lancet 2009;373:1301–9.