

DNASE1L3 regulation by transcription factor FOXP2 affects the proliferation, migration, invasion and tube formation of lung adenocarcinoma

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Abstract. Lung adenocarcinoma (LUAD) is prone to bone metastasis, resulting in poor prognosis. The present study aimed to detect the expression of deoxyribonuclease 1-like 3 (DNASE1L3) and forkhead-box P2 (FOXP2) in LUAD cells to investigate the role of DNASE1L3 in the regulation of proliferation, migration, invasion and tube formation of LUAD cells and how FOXP2 affects DNASE1L3 expression. The expression of DNASE1L3 and FOXP2 in LUAD cells was analyzed by reverse transcription-quantitative PCR (RT-qPCR) and western blotting. The transfection efficiency of DNASE1L3 overexpression plasmids, FOXP2 overexpression or interference plasmids into A549 cells was also confirmed by RT-qPCR and western blotting. The viability, proliferation, migration and invasion and tube formation of LUAD cells following transfection was in turn detected by MTT, EdU staining, wound healing, Transwell and tube formation assay. The expression of proteins associated with epithelial-mesenchymal transformation and tube formation was detected by western blotting. Binding between DNASE1L3 and FOXP2 was confirmed by dual-luciferase reporter assay and chromatin immunoprecipitation. Gene Expression Profiling Interactive Analysis database predicted that underexpression of DNASE1L3 in LUAD was associated with poor prognosis. DNASE1L3 expression was decreased in LUAD cells and overexpression of DNASE1L3 inhibited the proliferation, migration, invasion and tube formation of LUAD cells. Transcription factor FOXP2 positively regulated DNASE1L3 transcription in LUAD cells. FOXP2 was also underexpressed in LUAD cells and downregulation of FOXP2 promoted proliferation, migration, invasion and tube formation of LUAD cells, which was reversed by overexpression of DNASE1L3. In conclusion, DNASE1L3 was positively regulated by transcription factor

FOXP2 and overexpression inhibited proliferation, migration, invasion and tube formation of LUAD cells.

Introduction

Lung cancer ranks among the top malignancies in terms of morbidity and mortality globally, accounting for 11.4% of all cancer cases and 18% of all cancer-associated deaths (1). Lung cancer includes non-small cell lung cancer (NSCLC) and SCLC. Among the different types of NSCLC, including adenocarcinoma and squamous and large cell carcinoma, adenocarcinoma is the most common type (2). Lung adenocarcinoma (LUAD) is prone to bone metastasis and patients with bone metastasis have been shown to have poor prognosis. Additionally, the pain and bone breakage caused by bone metastasis decreases quality of life of patients (3). Molecular-targeted therapy and immunotherapy have been widely used in clinical practice as a supplement to traditional surgery and chemoradiotherapy; however, the 5-year survival rate of patients is still not ideal and more effective therapies need to be found (4,5). Therefore, it is of clinical significance to study the pathogenesis of LUAD metastasis and find effective treatments.

Deoxyribonuclease 1-like 3 (DNASE1L3) is a member of DNASE1 gene family and serves a key role in DNA degradation during cell death and apoptosis (6,7). DNASE1L3 has been shown to be involved in signal transduction in breast cancer, exhibiting intracellular signal cascade receptor activity and serving as a GTPase regulatory factor (8). DNASE1L3 has also been shown to suppress apoptosis and reprogram glucose metabolism, thus inhibiting hepatocellular carcinoma (HCC) progression (9). The senescence-associated secretory phenotypes are damaged by DNASE1L3 interacted with H2BE to suppress tumor angiogenesis in HCC (10). Underexpression of DNASE1L3 in colon cancer samples is a potential prognostic biomarker associated with immune invasion of colon cancer and overexpression of DNASE1L3 inhibits cell proliferation and motility (11). A previous study reported that DNASE1L3 is downregulated in LUAD (12). An additional study found that mRNA and protein expression levels of DNASE1L3 in patients with LUAD are significantly lower than those in normal tissue and low expression of DNASE1L3 is significantly correlated with higher pathological stage, T stage and poor prognosis, making it an independent factor for predicting the overall

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survival rate (13). The specific role of DNASE1L3 in LUAD, to the best of our knowledge, has not yet been reported.

Previous studies have shown that transcription factor forkhead-box P2 (FOXP2) is involved in the regulation of tumor invasion and metastasis (14-17). FOXP2 protein has been shown to promote cell proliferation, invasion and metastasis in breast cancer (14). FOXP2 has also been shown to activate TGF- β to promote migration and invasion of prostate cancer cells (15). Upregulated FOXP2 expression inhibits proliferation, migration, invasion and epithelial-mesenchymal transformation (EMT) and promotes cell apoptosis in NSCLC (16,17). All these findings imply that FOXP2 may function in opposing ways in different tissues.

Therefore, it was hypothesized that as DNASE1L3 is underexpressed in LUAD, overexpression of DNASE1L3 may inhibit malignant progression of LUAD. In addition, DNASE1L3 transcription may be positively regulated by FOXP2.

Materials and methods

Bioinformatic analysis. The expression of DNASE1L3 in LUAD (n=483) and adjacent tissue (n=347) as well as overall survival were analyzed using Gene Expression Profiling Interactive Analysis (GEPIA; gepia.cancer-pku.cn/) (18). The JASPAR database (jaspar.genereg.net/) predicted the potential binding between FOXP2 and DNASE1L3 promoter.

Cell culture. Human bronchial epithelial cell line (16HBE cells) and three LUAD cell lines (PC-9, NCI-H1975 and A549 cells) were provided by Ningbo Mingzhou Biotechnology Co., Ltd. Human umbilical vein endothelial cells (HUVECs) were obtained from PromoCell GmbH. The cells were cultured at 37°C in 5% CO₂ in RPMI-1640 medium (Sigma-Aldrich, Merck KGaA) with 10% FBS (Sigma-Aldrich, Merck KGaA). When cells reached the logarithmic growth phase, they were digested with 0.25% trypsin and collected for experiments.

Cell transfection. PcDNA3.1(+) Overexpression-negative control (empty vector plasmid; Oe-NC), Oe-DNASE1L3 (accession no. NM_001256560.2) or Oe-FOXP2 (accession no. NM_001172766.3), small interfering RNAs targeting FOXP2 (si-FOXP2; #1, 5'-GACATTCAGACAAATACAACATT-3'; #2, 5'-GACAATAAGCAACAGTTCAATGA-3') and negative control sequence (NC, 5'-AAGACAUUGUGU GUCCGCCTT-3'; both 50 nM) were designed and provided by Guangzhou RiboBio Co., Ltd. A549 cells in logarithmic growth phase were seeded in 6-well plates. When cells grew to a density of 80%, plasmids carrying the target gene and siRNAs were transfected into the cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h at 37°C in 5% CO₂ as previously described (19). Subsequent experiments were performed 48 h post-transfection.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The expression of DNASE1L3 and FOXP2 in LUAD cells or transfected LUAD cells was detected by RT-qPCR. A549 cells were collected and total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Next, total RNA was reverse-transcribed

into cDNA using a PrimeScript™ RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol and qPCR was conducted with SYBR-Green PCR Master Mix (MedChemExpress). The following thermocycling conditions were used: Initial denaturation at 94°C for 5 min, 36 cycles at 94°C for 20 sec, 54°C for 20 sec and 72°C for 20 sec. The primer sequences were as follows: DNASE1L3, forward, 5'-AGC CCTTTGTGGTCTGGTTC-3' and reverse, 5'-TCCTTAACG GATGTCTCTGGG-3'; FOXP2 forward, 5'-AATCTGCGA CAGAGACAATAAGC-3' and reverse, 5'-TCCACTTGT TTGCTGCTGTAAA-3' and GAPDH forward, 5'-GGAGCG AGATCCCTCCAAAAT-3' and reverse, 5'-GGCTGTTGT CATACTTCTCATGG-3'. Relative expression of DNASE1L3 and FOXP2 was quantified using the 2^{- $\Delta\Delta C_q$} method (20) and GAPDH served as the internal control.

Western blotting. The expression of DNASE1L3 and FOXP2 in LUAD cells or transfected LUAD cells and expression of E-cadherin, N-cadherin, Snail and vascular endothelial growth factor (VEGF) in transfected LUAD cells was determined by western blotting. Following transfection, A549 cells were homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology) to extract total protein. The protein concentration was detected by BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Protein samples (40 μ g/lane) were loaded into a 10% SDS-PAGE gel, which was then electrophorized and transferred to a PVDF membrane. The membrane was blocked with 5% BSA (Beyotime Institute of Biotechnology) at room temperature for 1 h before incubation with primary antibodies against DNASE1L3 (cat. no. ab152118; 1/1,000; Abcam), E-cadherin (cat. no. ab40772; 1/10,000; Abcam), N-cadherin (cat. no. ab76011; 1/5,000; Abcam), Snail (cat. no. ab216347; 1/1,000; Abcam), VEGF (cat. no. ab32152; 1/1,000; Abcam), FOXP2 (cat. no. ab16046; 1/1,000; Abcam) and GAPDH (cat. no. ab9485; 1/2,500; Abcam) at 4°C overnight. Afterwards, the membranes were incubated with HRP-conjugated Goat Anti-Rabbit IgG H&L secondary antibody (cat. no. ab97051; 1/2,000; Abcam) at room temperature for 1 h. A BeyoECL Plus kit (cat. no. P0018S; Beyotime Institute of Biotechnology) was used to visualize protein bands, which were quantified by ImageJ 1.8.0 software (National Institutes of Health).

MTT assay. The viability of transfected LUAD cells was analyzed by MTT assay. Transfected A549 cells (5x10³/well) were seeded into 96-well plates and incubated at 37°C with 5% CO₂ for 24, 48 and 72 h. Following incubation, 10 μ l MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA) was added to cells in each well, then incubated for 4 h at 37°C. The supernatant was discarded and 100 μ l DMSO was added to each well before plates were shaken at low speed for 10 min. The absorbance values at 490 nm were measured using a microplate reader.

EdU staining. EdU staining was used to determine proliferation of the transfected LUAD cells. The proliferation of A549 cells was determined using the Cell-Light EdU Apollo488 *In Vitro* Kit (cat. no. C10310-3; Guangzhou Ribobio Co., Ltd.). Briefly, A549 cells were incubated with EdU solution for 2 h at 37°C and fixed with PBS containing 4% paraformaldehyde at room temperature for 15 min. Subsequently, the nucleus was

stained with 0.01 mg/ml DAPI at 37°C for 30 min. Finally, five areas were randomly selected at x200 magnification to count the number of green fluorescence-positive cells under a fluorescence microscope (Olympus Corporation) using ImageJ (version no. 1.52; National Institutes of Health).

Wound healing assay. The migration of transfected LUAD cells was detected by wound healing assay. Following transfection, A549 cells were seeded into a six-well plate (3×10^5 cells/well) in RPMI-1640 medium and grown to 90% confluence at 37°C. A 100- μ l pipette tip was used to scratch a straight wound across the well and incubated at 37°C in serum-free medium. After 24 h, images of the migrated cells were captured using a light microscope (Olympus Corporation) at x100 magnification and scratch widths were recorded by ImageJ 1.8.0 software (National Institutes of Health). Multiple measurements were made along the scratch ($n=3$).

Transwell assay. The invasion of transfected LUAD cells was detected by Transwell assay. A549 cells were inoculated into 24-well invasion chambers (1×10^5 cells/well). The upper chamber was coated with Matrigel (8 μ m pores) at 37°C for 30 min. The lower chamber contained RPMI-1640 medium supplemented with 10% FBS as the chemoattractant. After incubation for 24 h at 37°C, the non-invasive cells inside the upper chamber were removed and the remaining cells were fixed with 4% paraformaldehyde for 15 min at room temperature and stained with 1% Giemsa for 5 min at room temperature. The invasive cells were observed by a light microscope (Olympus Corporation) at x100 magnification and the number of invasive cells was counted by a cell counter.

Tube formation assay. The tube formation of HUVECs in culture medium from transfected LUAD cells was determined by tube formation assay. Matrigel-coated 24-well Transwell plates (8 μ m pores) at 37°C for 30 min were used to conduct the tube formation assay. HUVECs were suspended in medium from control, si-NC, si-FOXP2, si-FOXP2+Oe-NC and si-FOXP2+Oe-DNASE1L3 groups at a density of 1×10^5 cells/ml. In total, ~100 μ l cell suspension was added to the surface of the Matrigel, which was incubated at 37°C for 6 h. The tube formation was visualized using a light microscope (Olympus Corporation) at x40 magnification.

Dual-luciferase reporter assay. Dual-luciferase reporter assay was performed to verify whether transcription factor FOXP2 interacted with the DNASE1L3 promoter. The wild-type (DNASE1L3-WT) or mutant (DNASE1L3-MUT) fragments of DNASE1L3 (accession no. NM_001256560.2) with the binding site of FOXP2 were constructed by Shanghai GenePharma Co., Ltd. WT or MUT sequence were cloned into the pGL3 luciferase reporter vector (Promega Corporation). A549 cells (5×10^5) were seeded in 24-well plates for 24 h at 37°C and co-transfected with WT or MT plasmid and Oe-NC or Oe-FOXP2 using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h at 37°C, the luciferase activity was detected using dual luciferase reporter assay kit (cat. no. E1910; Promega Corporation). Renilla luciferase activity was used for normalization.

Chromatin immunoprecipitation (ChIP). ChIP analysis was performed to confirm whether transcription factor FOXP2 bound to the DNASE1L3 promoter. ChIP analysis was carried out using ChIP Assay kit (Beyotime Institute of Biotechnology) according to manufacturer's protocol. Briefly, 1% formaldehyde was added to transfected A549 cells for 10 min at room temperature. The fixed cells were washed twice with phosphate-buffered saline and were lysed using a lysis buffer (0.1% SDS, 0.5% Triton X-100, 20 mM Tris-HCl, pH 8.1) that contained 150 mM NaCl and a protease inhibitor, after which chromatin fragments were obtained using sonication using a 10 sec on and 10 sec off mode for 12 cycles at 4°C. Samples were centrifuged at $13,000 \times g$ for 10 min at 4°C, and 100 μ l of supernatant was pre-absorbed by 2 μ g of anti-FOXP2 antibody (cat. no. #5337; 1/200; Cell Signaling Technology) or IgG control (cat. no. ab172730; 1:50; Abcam) overnight at 4°C. Samples were supplemented with protein agarose/sepharose (cat. no. #9863; Cell Signaling Technology) to precipitate the endogenous DNA-protein complex. The immunoprecipitated complex was centrifuged ($5,000 \times g$ for 1 min at 4°C) and washed with low salt, high salt, LiCl and TE buffers in the kit according to the manufacturer's protocols. The complex was eluted from the antibody using a solution of 1% SDS, 0.1 mol/l NaHCO₃ and 200 mmol/l NaCl. The complex was de-crosslinked at 65°C and the DNA fragment was recovered by phenol/chloroform extraction and purification. The enrichment of specific fragments was determined by RT-qPCR, as aforementioned.

Statistical analysis. Data from three independent replicates are presented as the mean \pm SD. GraphPad Prism 8.0.1 (GraphPad Software, Inc.) was used for the statistical analysis. Overall survival was calculated by Kaplan-Meier survival analysis. An unpaired Student's t test was applied for the comparison between two groups. Comparisons between >2 groups were made using one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

DNASE1L3 underexpression in LUAD is associated with poor prognosis. The GEPIA database indicated that the expression of DNASE1L3 in LUAD tissue was decreased (Fig. 1A) and low expression of DNASE1L3 was significantly associated with poor prognosis in patients with LUAD (Fig. 1B). RT-qPCR and western blotting were used to detect the mRNA and protein expression of DNASE1L3 in LUAD cells. DNASE1L3 was significantly downregulated in PC-9, NCI-H1075 and A549 cells compared with 16HBE cells and the lowest expression of DNASE1L3 was in the A549 cell line (Fig. 1C and D). Thereafter, A549 cells were chosen for the subsequent experiments.

Oe-DNASE1L3 inhibits proliferation of A549 cells. The transfection efficiency of Oe-DNASE1L3 in A549 cells was confirmed by RT-qPCR and western blotting. The viability and proliferation of Oe-DNASE1L3-transfected A549 cells were determined by MTT assay and EdU staining. When A549 cells were transfected with Oe-DNASE1L3, expression

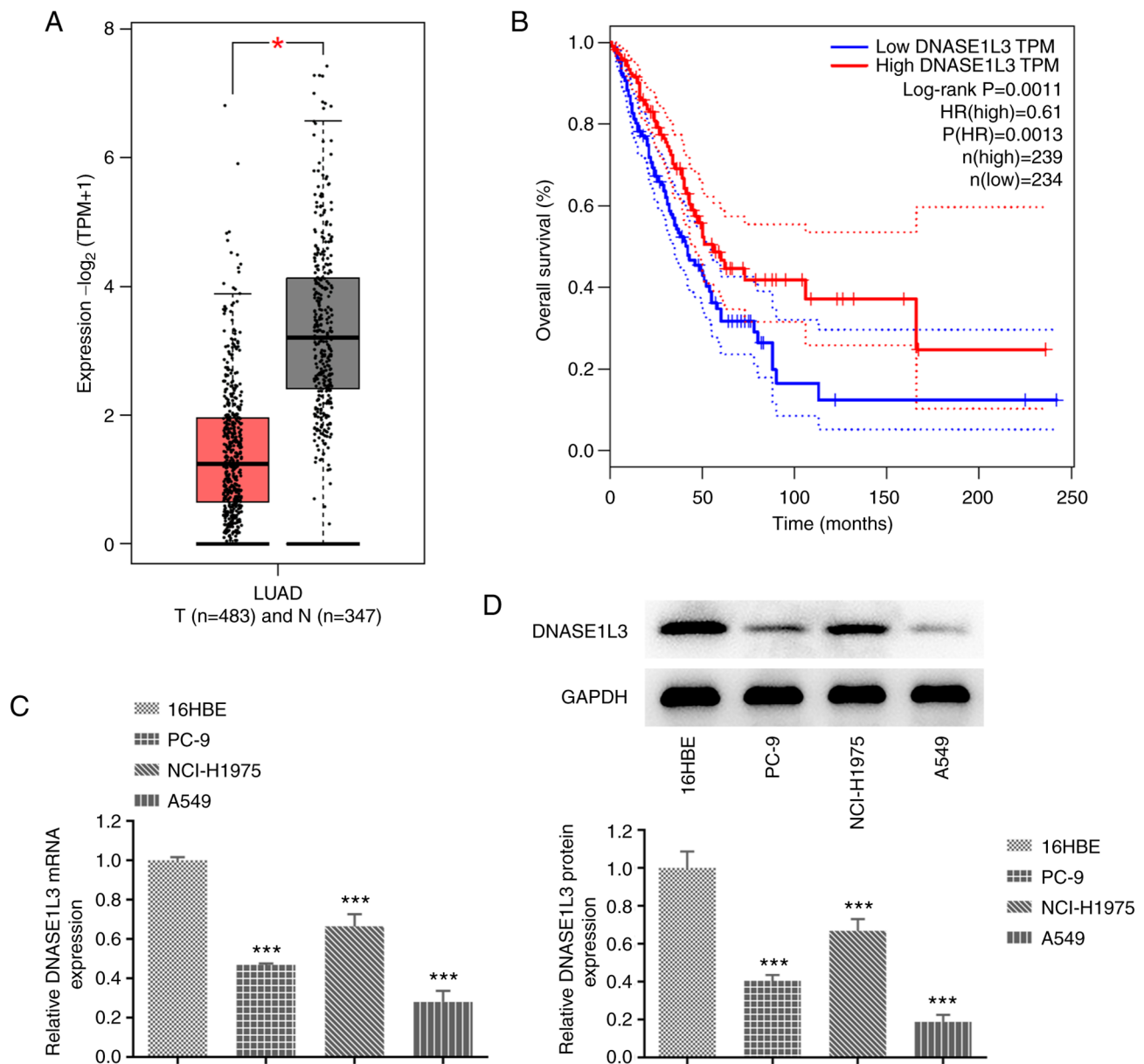


Figure 1. DNASE1L3 underexpression in LUAD is associated with poor prognosis. (A) DNASE1L3 expression in T and N was analyzed by the GEPIA database. * $P < 0.05$. (B) Association between DNASE1L3 expression and overall survival of patients with LUAD was predicted by GEPIA database. Solid line represents the survival curve and the dotted line represents 95% confidence interval. Expression of DNASE1L3 in human bronchial epithelium and multiple human LUAD cell lines was detected by (C) reverse transcription-quantitative PCR and (D) western blotting. Data from three independent repeats are presented as the mean \pm SD. *** $P < 0.001$ vs. 16HBE. DNASE1L3, deoxyribonuclease 1-like 3; LUAD, lung adenocarcinoma; GEPIA, Gene Expression Profiling Interactive Analysis; HR, hazard ratio; T, tumor; N, normal; TPM, Transcripts Per Kilobase Million.

of DNASE1L3 significantly increased (Fig. 2A and B). The viability of A549 cells was significantly decreased when cells were transfected with Oe-DNASE1L3 (Fig. 2C). Oe-DNASE1L3 significantly suppressed the proliferation of A549 cells (Fig. 2D and E).

Oe-DNASE1L3 inhibits migration, invasion and tube formation of A549 cells. The migration, invasion and tube formation of A549 cells transfected with Oe-DNASE1L3 were detected by wound healing, Transwell and tube formation assays, respectively. The expression of EMT- and tube formation-associated proteins in A549 cells transfected with Oe-DNASE1L3 was analyzed by western blotting. The migration rate and number of invaded cells were both significantly

decreased in the Oe-DNASE1L3 group by contrast with the Oe-NC group (Fig. 3A and B). The number of tubes was also significantly decreased in cells cultured in medium of A549 cells transfected with Oe-DNASE1L3 relative to the Oe-NC group (Fig. 3C). The expression of E-cadherin was significantly increased while the expression of N-cadherin, Snail and VEGF was significantly decreased in A549 cells transfected with Oe-DNASE1L3 relative to the Oe-NC group (Fig. 3D).

Transcription factor FOXP2 positively regulates DNASE1L3 transcription in A549 cells. The binding sites between FOXP2 and DNASE1L3 were predicted by the JASPAR database (Fig. 4A). FOXP2 expression was also significantly decreased

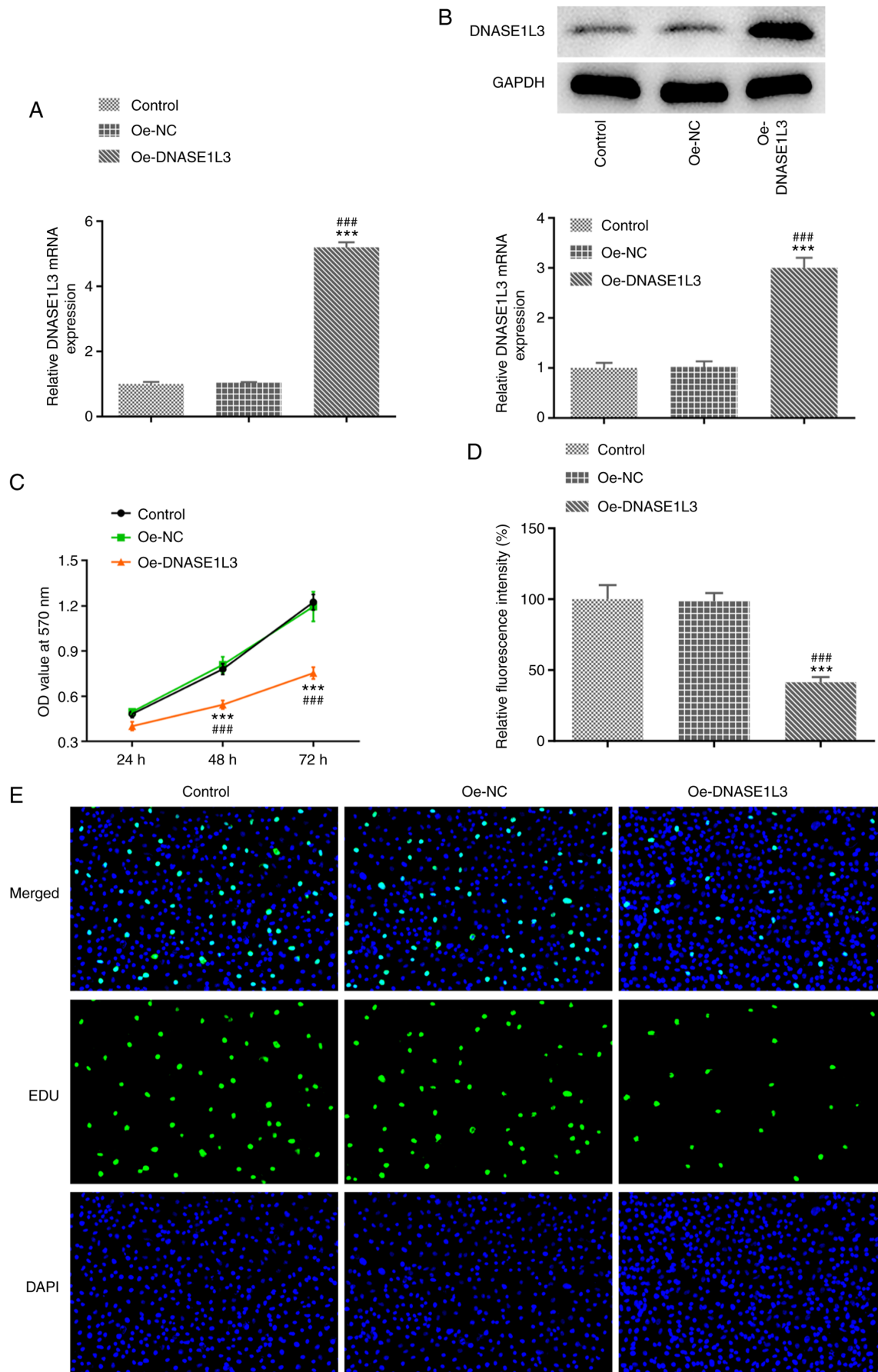


Figure 2. Oe-DNASE1L3 inhibits proliferation of A549 cells. Expression of DNASE1L3 in A549 cells transfected with Oe-DNASE1L3 was detected by (A) reverse transcription-quantitative PCR and (B) western blotting. (C) Viability of A549 cells transfected with Oe-DNASE1L3 was determined by MTT assay. Proliferation of A549 cells transfected with Oe-DNASE1L3 was (D) observed and (E) quantified by EdU staining. Magnification, x200. ***P<0.001 vs. control. ^{###}P<0.001 vs. Oe-NC. DNASE1L3, deoxyribonuclease 1-like 3; OD, optical density; Oe, overexpression; NC, negative control.

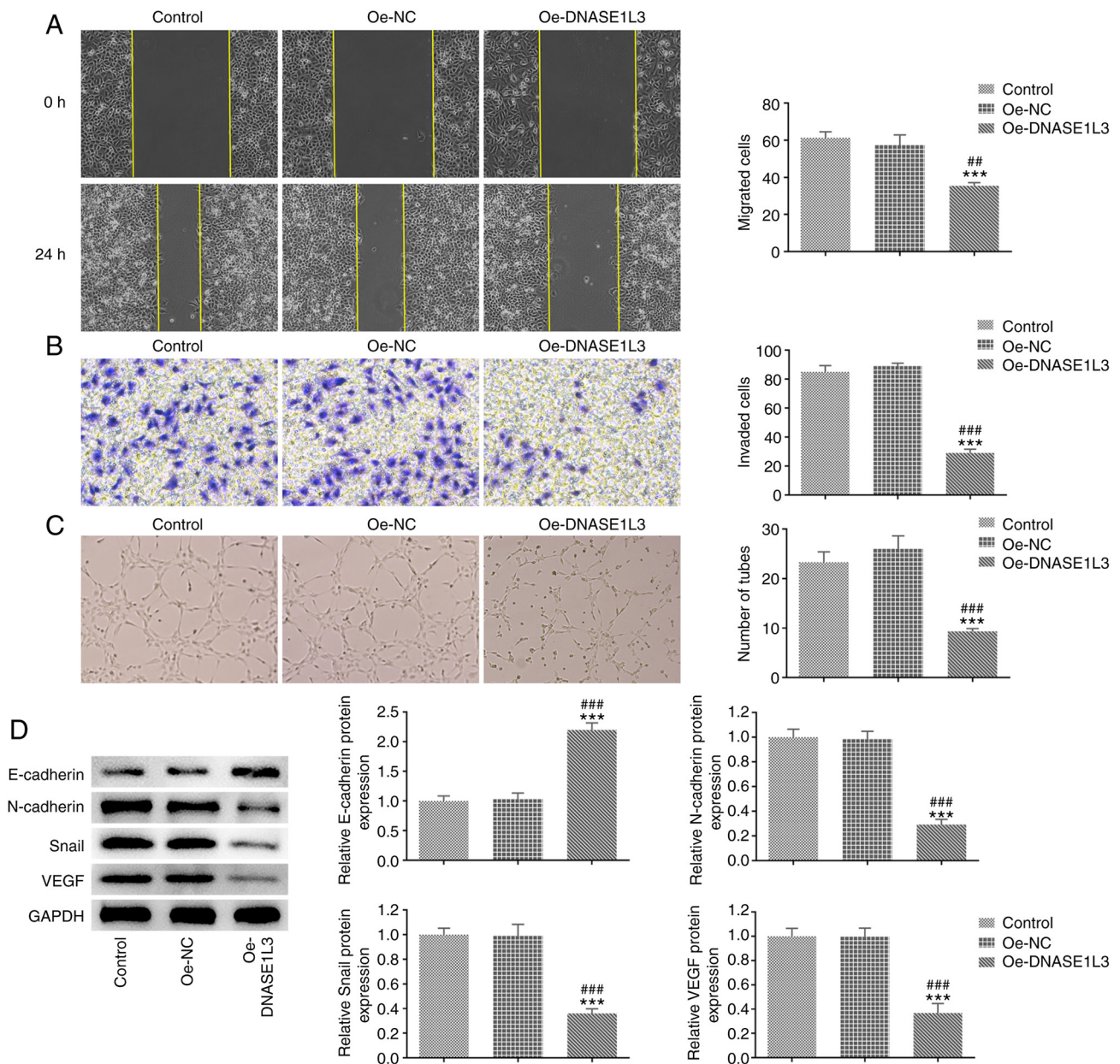


Figure 3. Oe-DNASE1L3 inhibits migration, invasion and tube formation of A549 cells. (A) Migration of A549 cells transfected with Oe-DNASE1L3 was detected by wound healing assay. Magnification, x100. (B) Invasion of A549 cells transfected with Oe-DNASE1L3 was detected by Transwell assay. Magnification, x100. (C) Tube formation of human umbilical vein endothelial cells cultured in medium from A549 cells transfected with Oe-DNASE1L3 was observed by tube formation assay. Magnification, x40. (D) Expression of epithelial-mesenchymal transformation- and tube formation-associated proteins in A549 cells transfected with Oe-DNASE1L3 was detected by western blotting. *** $P < 0.001$ vs. control. ** $P < 0.01$, *** $P < 0.001$ vs. Oe-NC. DNASE1L3, deoxyribonuclease 1-like 3; Oe, overexpression; NC, negative control; VEGF, vascular endothelial growth factor.

in A549 compared with 16HBE cells (Fig. 4B and C). The transfection efficiency of Oe-FOXP2 and si-FOXP2 in A549 cells was confirmed by RT-qPCR and western blotting. Dual-luciferase reporter assay and ChIP analysis were performed to verify the binding between FOXP2 and DNASE1L3. The expression of FOXP2 in A549 cells transfected with Oe-FOXP2 was significantly increased and significantly decreased in A549 cells transfected with si-FOXP2#1 and si-FOXP2#2. si-FOXP2#2 was selected for subsequent study as it displayed a more excellent interference efficacy (Fig. 4D and E). The luciferase activity was significantly increased in A549 cells co-transfected with DNASE1L3-WT and Oe-FOXP2 (Fig. 4F). The binding of FOXP2 to DNASE1L3

was detected by the addition of anti-FOXP2 antibody and the results elaborated that DNASE1L3 promoter was abundant in anti-FOXP2 antibody (Fig. 4G). These results indicated that FOXP2 may regulate DNASE1L3 expression. When A549 cells were transfected with Oe-FOXP2 and si-FOXP2, expression of FOXP2 was significantly increased and decreased, respectively (Fig. 4H and I).

Transcription factor FOXP2 regulates transcription of DNASE1L3 and promotes proliferation of A549 cells. The viability and proliferation of A549 cells transfected with si-FOXP2#2 and Oe-DNASE1L3 were detected by MTT assay and EdU staining, respectively. When A549 cells were

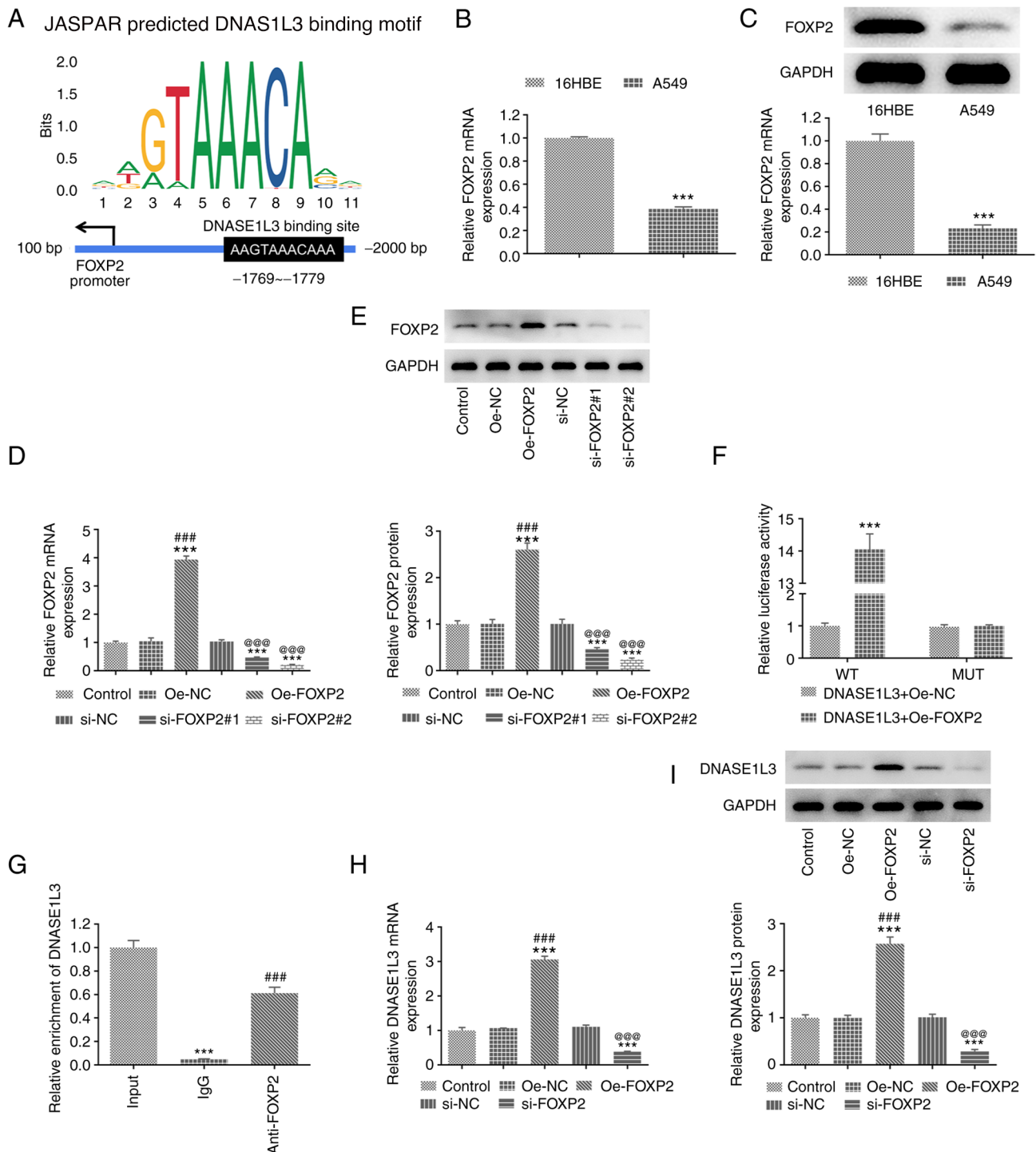


Figure 4. Transcription factor FOXP2 positively regulates DNASE1L3 transcription in A549 cells. (A) Binding between FOXP2 and the DNASE1L3 promoter region was predicted using the JASPAR database. Expression of FOXP2 in A549 cells was detected by (B) reverse transcription-quantitative PCR and (C) western blotting. ***P<0.001 vs. 16HBE. Expression of FOXP2 in A549 cells transfected with Oe-FOXP2, si-FOXP2#1 or si-FOXP2#2 was detected by (D) reverse transcription-quantitative PCR and (E) western blotting. ***P<0.001 vs. control. ***P<0.001 vs. Oe-NC. @@@P<0.001 vs. si-NC. (F) Luciferase activity of A549 cells transfected with DNASE1L3-WT and Oe-FOXP2 was detected by dual-luciferase reporter assay. ***P<0.001 vs. DNASE1L3-WT + Oe-NC. (G) Binding of FOXP2 and DNASE1L3 was confirmed by ChIP. ***P<0.001 vs. IgG. ***P<0.001 vs. Oe-NC. @@@P<0.001 vs. si-NC. Expression of DNASE1L3 in A549 cells transfected with Oe-FOXP2 or si-FOXP2 was detected by (H) reverse transcription-quantitative PCR and (I) western blotting. ***P<0.001 vs. control. ***P<0.001 vs. Oe-NC. @@@P<0.001 vs. si-NC. DNASE1L3, deoxyribonuclease 1-like 3; FOXP2, forkhead-box P2; Oe, overexpression; NC, negative control; si, small interfering; WT, wild-type; MUT, mutant.

transfected with si-FOXP2#2, viability significantly increased after 72 h. However, the viability of A549 cells was significantly decreased when si-FOXP2-transfected A549 cells were co-transfected with Oe-DNASE1L3 both at 48 and

72 h (Fig. 5A). Proliferation of A549 cells was significantly increased by suppressing FOXP2 expression and significantly decreased in cells co-transfected with si-FOXP2#2 and Oe-DNASE1L3 (Fig. 5B and C).

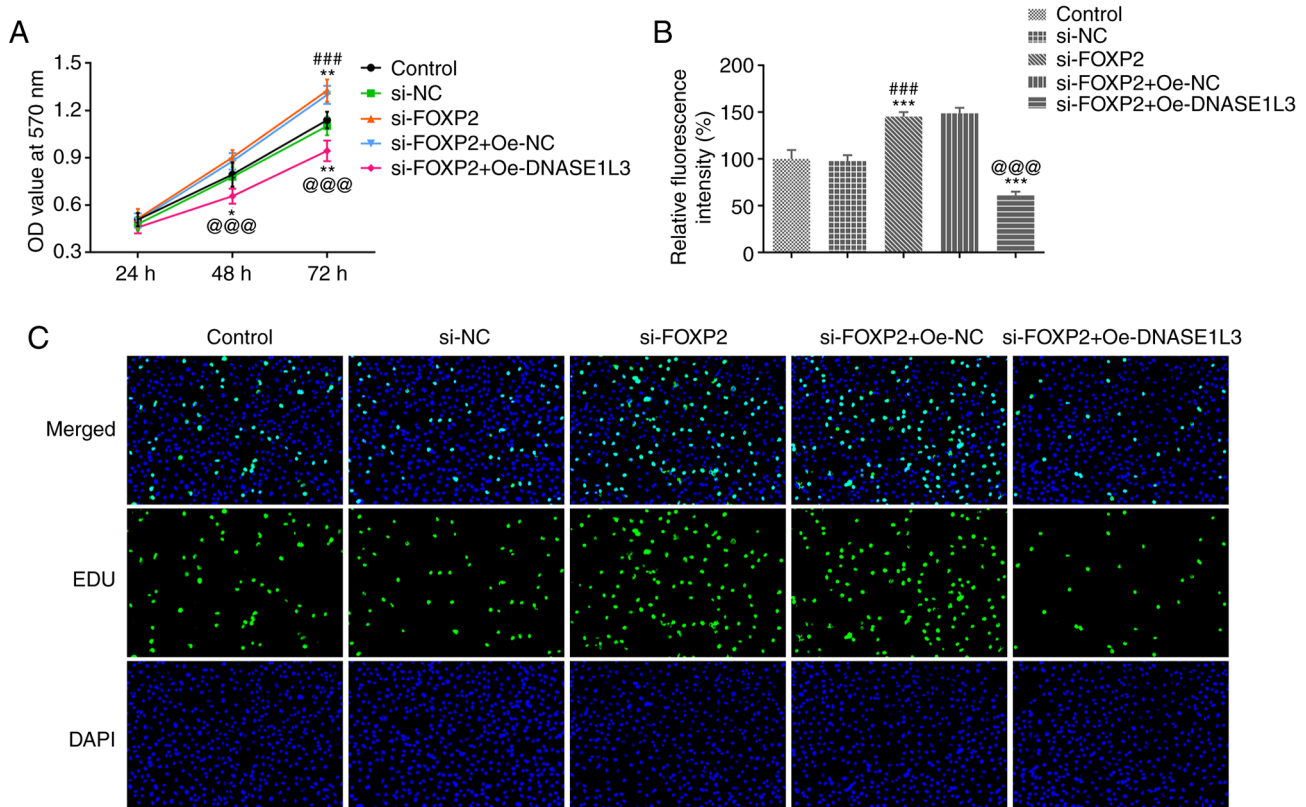


Figure 5. Transcription factor FOXP2 regulates transcription of DNASE1L3 and promotes the proliferation of A549 cells. (A) Viability of A549 cells transfected with si-FOXP2 and Oe-DNASE1L3 was determined by MTT. Proliferation of A549 cells transfected with si-FOXP2 and Oe-DNASE1L3 was (B) observed and (C) quantified by EdU staining. Magnification, x200. * $P < 0.05$, ** $P < 0.01$ vs. control, *** $P < 0.001$ vs. si-NC. @@@ $P < 0.001$ vs. si-FOXP2+Oe-NC. DNASE1L3, deoxyribonuclease 1-like 3; FOXP2, forkhead-box P2; OD, optical density; Oe, overexpression; NC, negative control; si, small interfering.

Transcription factor FOXP2 regulates transcription of DNASE1L3 and promotes the migration, invasion and tube formation of A549 cells. The migration, invasion and tube formation of A549 cells transfected with si-FOXP2#2 and Oe-DNASE1L3 were detected by wound healing, Transwell and tube formation assays, respectively. The expression of EMT- and tube formation-associated proteins in A549 cells transfected with si-FOXP2 and Oe-DNASE1L3 was analyzed by western blotting. Suppression of FOXP2 expression significantly improved migration and invasion of A549 cells and tube formation of HUVECs. These behaviors were all significantly decreased following transfection with Oe-DNASE1L3 (Fig. 6A-C). The expression of E-cadherin was significantly decreased while expression levels of N-cadherin, Snail and VEGF were significantly increased by si-FOXP2#2 transfection; these effects were significantly reversed by co-transfection with Oe-DNASE1L3 (Fig. 6D).

Discussion

LUAD, the most common type of lung cancer, grows and migrates faster than other subtypes and most patients are already in the middle and advanced stage when treated (21). The proliferation, invasion and migration of lung cancer cells are often accompanied by EMT progression (22,23). EMT describes epithelial cells that have morphologically transformed into mesenchymal cells and acquired migration ability, which serves a key role in the process of embryonic

growth, tissue remodeling and tumor metastasis (24,25). EMT is a key process in lung cancer cell migration, during which the cytoplasmic skeleton is rebuilt and connectivity between cells is weakened, thus promoting cell migration (26). VEGF α is a highly specific regulatory factor that induces tumor angiogenesis and promotes migration of vascular endothelial cells by increasing mitosis of blood tubules. Remodeling of extracellular matrix and increased vascular permeability are associated with occurrence, development and metastasis of lung cancer (27,28). DNASE1L3 suppresses tumor angiogenesis in HCC and overexpression of DNASE1L3 inhibits proliferation and motility of colon cancer cells (9,10). The present study showed that Oe-DNASE1L3 inhibited proliferation, invasion, migration and tube formation of A549 cells by suppressing EMT progression and VEGF expression.

The loss or increase of expression of FOX protein may change the fate of cells and promote the occurrence of tumors and the progression of cancers through modulation of gene expression or signaling. FOXP2 is expressed in a number of cell types; however, it is underexpressed in biopsies of breast, liver and gastric cancer (29-31). In the present study, FOXP2 expression was also decreased in A549 cells. Underexpression of FOXP2 results in increased invasion of tumor cells and vimentin expression and decreased E-cadherin expression (30). In HCC, microRNA (miR)-196b promotes the migration and invasion of tumor cells by directly binding to the 3' untranslated region of FOXP2 mRNA to inhibit epitaxy (32). Additionally, a previous study on pancreatic ductal carcinoma

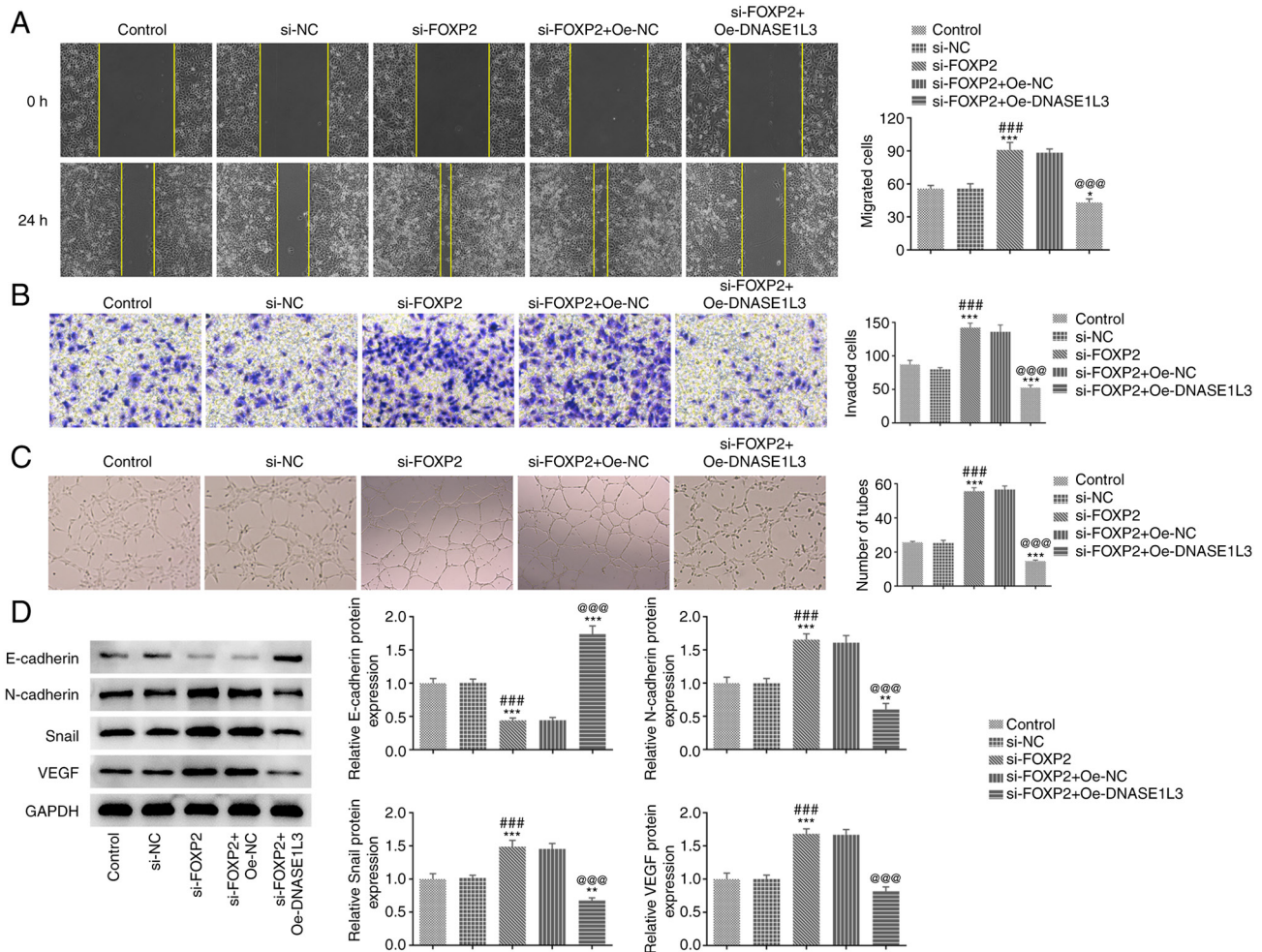


Figure 6. Transcription factor FOXP2 regulates transcription of DNASE1L3 and promotes migration, invasion and tube formation of A549 cells. (A) Migration of A549 cells transfected with si-FOXP2 and Oe-DNASE1L3 was detected by wound healing assay. Magnification, x100. (B) Invasion of A549 cells transfected with si-FOXP2 and Oe-DNASE1L3 was determined by Transwell assay. Magnification, x100. (C) Tube formation of human umbilical vein endothelial cells cultured in the medium from A549 cells transfected with si-FOXP2#2 and Oe-DNASE1L3 was observed by tube formation assay. Magnification, x40. (D) Expression of epithelial-mesenchymal transformation- and tube formation-associated proteins in A549 cells transfected with si-FOXP2#2 and Oe-FOXP2 was detected by western blotting. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control. ### $P < 0.001$ vs. si-NC. @@@ $P < 0.001$ vs. si-FOXP2#2+Oe-NC. DNASE1L3, deoxyribonuclease 1-like 3; FOXP2, forkhead-box P2; Oe, overexpression; NC, negative control; si, small interfering; VEGF, vascular endothelial growth factor.

showed miR-23a significantly promotes proliferation and invasion of tumor cells and subsequent restoration of FOXP2 expression limits the effect of miR-23a on proliferation and invasion. This suggests that FOXP2 serves a role in inhibiting tumor proliferation and invasion (33). FOXP2 has been found to regulate angiogenesis of U87 glioma-exposed endothelial cells (34). The present study found that underexpression of FOXP2 promoted the proliferation, invasion, migration and tube formation in A549 cells by promoting EMT progression and VEGF expression. Oe-DNASE1L3 reversed the effect of downregulation of FOXP2 on malignant behaviors of A549 cells, indicating that FOXP2 positively regulates DNASE1L3 transcription.

In conclusion, the present study illustrated the role of DNASE1L3 in LUAD and showed that DNASE1L3 was positively regulated by transcription factor FOXP2, which in turn inhibited proliferation, migration, invasion and tube formation of LUAD cells. The present study is limited by the use of only the A549 cell line; other cell lines and *in vivo* models should be assessed to confirm the results in future research.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FM designed and conceived the study, performed experiments and wrote the manuscript. FM, XY and PX analyzed the data. XY and PX edited the manuscript. FM, XY and PX confirm

the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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