FOXP2 inhibits the aggressiveness of lung cancer cells by blocking TGFβ signaling

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Abstract. Lung cancer is associated with high morbidity and mortality rates. Forkhead box P2 (*FOXP2*) functions as an antitumor gene in various cancers. However, its role in lung cancer remains to be elucidated. The present study explored the potential role of *FOXP2* in lung cancer. mRNA levels and protein expression were determined using RT-qPCR and western blotting, respectively. Functional analysis was performed using the CCK-8, Transwell and TUNEL assays. *FOXP2* expression was downregulated in lung cancer. Notably, FOXP2 suppressed the proliferative, migratory and invasive abilities of lung cancer cells and promoted tumor cell apoptosis. In addition, FOXP2 blocked TGFβ signaling. However, SRI-011381-stimulated activation of TGFβ signaling reversed the effects of overexpressed *FOXP2* and promoted the aggressiveness of lung cancer cells. *FOXP2* functions as an antitumor gene in lung cancer cells. FOXP2 suppressed the malignant behavior of lung cancer by inactivating TGFβ signaling.

Introduction

Lung cancer is one of the most common malignant tumors worldwide (1). The annual global incidence of lung cancer is over 1,800,000 (2). Various factors, such as smoking, habits and environment, contribute to the morbidity and mortality of lung cancer (3). In developed countries, lung cancer mortality has decreased since the 1990s (4). However, in China, patients with lung cancer have a higher mortality burden (5). Although great advances have been made in lung cancer treatment, such as chemotherapy, radiotherapy and surgery, the long-term overall survival rates remain unsatisfactory (6) due to limited access to diagnosis and treatment (7). Therefore, the identification of novel diagnostic markers for lung cancer is vital.

Forkhead box P2 (*FOXP2*), a transcription factor (8), is located on chromosome 7q31 and is a key regulator of metabolism, development and differentiation (9). *FOXP2* is involved in embryonic and organ development, including that of the heart, lungs and central nervous system (10). Abnormal *FOXP2* expression contributes to the pathogenesis of lung disorders, including lung cancer. For instance, FOXP2 alleviates LPS-induced apoptosis in human pulmonary alveolar epithelial cells and protects against acute lung injury (11). *FOXP2* expression is decreased in patients with lung cancer (12). Notably, FOXP2-mediated upregulation of *DNASE1L3* suppresses tumor cell proliferation and angiogenesis in lung adenocarcinoma (13). However, its role in lung cancer remains to be elucidated.

TGFβ regulates cell proliferation, migration and differentiation (14). However, TGFβ is frequently deregulated in carcinogenesis (15). TGFβ activation induced by tumor and stromal cells promotes tumor growth and metastasis (16). In addition, TGFβ signaling drives epithelial-to-mesenchymal transition processes (17), which may contribute to the chemoresistance and immune surveillance of tumor cells (18). Activated TGFβ ligand coordinates with its receptors to phosphorylate SMADs, which promotes nuclear translocation of SMADS to regulate the expression of the TGFβ target (19). In lung cancer, hypoxia-inducible factor 1-α-mediated activation of TGFβ/SMAD signaling accelerates tumor cell glycolysis and growth (20). Epigenetically stimulated *TGFβ2* transcription enhances the radioresistance of lung cancer (21). However, the roles of TGFβ/SMAD signaling in lung cancer are still not fully understood.

The present study investigated the potential role of *FOXP2* in lung cancer. Gene and protein expression were determined using RT-qPCR and western blotting, respectively. Functional analysis was performed using the CCK-8, colony formation, Transwell and TUNEL assays.

Materials and methods

Sampling. A total of 20 lung cancer tissues and adjacent tissues (>5 cm away from the tumor) [10 males and 10 females; 6 patients aged <60 years old (45-59 years old) and 14 patients

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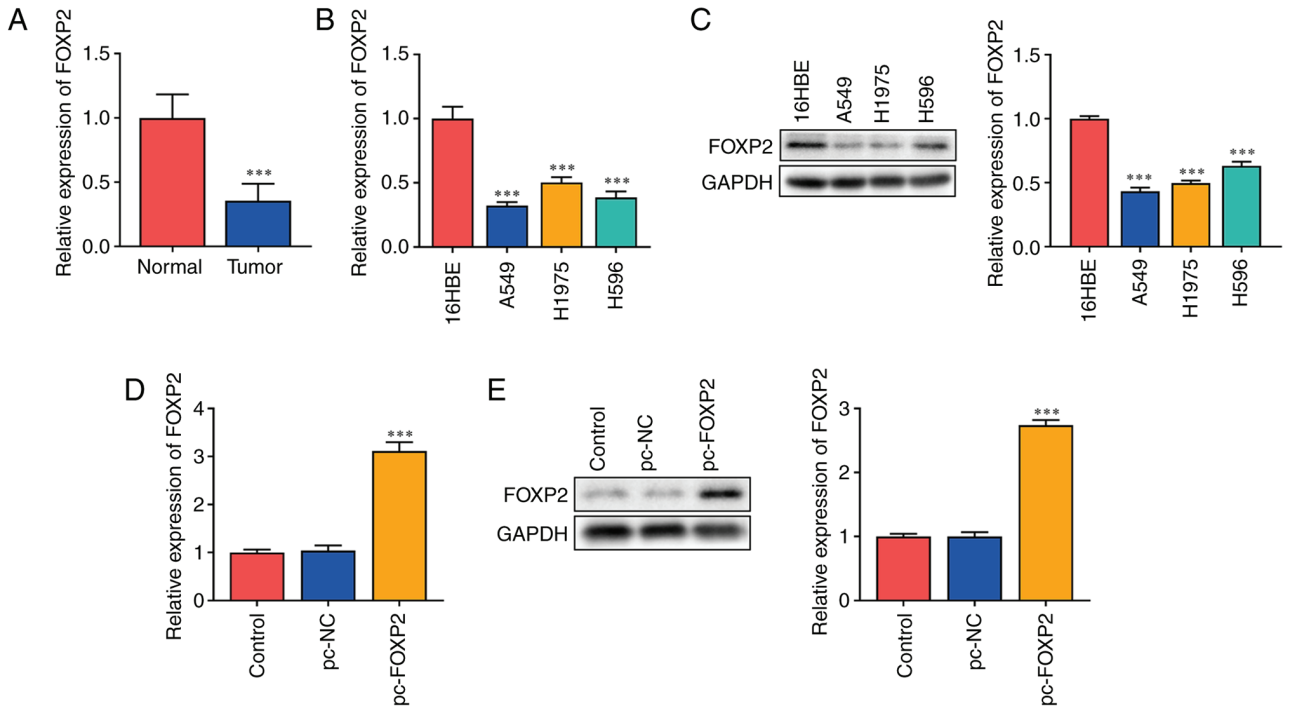

Figure 1. FOXP2 is downregulated in lung cancer. (A) FOXP2 mRNA expression in clinical samples were determined using RT-qPCR. ***P<0.001 vs. Normal. (B) FOXP2 mRNA expression in tumor cells were determined using RT-qPCR. ***P<0.001 vs. 16HBE. (C) FOXP2 protein in tumor cells were determined using western blotting. ***P<0.001 vs. 16HBE. (D) FOXP2 mRNA expression in tumor cells were determined using RT-qPCR after transfection with pc-FOXP2. ***P<0.001 vs. pc-NC. (E) FOXP2 protein expression in tumor cells were determined using western blotting after transfection with pc-FOXP2. ***P<0.001 vs. pc-NC. (A) n=20, (B-E) n=3. FOXP2, forkhead box P2; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control.

aged ≥60 years old] were collected from patients hospitalized at Changzhou First People's Hospital (Jiangsu, China). The tissues were immediately frozen in liquid nitrogen and stored at -80˚C for further processing. All diagnoses of non-small cell lung cancer were confirmed using pathological assays, including computed tomography, nuclear magnetic resonance imaging and immunohistochemistry. Patients who had previously received chemotherapy or radiotherapy were excluded from the present study. The present study was approved by the Ethics Committee of Changzhou First People's Hospital [Jiangsu, China; approval no. (2019)003].

Cell culture. Human lung cancer (A549, H1975 and H596) and human bronchial epithelial (16HBE) cell lines were provided by ATCC. Cells were cultured in RPMI-1640 medium (HyClone; Cytiva) containing 10% FBS at 37˚C in an incubator (Thermo Fisher Scientific, Inc.) with 5% CO_2. Cells were treated with 10 μM of SRI-011381 (MedChemExpress), an agonist of TGFβ signaling.

Cell transfection. pcDNA3.1 and pcDNA3.1-FOXP2 were obtained from Shanghai GenePharma Co., Ltd. A549 Cells with good growth state were taken for seed plate and transfected when the cell density reached 60%. The culture medium was replaced with a non-antibiotic medium 12 h before transfection. Cells were divided into the following groups: Control, untreated; pc-negative control (NC), transfected with 5 μl of Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and 20 μM pcDNA3.1 for 6 h at 37˚C; pc-FOXP2, transfected with 5 μl of Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and 20 μM pcDNA3.1-FOXP2 for 6 h at 37˚C and then replaced with complete medium and culture continued for 24-48 h.

Reverse transcription-quantitative (RT-q) PCR. All experimental operations were performed according to the manufacturer's protocols. When the cell density reached 1x10^6, total RNA was extracted from the cells. cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (cat. no. K1622; Thermo Fisher Scientific, Inc.). The mRNA expression was determined using PCR with the FastStart Universal SYBR Green Master (Rox) Kit (cat. no. 04913914001; Roche Diagnostics). mRNA expression was normalized to that of *GAPDH*. Results were measured using the $2^{-\Delta\Delta Cq}$ method (22). The PCR conditions were as follows: Pre-denaturation at 95˚C for 1 min, followed by denaturation at 95˚C for 15 sec, and annealing and extension at 60˚C for 30 sec, for 40 cycles. This was repeated three times for each set. The primer sequences were: *GAPDH*, F: 5' AGA AGGTGGTGAAGCAGGCGTC 3' and R: 5' AAAGGTGGA GGAGTGGGTGTCG 3'; and *FOXP2*, F: 5'-GATGCAACA ACTCCAGCAG-3' and R: 5'-AGGACTTAAGCCAGCTTG AG-3'.

Western blotting. Cells in good condition were collected and the cell culture medium was discarded. Thereafter, cells were washed twice with PBS, RIPA lysis buffer (cat. no. BL504A; Biosharp Life Sciences) was added and the cells were shaken on ice for 15 min. The supernatant was centrifuged at 4˚C, at 10,000 x g, for 5 min, and then the sample was heated at 100˚C for 10 min. The protein concentration was determined using the BCA method. The protein (20 μg/lane) was

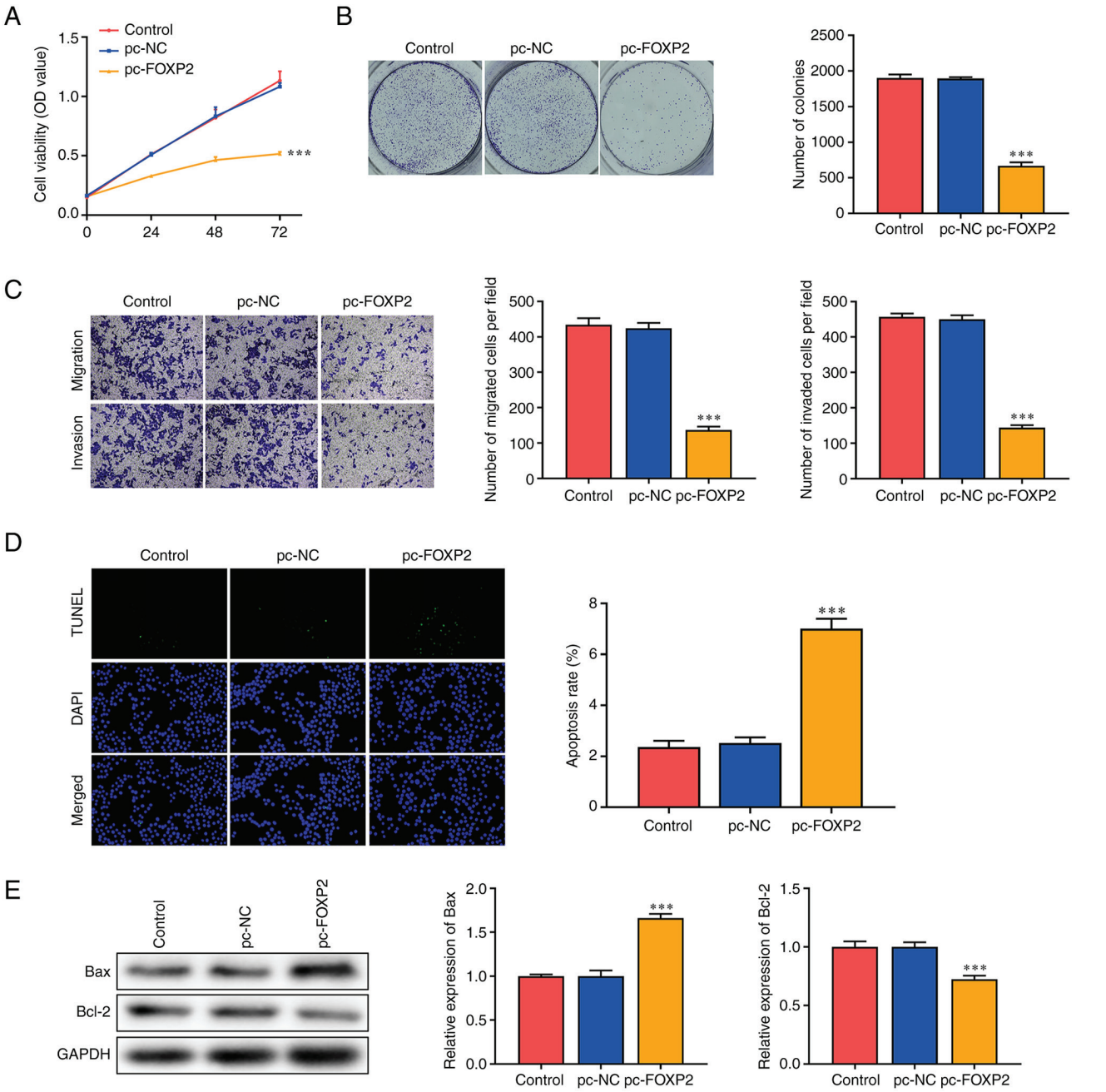

Figure 2. Overexpressed FOXP2 suppresses the aggressiveness of A549 cells. (A) Cell viability of A549 cells was determined using CCK-8 assay. (B) Cell proliferation was determined using colony formation assay. (C) Cell migrative and invasive ability was determined using Transwell assay. (D) Cell apoptosis was determined using TUNEL assay. (E) Bcl-2 and Bax protein expression was determined using western blotting. ***P<0.001 vs. pc-NC. n=3. FOXP2, forkhead box P2; NC, negative control.

isolated using SDS-PAGE (10%; 120 V) and transferred onto PVDF membranes (MilliporeSigma). Thereafter, the PVDF membranes were treated with 5% non-fat milk at room temperature to block them for 30 min, and incubated with primary antibodies at 4˚C overnight: FOXP2 (cat. no. ab16046; 1:1,000), BAX (cat. no. ab32503; 1:2,500), BCL-2 (cat. no. ab182858; 1:2,000), p-SMAD3 (cat. no. ab52903; 1:2,000), SMAD3 (cat. no. ab208182; 1:1,000), SMAD4 (cat. no. ab40759; 1:5,000), TGFβR1 (cat. no. ab235578; 1:1,000), zinc finger E-box binding homeobox 1 (ZEB1; cat. no. ab203829; 1:500), zinc finger protein SNAI1 (SNAIL; cat. no. ab216347; 1:1,000) and GAPDH (cat. no. ab181602; 1:5,000) and then with HRP-labeled secondary antibody incubated at room temperature for 1 h. (cat. no. ab205718; 1:10,000). All antibodies were provided by Abcam. Proteins were visualized using an enhanced chemiluminescence kit (Tanon Science & Technology Co., Ltd.). Finally, ImageJ (National Institutes of Health) was used to analyze the gray value of the images.

CCK-8 assay. The cells were plated in 24-well plates at a density of $1x10^5$ cells per well and incubated for 0, 24, 48 and 72 h after transfection at 37˚C. The cells were then cultured with CCK-8 (10 μl; Beijing Solarbio Science & Technology Co., Ltd.) and cultured for another 4 h at 37˚C. Subsequently, absorbance values were determined using a microplate reader at a wavelength of 450 nm.

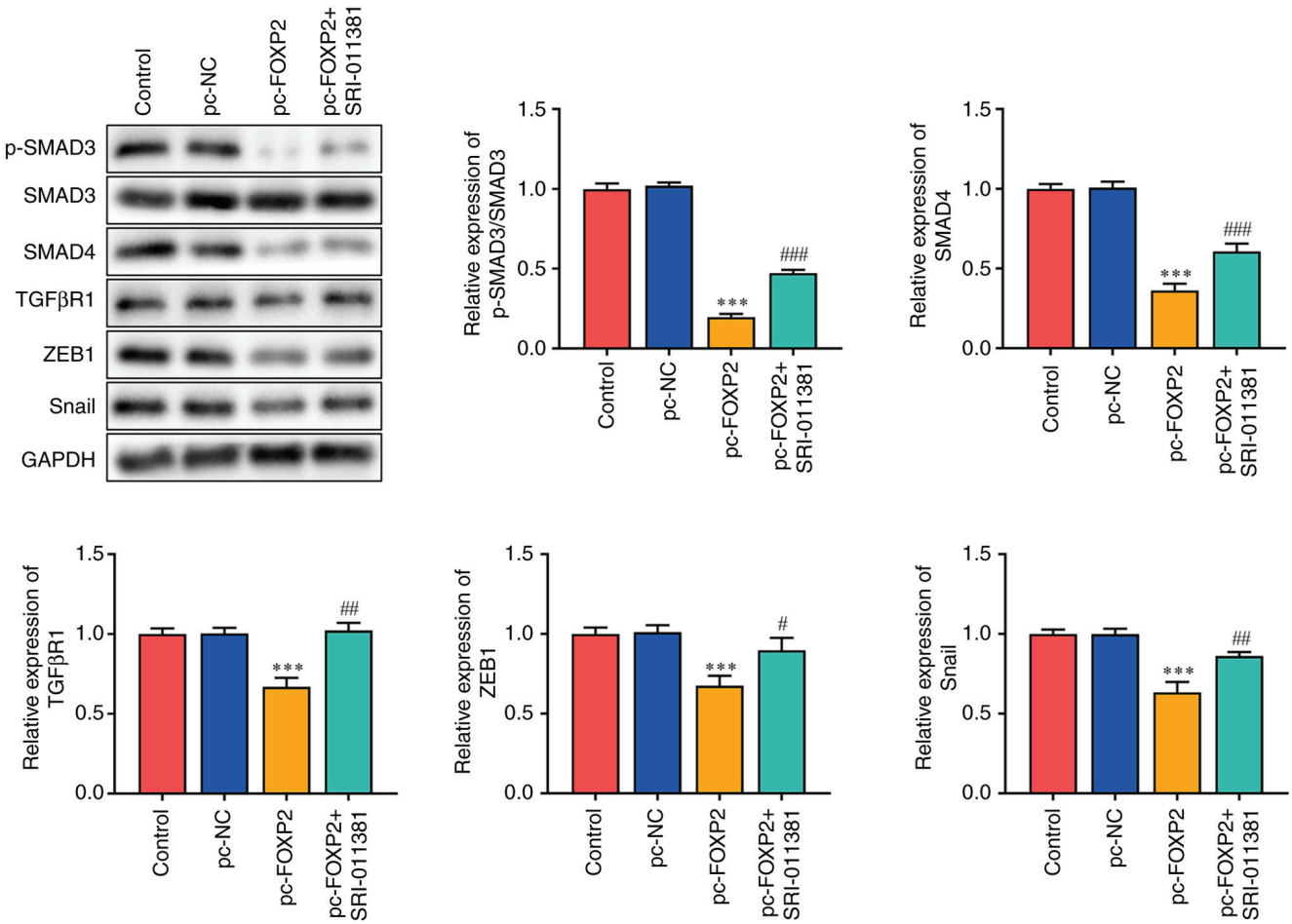

Figure 3. FOXP2 blocks TGFβ/SMAD signaling in lung cancer. The protein expression of TGFβ signaling was determined using western blotting. ***P<0.001 vs. pc-NC, #P<0.05, ##P<0.01, ###P<0.001 vs. pc-FOXP2. n=3. FOXP2, forkhead box P2; NC, negative control.

Colony formation assay. Cells were plated in a 96-well plate precoated with soft agar and cultured at 37˚C in a 5% CO_2 incubator for 14 days. After fixing with 100% methanol at room temperature for 30 min, cells were stained with 1% crystal violet at room temperature for 15 min. Images were captured under a microscope (Leica Microsystems GmbH). Colonies with diameters >2 mm in the predetermined fields of interest were counted (magnification, x200).

Transwell assay. Transwell chambers were pre-coated with Matrigel (BD Biosciences) and placed on ice for 30 min to form an even coating. Homogeneous serum-free cell suspensions ($5x10^5$ cells/well) were added to the upper chambers and the lower chambers were supplemented with 10% FBS. Transwell culture dishes were placed in a 5% CO_2 cell incubator at 37˚C for 24 h. Cells in the lower chamber were fixed and stained with 1% crystal violet (Beyotime Institute of Biotechnology). Finally, the number of migrated or invaded cells in predetermined fields of interest was calculated based on images captured using a microscope (CKX53; Olympus Corporation; magnification, x400).

TUNEL assay. Cells were harvested, fixed in 4% paraformaldehyde (MilliporeSigma) at room temperature for 15 min and permeabilized with 0.25% Triton-X100 (Dalian Meilun Biotechnology Co., Ltd.). Thereafter, cells were stained using an *in situ* cell death detection kit (MilliporeSigma). Images were visualized using a fluorescence microscope (Nikon Corporation). The cell death rate was calculated as TUNEL-positive cells/total cells x100.

Statistical analysis. Statistical analyses were performed using GraphPad software, version 9.5.1 (GraphPad; Dotmatics). Data were presented as mean ± standard deviation. Student's t-test was performed to analyze the differences between two groups, whereas one-way analysis of variance and Tukey's post hoc test were applied for multigroup analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

FOXP2 is downregulated in lung cancer. The present study hypothesized that *FOXP2* functions as an antitumor gene in lung cancer. It was found that *FOXP2* mRNA expression significantly decreased in patients with lung cancer (Fig. 1A). In addition, mRNA (Fig. 1B) and protein (Fig. 1C) expressions of FOXP2 were markedly decreased in lung cancer cells. A549 cells with a significant difference in *FOXP2* expression were used for subsequent experiments. The potential role of *FOXP2* in lung cancer was further investigated. A549 cells were transfected with *FOXP2* overexpression

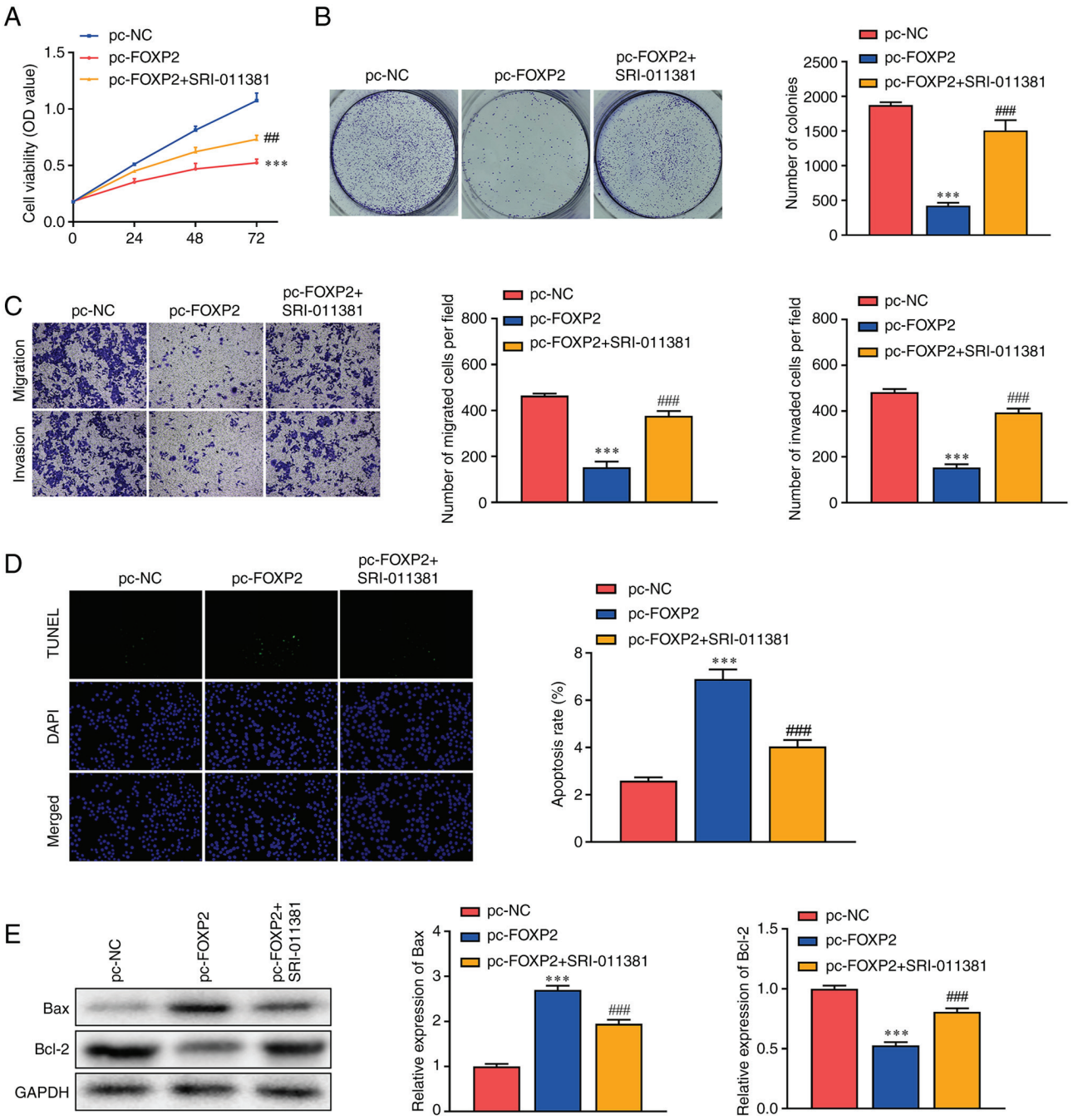

Figure 4. Activation of TGFβ/SMAD signaling promotes the malignant behaviors of lung cancer. (A) Cell viability of A549 cells was determined using CCK-8 assay. (B) Cell proliferation was determined using colony formation assay. (C) Cell migrative and invasive ability was determined using Transwell assay. (D) Cell apoptosis was determined using TUNEL assay. (E) Bcl-2 and Bax protein expression was determined using western blotting. ***P<0.001 vs. pc-NC, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. pc-FOXP2. n=3.

plasmids. FOXP2 expression in the pc-FOXP2 group was significantly increased at both the mRNA and protein levels (Fig. 1D and E), suggesting that the cells were successfully transfected.

FOXP2 overexpression suppresses the aggressiveness of A549 cells. Functional assays were performed to determine the effects of *FOXP2* on lung cancer cell function. The overexpression of *FOXP2* significantly suppressed the viability of A549 cells compared with that in the pc-NC group (Fig. 2A). In addition, *FOXP2* overexpression markedly inhibited colony formation in A549 cells (Fig. 2B). Migratory and invasive abilities were significantly suppressed in the pc-FOXP2 group (Fig. 2C). *FOXP2* overexpression significantly increased the TUNEL-positive cells (Fig. 2D). In addition, *FOXP2* overexpression increased BAX expression and suppressed BCL-2 expression (Fig. 2E). These findings suggested that *FOXP2* overexpression suppressed the malignant behavior of lung cancer cells.

FOXP2 blocks TGFβ/SMAD signaling in lung cancer. TGFβ/SMAD is involved in the carcinogenesis of lung cancer (23-25). FOXP2 overexpression suppresses tumor aggressiveness. Therefore, it was hypothesized that *FOXP2*

may inhibit the development of lung cancer by targeting TGFβ signaling. It was found that *FOXP2* overexpression significantly inhibited SMAD3 phosphorylation and suppressed the protein expression of SMAD4, TGFβR1, ZEB1 and SNAIL. However, the TGFβ/SMAD signaling agonist SRI-011381 reversed the effects of pc-FOXP2 (Fig. 3).

Activation of TGFβ/SMAD signaling promotes the malignant behaviors of lung cancer. Rescue assays were conducted to verify the role of FOXP2 and TGFβ/SMAD in lung cancer. Following exposure to SRI-011381, a TGFβ/SMAD signaling agonist, the inhibition of cell viability induced by *FOXP2* overexpression was alleviated (Fig. 4A). In addition, SRI-011381 treatment significantly abrogated the effects of *FOXP2* overexpression and increased the number of tumor cell clones (Fig. 4B). This was consistent with the results of the Transwell assay. In addition, the inhibition of tumor cell migration and invasion induced by *FOXP2* overexpression was markedly abrogated by SRI-011381 (Fig. 4C). In addition, SRI-011381 treatment significantly alleviated the effects of *FOXP2* overexpression and suppressed apoptosis in A549 cells (Fig. 4D). SRI-011381 treatment significantly dampened the effects of *FOXP2* overexpression, increased BCL-2 protein expression and downregulated BAX. These findings suggested that FOXP2 may suppress the aggressiveness of lung cancer by targeting TGFβ/SMAD signaling.

Discussion

In the present study, *FOXP2* expression was downregulated in lung cancer. Notably, *FOXP2* overexpression suppressed the proliferative, migratory and invasive abilities of lung cancer cells and promoted tumor cell apoptosis. In addition, FOXP2 blocked TGFβ signaling, the activation of which enhances malignant behaviors in tumor cells.

Increasing evidence indicates that *FOXP2* functions as an oncogene in various types of cancer. For instance, circST3GAL6-mediates upregulation of *FOXP2* promotes apoptosis and autophagy in gastric cancer (26). *FOXP2* overexpression inhibits the migration of colon cancer (27). However, the role of *FOXP2* in cancer remains unclear. Activation of HN1L/FOXP2 signaling-mediated stemness promotes tumor growth and migration in prostate cancer (28). In addition, *FOXP2* overexpression promotes the migration and invasion of colorectal cancer cells (29), suggesting that it may also function as an oncogene. Therefore, identifying the exact role of *FOXP2* in lung cancer is vital. In the present study, *FOXP2* expression was decreased in lung cancer cells. In addition, *FOXP2* overexpression suppressed the proliferative, migratory and invasive abilities of lung cancer cells, suggesting that *FOXP2* may function as an antitumor gene in lung cancer. These findings were consistent with those of previous studies (12,13).

FOXP2 alters cellular functions by regulating the expression of its targets (30). For instance, FOXP2 epigenetically activates RPS6KA6 to enhance tumor cell apoptosis in thyroid cancer (31). FOXP2 interacts with caspase-1 to drive tumor cell pyroptosis in colorectal cancer (23). In the present study, FOXP2 blocked TGFβ signaling, which plays a key role in the pathogenesis of cancers. However, its role varies with the stages of tumors. At the early stages, TGFβ signaling functions as a tumor suppressor and promotes cell cycle arrest (24). However, the enrichment of proinflammatory TGFβ induces the degradation of epithelial functions and the acquisition of mesenchymal features (25), promoting tumor cell migration and invasion. In addition, the continuous release of TGFβ contributes to the immune evasion of tumor cells by recruiting macrophages, cancer-associated fibroblasts and neutrophils (32). In the present study, SRI-011381-mediated activation of TGFβ/SMAD signaling promoted the proliferation, migration and invasion of lung cancer cells and suppressed tumor cell apoptosis. These findings suggested that FOXP2 suppressed the aggressiveness of lung cancer cells by targeting TGFβ/SMAD signaling.

The present study had some limitations. First, it included only 20 participants and did not distinguish the malignant degree of lung cancer. Future studies with a larger sample size are needed to confirm the results and further studies are needed to investigate the correlation between FOXP2 expression and malignant degree of lung cancer. Second, avoiding recollection bias when obtaining past information was difficult. In addition, some of the clinical data were missing. Therefore, well-designed studies are warranted in the future.

In conclusion, *FOXP2* functions as an antitumor gene in lung cancer. FOXP2 suppressed the proliferation, migration and invasion of lung cancer cells and promoted apoptosis in lung cancer cells by blocking TGFβ/SMAD signaling. Therefore, FOXP2/TGFβ/SMAD signaling may be a potential target for lung cancer.

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Availability of data and material

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

ZYL conceived and designed the study. WYS, SH and LZ performed the literature search, performed the experiments and data extraction. HB analyzed and interpreted the data. WYS and HB drafted the manuscript. ZYL, WYS and SH confirm the authenticity of all the raw data and revised the final version of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Changzhou First People's Hospital [Jiangsu, China; approval no. (2019)003]. All patients signed informed consent forms.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflicts of interest.

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