Original Article

Hsa_circ_0006427 Suppresses Multiplication, Migration and Invasion of Non-Small Cell Lung Cancer Cells through miR-346/VGLL4 Pathway

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Abstract

Objective: Circular RNAs (circRNAs) are identified as key modulators in cancer biology. Nonetheless, the role of *circ_0006427* in non-small cell lung cancer (NSCLC) and its modulatory mechanism are undefined. This study aimed to investigate the potential function and mechanism of *circ_0006427* in NSCLC.

Materials and Methods: In this experimental study, *circ_0006427*, *miR-346* and vestigial like family member 4 (*VGLL4*) mRNA expressions were analyzed in NSCLC tissues and cells, using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Multiplication, migration and invasion of NSCLC cells were examined using the CCK-8 method and Transwell experiment, respectively. Dual-luciferase reporter gene experiments were conducted to identify the paring relationship between *circ_0006427* and *miR-346*. Western blot was employed to determine expressions of *VGLL4* and epithelial-mesenchymal transition (EMT) markers on protein levels. Immuno-histochemistry (IHC) method was adopted to assess VGLL4 protein expression in NSCLC tissues.

Results: *Circ_0006427* expression was down-regulated in NSCLC tissues and cells, and *circ_0006427* suppressed multiplication, migration, invasion and EMT of NSCLC cells. *miR-346* expression was upregulated in NSCLC tissues and cells, and *miR-346* worked as a target of *circ_0006427*. *VGLL4* was down-regulated in NSCLC tissues and cells, and knockdown of *VGLL4* accelerated multiplication, migration, invasion and EMT of NSCLC cells. *Circ_0006427* enhanced *VGLL4* expression by competitively binding with *miR-346*.

Conclusion: *Circ_0006427/miR-346/VGLL*4 axis regulated NSCLC progression.

***Keywords**:* circRNA, microRNA, Non-Small Cell Lung Cancer, Vestigial-Like Family Member 4

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Introduction

Lung cancer takes up approximately 20% of all cancer-related deaths (1). Non-small cell lung cancer (NSCLC) is the main pathological type of lung cancer, composed of about 85% of all lung cancer cases (2). Despite the significant diagnostic and therapeutic progress, 5-year overall survival rate of NSCLC patients is still below 20%, due to metastasis and recurrence of malignancy (3). Therefore, it is imperative to better comprehend the underlying molecular mechanisms involved in NSCLC progression to develop novel and effective therapies and to improve prognosis.

Circular RNA (circRNA) is a kind of non-coding RNA (ncRNA) widely found in eukaryocytes (4). Unlike linear RNAs, circRNAs are covalently closed loop molecules that do not have a 5' or 3' end; so they are resistant to degradation and more stable than linear RNAs (5). circRNAs not only function as competitive endogenous RNAs (ceRNAs) to modulate the availability of microRNAs (miRNAs), but also modulate gene transcription and translation via interacting with proteins (6-8). Reportedly, circRNA partakes in regulating multiplication, cell cycle, apoptosis and metastasis of cancer cells (9-11). *Circ_0006427* is a newly discovered circRNA, linked to tumorigenesis. Its expression is downregulated in lung adenocarcinoma tissues and cell lines, and it can impede disease progression by modulating *miR-6783-3p/DKK1* axis (12). Nevertheless, function of *circ_0006427* in NSCLC progression and the potential mechanisms remain to be further elucidated.

miRNAs are crucial regulators in cancer biology (13, 14). *miR-346* is reported to accelerate hepatocellular carcinoma progression by suppressing *BRMS1* expression (15). Furthermore, expression of *miR-346* is validated to be upregulated in NSCLC, and *miR-346* facilitates growth and metastasis of NSCLC cells by modulating the XPC/ERK/Snail/E-cadherin pathway (16). Vestigial-like family member 4 (*VGLL4*) is a tumor suppressor that competitively binds to TEA domain-containing transcription factors (TEADs) to block its interaction with Yes kinase-associated protein (YAP), and suppresses lung cancer progression (17, 18). In this study, we aimed to investigate the effect of circ_0006427 on NSCLC cell proliferation, migration and invasion, and to explore the

mechanism of its interaction with the miR-346/VGLL4 axis in NSCLC.

Materials and Methods

Clinical specimens

In this experimental study, NSCLC tissues and matched paracancerous tissues (n=50) were obtained from Zhejiang hospital (Hangzhou, China). All subjects were pathologically diagnosed after surgery and all of the patients signed the informed consent form. This study was endorsed by the Research Ethics Committee of Zhejiang Hospital (ZJH20170133), and the procedures were designed according to the guidance of the Declaration of Helsinki. Tissue samples were obtained during surgery and preserved in liquid nitrogen until RNA extraction.

Cell culture and transfection

Normal human bronchial epithelial cell (HNBEC) line, BEAS-2B, and lung cancer cell lines (L9981, A549, H292, NCI-H460, and H460) were obtained from the China Center for Type Culture Collection (CCTCC, Wuhan, China). All cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, USA) containing 10% fetal bovine serum (FBS, Sijiqing, China), 100 U/ml penicillin and 100 U/ml streptomycin (Hyclone, USA) at 37˚C in 5% CO_2.

For *circ_0006427* overexpression, the pcDNA 3.1 vectors (pcDNA, Invitrogen, USA), containing the fragment of *circ_0006427*, were constructed by RiboBio (Guangzhou, China), namely *circ_0006427*/pcDNA 3.1 *(circ_0006427*). shRNA against *VGLL4* (*VGLL4* shRNA, sh-*VGLL4*) and the corresponding negative control (sh-NC), as well as miR-346 mimic and the negative control (NC-mimic) were available from GenePharma (Shanghai, China). NSCLC cells were transfected using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's instructions.

Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from NSCLC tissues and cells using TRIzol reagent (Takara, Japan). Next, 1 µg total RNA was reversely transcribed into cDNA using PrimeScript RT Master Mix (Takara, Japan). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was executed on ABI 7500 system (Applied Biosystems, USA) employing TransStart Tip Green qPCRSuperMix (Transgen Biotech, China). *β-ACTIN* was the endogenous control for *circ_0006427* and *VGLL4*, while *U6* was the endogenous control for *miR-346*. $2^{-\Delta\Delta Ct}$ method was utilized to calculate relative expression of the genes. The specific primer sequences were as follows:

circ_0006427-

F: 5′-ACAGCTACCGGATGAATGCT-3′
R: 5′-AGGGCTTCAGCTGTTTCTCA-3′

VGLL4-

F: 5′-AACTGCAACCTCTCGCACTG-3′
R: 5′-GCTCGGGCTCCTTGTAATTCT-3′

miR-346-

F: 5′-TGTCTGCCCGCATGCCT-3′
R: 5′-AGTGCAGGGTCCGAGGTATT-3′

β-ACTIN-

F: 5′-CTGTCACCTTCACCGTTCCAGTTT-3′
R: 5′-AGGGGCCATCCACAGTCTTC-3′

U6-

F: 5′-GCTTCGGCAGCACATATACTAAAAT-3′
R: 5′-CGCTTCACGAATTTGCGTGTCAT-3′

Subcellular fractionation assay

Cytoplasmic and nuclear RNA purification kit (Thermo Fisher Scientific, USA) was used to isolate and extract cytoplasmic and nuclear RNA from A549 and H292 cells. Then, expression of *circ_0006427* in the cytoplasm and nucleus of A549 and H292 cells was measured by qRT-PCR. *β-ACTIN* and *U6* were used as cytoplasmic and nuclear controls, respectively.

Cell multiplication experiment

NSCLC cells were transfected for 48 hours, and then their cell viability was detected by cell counting kit-8 (CCK-8, Beyotime, China) assay. In brief, NSCLC cells were inoculated in 96-well plates (3×10^3 cells/well). After 12 hours, 24 hours, 48 hours, 72 hours and 96 hours of culture, 10 µl CCK-8 solution was supplemented to each well and incubated at 37°C for 2 hours. Cell viability of NSCLC cells was subsequently assessed at 450 nm using a spectrophotometer (Bio-Rad, USA).

Transwell experiment

NSCLC cells were resuspended with fresh medium containing 1% FBS. To detect cell migration, NSCLC cells were inoculated in the upper compartment of the Transwell system (Costar, USA). DMEM containing 10% FBS was replenished to the bottom compartment. After culturing the cells were for 24 hours, the remained NSCLC cells on the top surface of filter were removed by a cotton swab. The cells on the below surface of filter were fixed and stained, followed by observing under a microscope. To detect cell invasion, the filters were pre-coated with Matrigel (Millipore, USA) before the inoculation of cells, and the other procedures were performed the same as the migration assay.

Western blot

NSCLC cells were lysed in RIPA lysis buffer (Beyotime, China). The proteins were separated via SDS-PAGE and electrophoretically transferred to PVDF membranes (Millipore, USA). The membranes were then blocked with 5% skimmed milk for 2 hours and they were incubated

with the primary antibodies including anti-*VGLL4* (Abcam, China, 1:1000), E-cadherin (Abcam, China, 1:1000), Vimentin (Abcam, China, 1:1000), and *β-ACTIN* (Abcam, China, 1:1000) at 4°C overnight, respectively. Following that, the membranes were rinsed with TBST buffer and incubated with horseradish peroxidase (HRP)-coupled secondary antibodies (Beyotime, China) for 2 hours. Ultimately, development of protein bands was performed with ECL kit (Amersham Pharmacia Biotech, UK), and the grayscale values of each protein were analyzed using Image J.

Bioinformatics analysis

The binding sites between *circ_0006427* and *miR-346*, or *miR-346* and *VGLL4* were predicted using CircInteractome database (https://circinteractome.nia.nih.gov/) and StarBase (http://starbase.sysu.edu.cn/) database.

Dual-luciferase reporter experiment

The wild-type (WT) or mutant (MUT) *circ_0006427* and *VGLL4* 3′UTR sequences containing the binding site for *miR-346* were cloned into the pGL3 vector (Promega, USA) to construct the reporter vectors: *circ_0006427*-WT, *VGLL4*-WT, *circ_0006427*-MUT and *VGLL4*-MUT. A549 and H292 cells were co-transfected with *miR-346* mimics or NC-mimics with the reporter vectors, respectively. 48 hours after the transfection, luciferase activity of the cells in each group was examined with a dual-luciferase reporter gene reporter system (Promega, USA).

IHC experiment

In brief, samples were fixed in formalin and embedded in paraffin. Xylene was used for paraffin section dewaxing. After rehydrating the sections, antigen repair was performed. Next, the sections were incubated with anti-*VGLL4* antibody (Abcam, China, 1:100) overnight at 4°C. After rinsing with PBS 5 times, secondary antibody IgG (Beyotime, China, 1:400) was supplemented, with which the sections were incubated for 30 minutes. Then, the sections were rinsed with PBS three times. Next, the tissue sections were stained with DAB solution. Finally, staining intensity was photographed using an Olympus BX51 microscope (Olympus, Japan) and scored by two independent pathologists. The scoring system was based on the staining intensity and staining extent. The staining intensity was classified as 0 (negative), 1 (weak), 2 (medium) and 3 (strong). The degree of staining extent depended on the percentage of positive cells, classified into 0 (<5%), 1 (5-25%), 2 (26-50%), 3 (51-75%) and 4 (>75%). Then, these two scores were multiplied and IHC results were classified as 0-3 (negative) and 4-12 (positive).

Statistical analysis

Each experiment was conducted in triplicate. Statistical analysis was implemented using SPSS (version 25.0, SPSS Inc., USA). All data were expressed as mean ± standard deviation (SD). Student's t tests or one-way ANOVA and post-hoc Dunnett's test were applied to analyze the differences. Pearson's correlation analysis was employed to analyze the correlations among *circ_0006427*, *miR-346* and *VGLL4* in NSCLC samples. Correlations between *circ_0006427* expression and clinicopathological parameters were analyzed using the Chi-square test. $P<0.05$ was considered to be statistically significant.

Results

circ_0006427 was downregulated in NSCLC tissues and cells

To assess expression of *circ_0006427* in NSCLC tissues and cells, qRT-PCR was employed. The data displayed that *circ_0006427* was markedly downregulated in NSCLC tissues relative to that in paracancerous tissues (Fig.1A). Similarly, *circ_0006427* expression was unveiled to be downregulated in NSCLC cell lines compared to that in HNBEC line BEAS-2B (Fig.1B). Furthermore, the subcellular distribution of *circ_0006427* was analyzed. Findings showed that *circ_0006427* was mainly located in the cytoplasm of A549 and H292 cells (Fig.1C). Additionally, the relationship between *circ_0006427* expression and clinicopathologic indicators of NSCLC was analyzed by the Chi-square test. The data manifested that reduced level of *circ_0006427* in NSCLC tissues was associated with larger tumor size and positive lymph node metastasis (Table S1, See Supplementary Online Information at www.celljournal.org).

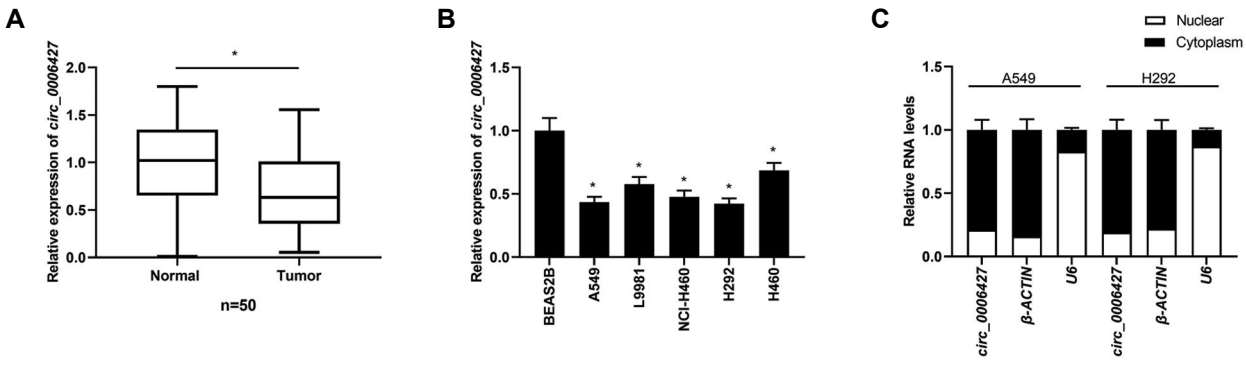

Fig.1: *circ_0006427* was down-regulated in NSCLC. **A.** *circ_0006427* expression in NSCLC tissues and paracancerous tissues was examined by qRT-PCR. **B.** *circ_0006427* expression in NSCLC cells and BEAS-2B cells was detected by qRT-PCR. **C.** Subcellular fractionation assay was used to measure the expression levels of *circ_0006427*, *U6* and *β-ACTIN* in the nuclear and cytoplasm of A549 and H292 cells. All experiments were repeated thrice with triplicate samples in each experiment. *; P<0.05, NSCLC; Non-small cell lung cancer, and qRT-PCR; Quantitative reverse transcription polymerase chain reaction.

Upregulation of *circ_0006427* expression repressed multiplication, migration, invasion and EMT of NSCLC cells

To elucidate the biological function of *circ_0006427* in NSCLC, A549 and H292 cells with the *circ_0006427* lowest expression were transfected with over-expressed *circ_0006427* plasmid to construct an overexpressed model of *circ_0006427* (Fig.2A). The multiplication of NSCLC cells was examined by CCK-8 method, and cell migration and invasion were detected by Transwell experiment. The data implied that upregulation of *circ_0006427* remarkably impeded the multiplication, migration and invasion of NSCLC cells (Fig.2B-F). Moreover, EMT-related protein expression in NSCLC cells was assessed by Western blot, and the data unmasked that upregulation of *circ_0006427* markedly augmented E-cadherin protein expression and inhibited Vimentin protein expression (Fig.2G, H). These data suggested that *circ_0006427* overexpression impeded multiplication, migration, invasion and EMT process of NSCLC cells.

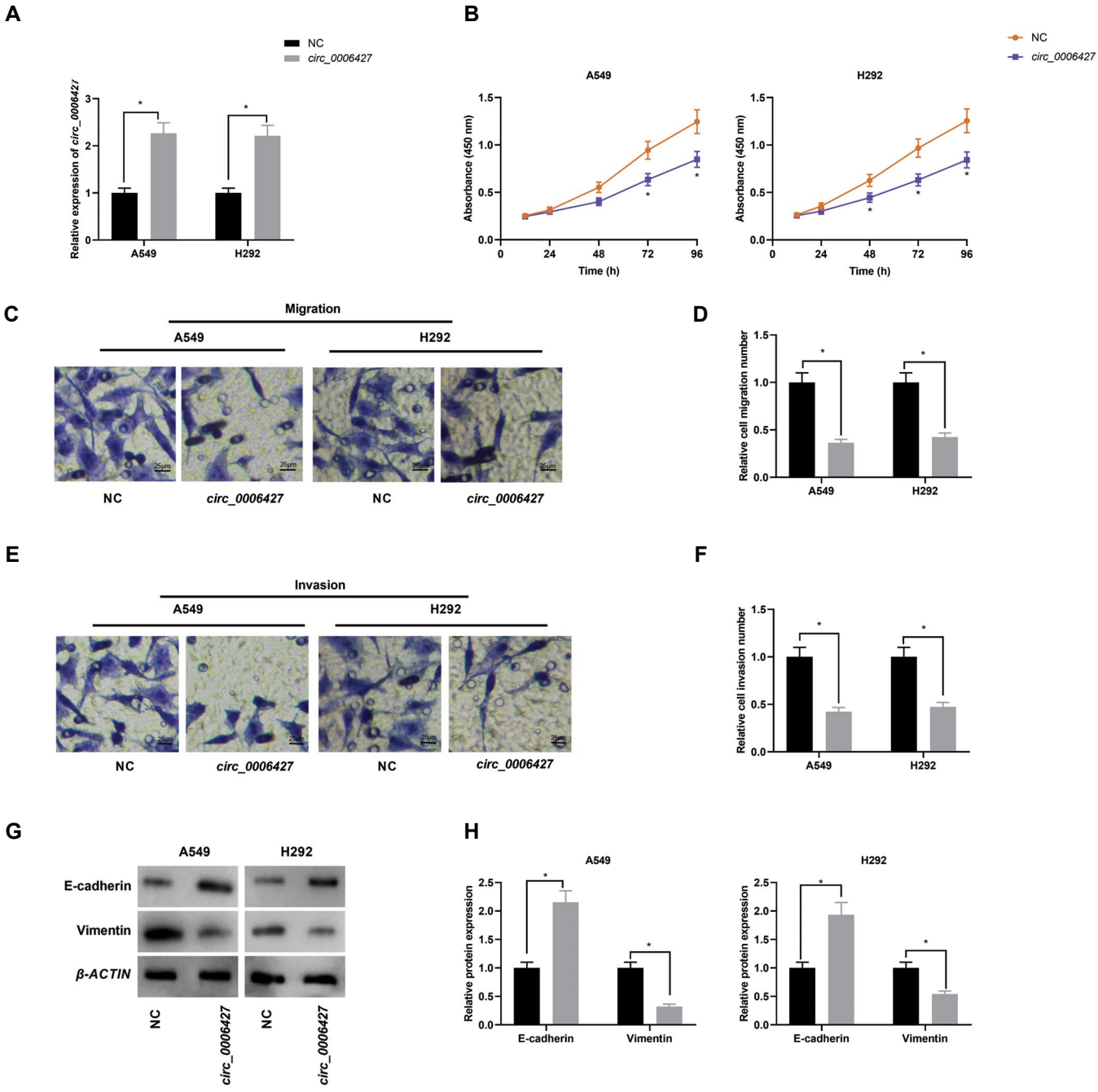

Fig.2: *circ_0006427* overexpression restrained multiplication, migration, invasion and EMT of NSCLC cells. **A.** *circ_0006427* expression in A549 and H292 cells after *circ_0006427* overexpression was detected using qRT-PCR. **B.** The CCK-8 method was employed to detect NSCLC cell multiplication. **C-F.** Transwell experiments were utilized to detect NSCLC cell migration and invasion. **G** and **H.** Western blot was performed to determine E-cadherin and Vimentin protein expressions in NSCLC cells. Original blots are shown in Figure S2 (See Supplementary Online Information at www.celljournal.org), Figure 2G. All experiments were repeated thrice with triplicate samples in each experiment. *; P<0.05, EMT; Epithelial-mesenchymal transition, NSCLC; Non-small cell lung cancer, and NC; Negative control.

circ_0006427 acted as a molecular sponge for *miR-346* in NSCLC cells

The potential binding sites between *circ_0006427* and *miR-346* were projected by bioinformatics analysis (Fig.3A). Subsequently, the relationship between *circ_0006427* and *miR-346* was examined by dual-luciferase reporter gene experiments, the data of which demonstrated that *miR-346* mimics substantially diminished the luciferase activity of *circ_0006427* WT reporter, while did not notably repress the luciferase activity of *circ_0006427* MUT reporter (Fig.3B). Moreover, *miR-346* was remarkably overexpressed in NSCLC tissues and cell lines (Fig.3C, D). Findings of qRT-PCR uncovered that *miR-346* expression was markedly downregulated by *circ_0006427* overexpression in NSCLC tissues (Fig.3E). Additionally, *circ_0006427* expression in NSCLC tissues was negatively associated with *miR-346* expression (Fig.3F). Collectively, these data implied that *circ_0006427* negatively regulated *miR-346* in NSCLC.

miR-346 overexpression reversed the functions of *circ_0006427* in NSCLC cells

To delve into whether *circ_0006427* is involved in NSCLC progression through repressing *miR-346* expression, *miR-346* mimics or NC-mimics were transfected into overexpressed *circ_0006427* cells, A549 and H292 cells respectively (Fig.4A). Cell multiplication, migration and invasion were further examined by CCK-8 method and Transwell experiment. Findings demonstrated that suppressing effects of *circ_0006427* overexpression on cell multiplication, migration and invasion were partially attenuated by *miR-346* mimics (Fig.4B-D). Furthermore, Western blot demonstrated that E-cadherin expression was decreased and Vimentin expression was increased in *circ_0006427*+NC-mimics group remarkably compared to those in *circ_0006427+miR-346* group (Fig.4E, F). These results substantiated that *circ_0006427* participated in modulating multiplication, migration, invasion and EMT of NSCLC cells through suppressing *miR-346* expression.

Knockdown of *VGLL4* enhanced NSCLC cell multiplication, migration, invasion and EMT

As a tumor suppressor protein, *VGLL4* associated with lung cancer progression (18). This work examined *VGLL4* expression in NSCLC tissues and paracancerous tissues through IHC and qRT-PCR analyses. Consistently, our data unearthed that *VGLL4* was markedly down-regulated in NSCLC tissues (Fig.5A, B). Similarly, *VGLL4* expression was remarkably reduced in NSCLC cell lines compared to the BESA-2B cells (Fig.5C, D). To fathom tumor-suppressive function of *VGLL4* in NSCLC progression, sh-*VGLL4* was transfected into A549 and H292 cells. As shown, *VGLL4* expression was markedly repressed in NSCLC cells transfected with sh-*VGLL4* (Fig.5E). Data of CCK-8 and Transwell experiments showed knockdown of *VGLL4* remarkably enhanced multiplication, migration and invasion of A549 and H292 cells (Fig.5F-I). Moreover, Western blot revealed that knockdown of *VGLL4* repressed E-cadherin expression in A549 and H292 cells, but upregulated Vimentin expression (Fig.5J, K). These findings elucidated that *VGLL4* played a tumor-suppressive role in NSCLC.

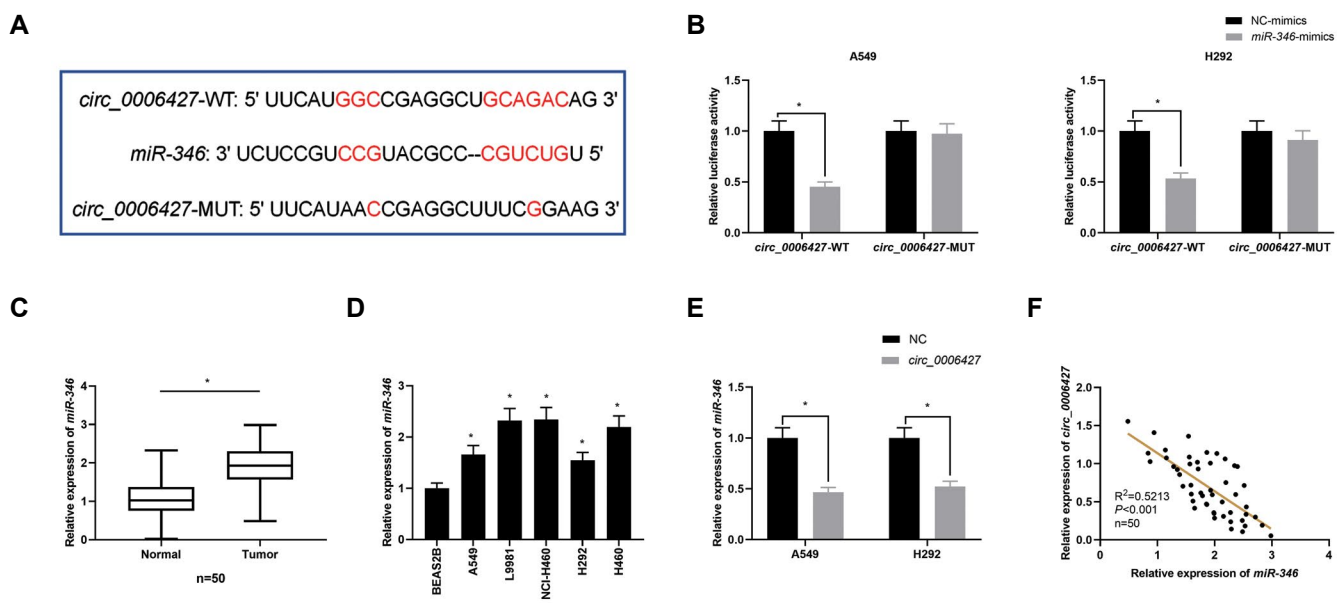

Fig.3: *miR-346* was target of *circ_0006427*. **A.** Bioinformatics analysis predicted binding sequence between *miR-346* and circ_0006427. **B.** Dual-luciferase reporter gene experiments were employed to verify binding relationship between *miR-346* and *circ_0006427*. **C, D.** *miR-346* expression in NSCLC tissues and cells was detected by qRT-PCR. **E.** *miR-346* expression in NSCLC cells overexpressing *circ_0006427* was detected by qRT-PCR. **F.** Pearson's correlation analysis was adopted to analyze the correlation between *miR-346* expression and *circ_0006427* expression in NSCLC tissues. All experiments were repeated thrice with triplicate samples in each experiment. *; P<0.05, NSCLC; Non-small cell lung cancer, qRT-PCR; Quantitative reverse transcription polymerase chain reaction, WT; Wild type, MUT; Mutant, NC-mimics; Mimics negative control, and NC; Negative control.

Circ_0006427 elevated *VGLL4* expression by competitively repressing *miR-346* expression in NSCLC cells

Next, bioinformatics analysis indicated that *VGLL4* 3′UTR contained a binding site of *miR-346* (Fig.6A). Subsequent dual-luciferase reporter gene experiments validated this binding relationship (Fig.6B). Effects of the *circ_0006427/miR-346* axis on *VGLL4* expression were then investigated. Data of qRT-PCR analysis and Western blot suggested that *circ_0006427* overexpression up-regulated *VGLL4* mRNA and protein expressions in NSCLC cells. This upregulation was remarkably reversed by the co-transfection of *miR-346* mimics (Fig.6C, D). Furthermore, the expression of *VGLL4* mRNA in NSCLC tissues was positively associated with *circ_0006427* expression and negatively correlated with *miR-346* expression (Fig.6E, F). From these data, we concluded that *circ_0006427* could upregulate *VGLL4* expression in NSCLC cells through repressing *miR-346* expression.

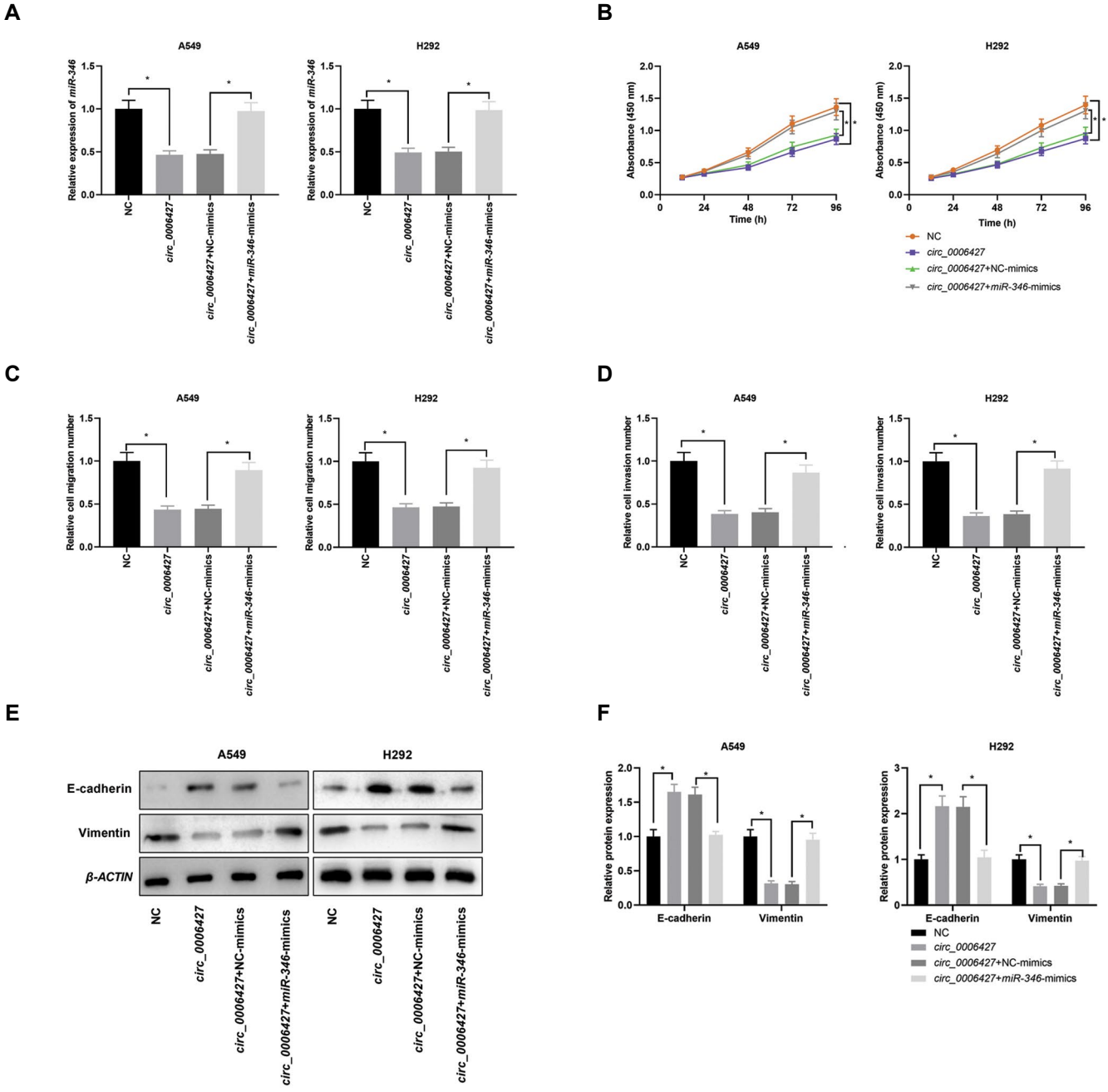

Fig.4: *circ_0006427* targeted *miR-346* and modulated multiplication, migration, invasion and EMT of NSCLC cells. **A.** *miR-346* expression in A549 and H292 cells transfected with *circ_0006427* overexpression plasmid or co-transfected with *miR-346* mimics was detected by qRT-PCR. **B.** CCK-8 assay was utilized to detect the multiplication of NSCLC cells after the transfection. **C, D.** Transwell experiments were exploited to detect migration and invasion of NSCLC cells after the transfection. **E** and **F.** Western blot was applied to determine E-cadherin and Vimentin protein expressions in NSCLC cells. Original blots are shown in Figure S2 (See Supplementary Online Information at www.celljournal.org), Figure 4E. All experiments were repeated thrice with triplicate samples in each experiment. *; P<0.05, EMT; Epithelial-mesenchymal transition, NSCLC; Non-small cell lung cancer, qRT-PCR; Quantitative reverse transcription PCR, NC; Negative control, and NC-mimics; Mimics negative control.

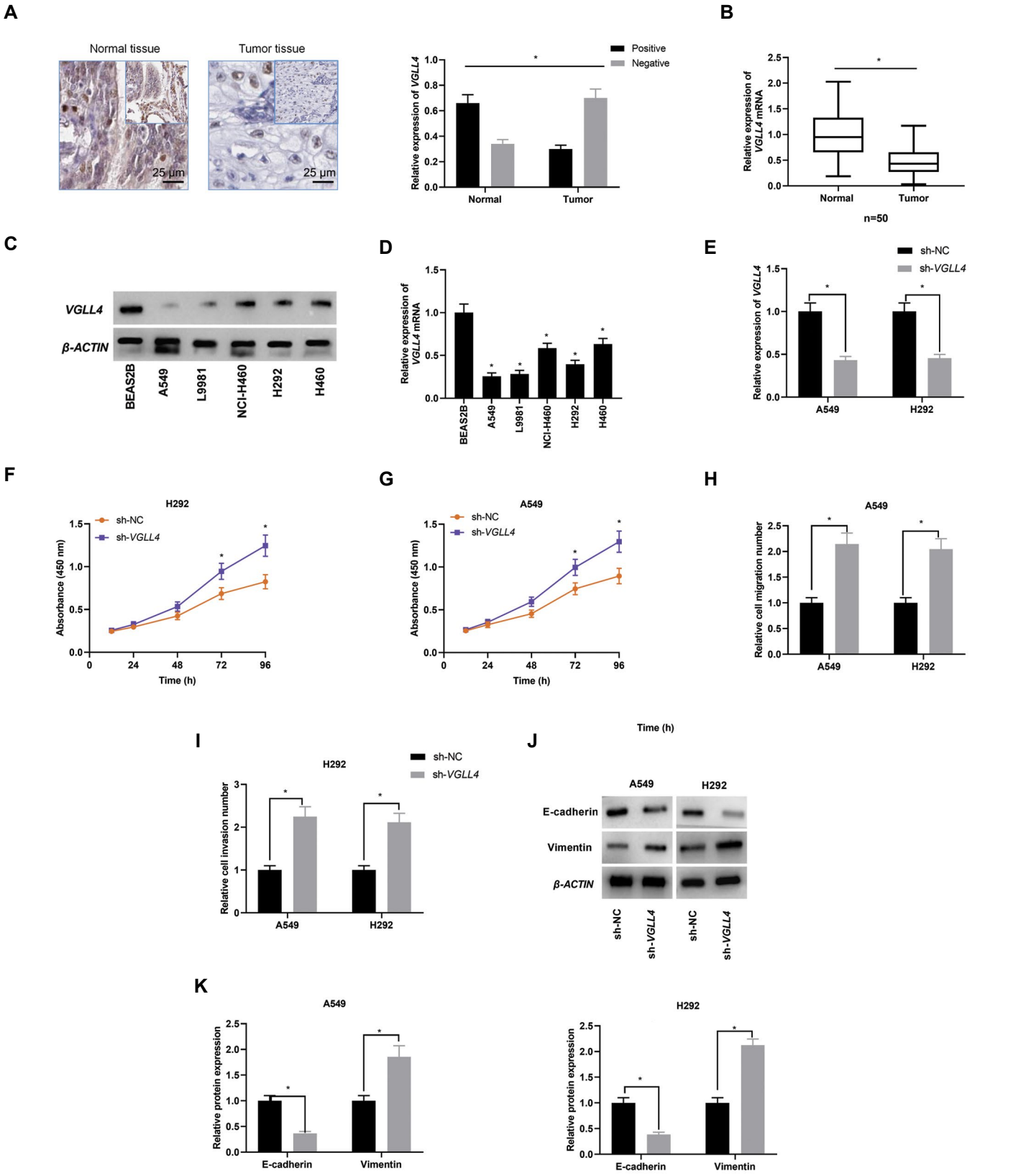

Fig.5: Knockdown of *VGLL4* impeded multiplication, migration, invasion and EMT of NSCLC cells. **A, B.** IHC and qRT-PCR methods were employed to detect *VGLL4* protein and mRNA expressions in NSCLC tissues and paracancerous tissues. **C, D.** Western blot and qRT-PCR were implemented to detect VGLL4 protein and mRNA expressions in NSCLC cells and BEAS2B cells. Original blots are shown in Figure S2 (See Supplementary Online Information at www.celljournal.org), Figure 5C. **E.** *VGLL4* expression in NSCLC cells transfected with sh-*VGLL4* was detected by qRT-PCR. **F, G.** CCK-8 assay was employed to monitor NSCLC cell multiplication. **H, I.** Transwell experiments were executed to detect NSCLC cell migration and invasion. **J, K.** Western blot was used to determine E-cadherin and Vimentin protein expressions in NSCLC cells transfected with sh-VGLL4. Original blots are shown in Figure S2 (See Supplementary Online Information at www.celljournal.org), Figure 5J. All experiments were repeated thrice with triplicate samples in each experiment. *; P<0.05, EMT; Epithelial-mesenchymal transition, NSCLC; Non-small cell lung cancer, IHC; Immunohistochemical, and qRT-PCR; Quantitative reverse transcription polymerase chain reaction.

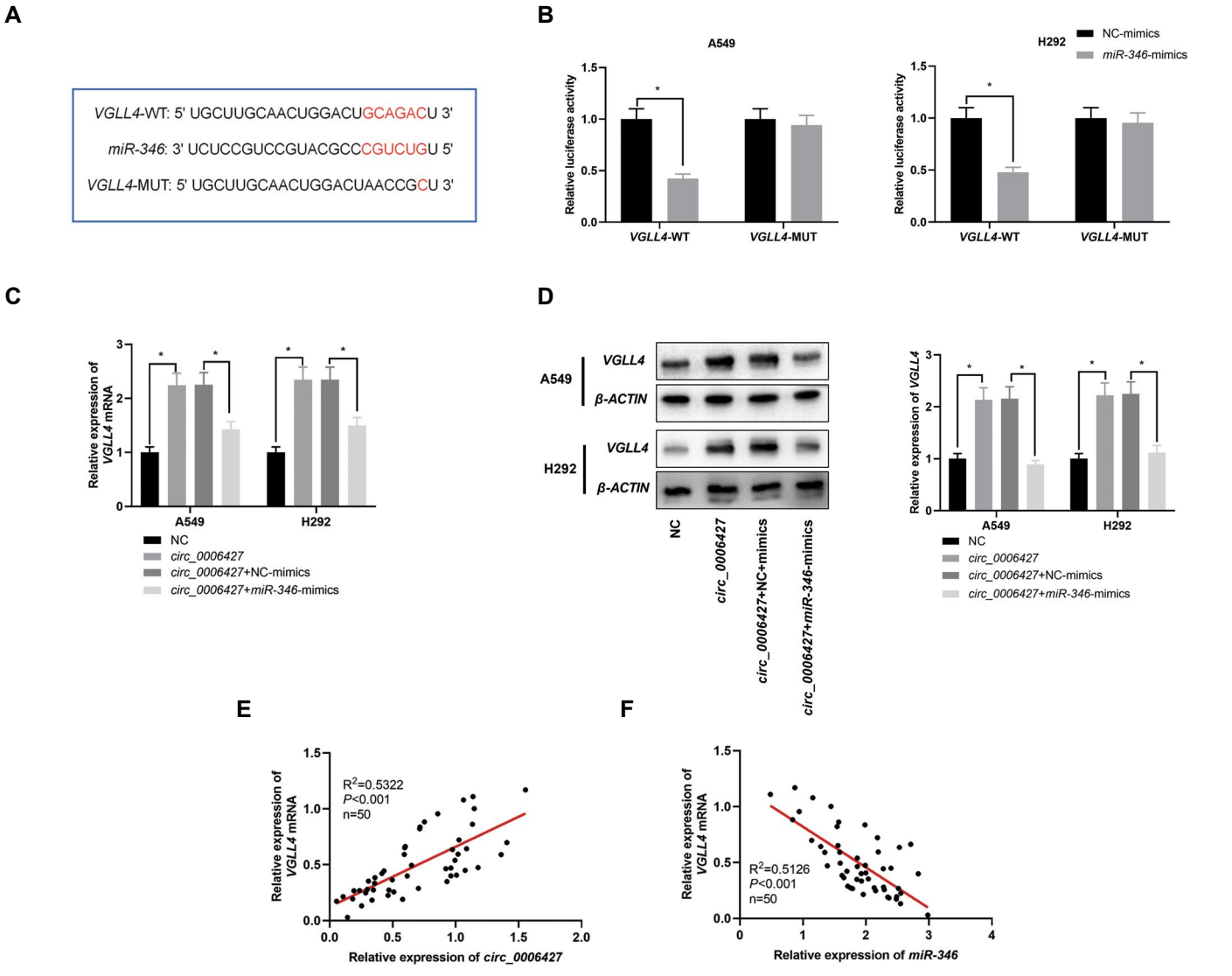

Fig.6: *VGLL4* was the target of *miR-346* in NSCLC cells and positively regulated by *circ_0006427*. **A.** Bioinformatics analysis predicted binding sequence between *miR-346* and *VGLL4*. **B.** Binding relationship between *miR-346* and *VGLL4* was verified using the dual-luciferase reporter gene assay. **C, D.** *VGLL4* mRNA and protein expressions in A549 and H292 cells transfected with *circ_0006427* overexpression plasmid or co-transfected with *miR-346* mimics were detected by qRT-PCR and Western blot. Original blots are shown in Figure S2 (See Supplementary Online Information at www.celljournal.org), Figure 6D. **E** and **F.** Pearson's correlation analysis was applied to determine correlation of *VGLL4* mRNA with *circ_0006427* or *miR-346* expression in NSCLC tissues. All experiments were repeated thrice with triplicate samples in each experiment. *; P<0.05, NSCLC; Non-small cell lung cancer, qRT-PCR; Quantitative reverse transcription polymerase chain reaction, WT; Wild type, MUT; Mutant, NC-mimics; Mimics negative control, and NC; Negative control.

Discussion

circRNAs figure prominently in the regulation of diverse biological processes (19，20). In recent years, circRNAs are proven to participate in the tumorigenesis and development of several cancers, including NSCLC (21). For example, *circ_0008305* restrains TGF-β-induced NSCLC cell invasion and EMT by modulating *TIF1γ* expression (22); downregulation of *circ_0067934* expression suppresses multiplication, migration and invasion of NSCLC cells. It is linked to unfavorable prognosis of patients (23). Notably, *circ_0006427* expression is downregulated in lung adenocarcinoma, and *circ_0006427* overexpression represses multiplication, migration, invasion and EMT of lung adenocarcinoma cells (12). Consistently, in this work, *circ_0006427* was unearthed to be remarkably under-expressed in both NSCLC tissues and cell lines; *circ_0006427* low expression was related to larger tumor size and positive LNM of NSCLC patients. Functionally, *circ_0006427* was verified to be tumor-suppressive in NSCLC progression by restraining cell multiplication, migration, invasion and EMT.

The role of miRNAs in tumor progression is extensively studied (24, 25). For instance, *miR-421* is highly expressed in NSCLC. It accelerates cell multiplication and cell cycle progression (26). Similarly, *miR-619-5p* in tumor-derived exosome facilitates growth and metastasis of NSCLC cells by impeding *RCAN1.4* expression (27). Accumulating research demonstrated that circRNAs can play "molecular sponge" role by antagonizing miRNAs to modulate expression of the target genes, thus playing an essential regulatory role in tumor progression. For

instance, *circ-ZKSCAN1* facilitates NSCLC progression by targeting *miR-330-5p* to regulate *FAM83A* expression and inactivate the MAPK signaling pathway (28); *circ_0000003* enhances multiplication and metastasis of NSCLC cells by regulating *miR-338-3p*/*IRS2* axis (29). In this work, it was revealed that there was a pairing relationship between *circ_0006427* and *miR-346* in NSCLC progression and *circ_0006427* could work as a molecular sponge for *miR-346*. Meanwhile, *miR-346* expression was markedly upregulated in NSCLC tissues and cell lines, which is consistent with the previous reports (16). Additionally, *miR-346* overexpression remarkably attenuated tumor-suppressive effect caused by *circ_0006427* overexpression. These results implied that *circ_0006427* could exert tumor-suppressive effects in NSCLC progression via sponging *miR-346*.

VGLL4, a member of the VGLL family, is an antagonist of the proto-oncogenic protein YAP (17). Unlike the other VGLL family proteins, *VGLL4* has two TDU domains (18). The N-terminal of *VGLL4* protein can bind to ubiquitin-specific protease 11, thus improving stability of the *VGLL4* protein by facilitating its deubiquitination (30). The role of *VGLL4* varies among cells and tissues. *VGLL4* overexpression is reported to enhance the colony-formation ability of human embryonic stem cells (31). Absence of *VGLL4* impedes *PD-L1* expression and tumor immune escape (32). Additionally, aberrant expression of *VGLL4* occurs frequently in diverse cancers and *VGLL4* is closely related to cell multiplication, migration, invasion and EMT. This can suppress cancer progression by modulating multiple signaling pathways, such as YAP and Wnt/β-catenin (33, 34). Importantly, *VGLL4* is tumor-suppressive in lung cancer, competing with YAP for binding to *TEAD4* and repressing transcription of *TEAD4* downstream genes (18, 35). In this work, we demonstrated that *VGLL4* expression was remarkably downregulated in NSCLC tissues and cells. Simultaneously, knockdown of *VGLL4* markedly enhanced multiplication, migration, invasion and EMT of NSCLC cells. Interestingly, *VGLL4* expression is validated to be linked to multiple miRNAs, such as *miR-130b*, *miR-222* and *miR-130a* (35-37). In this work, *VGLL4* was uncovered to be a direct target of *miR-346* in NSCLC cells. Further experimental data demonstrated that *circ_0006427* could modulate *VGLL4* expression by competitively binding to *miR-346*. Hence, we concluded that *circ_0006427/miR-346/VGLL4* axis could probably take part in NSCLC development.

Conclusion

This work revealed that *circ_0006427* was remarkably downregulated in NSCLC tissues and cells. *Circ_0006427* overexpression restrains multiplication, migration, invasion and EMT of NSCLC cells. Mechanistically, *circ_0006427* modulates *VGLL4* expression via sponging *miR-346*. These data implicated that *circ_0006427/miR-346/VGLL4* regulatory axis could be an essential factor in NSCLC progression.

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Authors' Contributions

X.Z., M.Sh.; Conceived and designed the experiments. J.S., L.W., X.Z.; Performed the experiments. L.W., J.S.; Analyzed the data. J.S., M.Sh., X.Z.; Wrote the manuscript. All authors read and approved the final manuscript.

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(6): 394-424.
2. Radovic M, Kanesvaran R, Rittmeyer A, Früh M, Minervini F, Glatzer M, et al. Multidisciplinary treatment of lung cancer in older patients: a review. J Geriatr Oncol. 2019; 10(3): 405-410.
3. Testa U, Castelli G, Pelosi E. Lung cancers: molecular characterization, clonal heterogeneity and evolution, and cancer stem cells. Cancers (Basel). 2018; 10(8): 248.
4. Meng S, Zhou H, Feng Z, Xu Z, Tang Y, Li P, et al. CircRNA: functions and properties of a novel potential biomarker for cancer. Mol Cancer. 2017; 16(1): 94.
5. Kulcheski FR, Christoff AP, Margis R. Circular RNAs are miRNA sponges and can be used as a new class of biomarker. J Biotechnol. 2016; 238: 42-51.
6. Liu T, Ye P, Ye Y, Lu S, Han B. Circular RNA hsa_circRNA_002178 silencing retards breast cancer progression via microRNA-328-3p-mediated inhibition of COL1A1. J Cell Mol Med. 2020; 24(3): 2189-2201.
7. Li X, Wang J, Zhang C, Lin C, Zhang J, Zhang W, et al. Circular RNA circITGA7 inhibits colorectal cancer growth and metastasis by modulating the Ras pathway and upregulating transcription of its host gene ITGA7. J Pathol. 2018; 246(2): 166-179.
8. AbouHaidar MG, Venkataraman S, Golshani A, Liu B, Ahmad T. Novel coding, translation, and gene expression of a replicating covalently closed circular RNA of 220 nt. Proc Natl Acad Sci USA. 2014; 111(40): 14542-14547.
9. Xu Q, Deng B, Li M, Chen Y, Zhuan L. circRNA-UBAP2 promotes the proliferation and inhibits apoptosis of ovarian cancer though miR-382-5p/PRPF8 axis. J Ovarian Res. 2020; 13(1): 81.
10. Wang K, Sun Y, Tao W, Fei X, Chang C. Androgen receptor (AR) promotes clear cell renal cell carcinoma (ccRCC) migration and invasion via altering the circHIAT1/miR-195-5p/29a-3p/29c-3p/CDC42 signals. Cancer Lett. 2017; 394: 1-12.
11. Fan L, Cao Q, Liu J, Zhang J, Li B. Circular RNA profiling and its potential for esophageal squamous cell cancer diagnosis and prognosis. Mol Cancer. 2019; 18(1): 16.
12. Yao Y, Hua Q, Zhou Y. CircRNA has_circ_0006427 suppresses the progression of lung adenocarcinoma by regulating miR-6783-3p/DKK1 axis and inactivating Wnt/β-catenin signaling pathway. Biochem Biophys Res Commun. 2019; 508(1): 37-45.
13. Thomson DW, Dinger ME. Endogenous microRNA sponges: evidence and controversy. Nat Rev Genet. 2016; 17(5): 272-283.
14. Long Z, Wang Y. miR-195-5p suppresses lung cancer cell proliferation, migration, and invasion Via FOXK1. Technol Cancer Res Treat. 2020; 19: 1533033820922587.
15. Guo Z, Li J, Sun J, Sun L, Zhou Y, Yu Z. miR-346 Promotes HCC progression by suppressing breast cancer metastasis suppressor 1 expression. Oncol Res. 2018; 26(7): 1073-1081.
16. Sun CC, Li SJ, Yuan ZP, Li DJ. MicroRNA-346 facilitates cell growth and metastasis, and suppresses cell apoptosis in human non-small cell lung cancer by regulation of XPC/ERK/Snail/E-cadherin pathway. Aging (Albany NY). 2016; 8(10): 2509-2524.
17. Lin Z, Guo H, Cao Y, Zohrabian S, Zhou P, Ma Q, et al. Acetylation of VGLL4 regulates hippo-YAP signaling and postnatal cardiac growth. Dev Cell. 2016; 39(4): 466-479.
18. Zhang W, Gao Y, Li P, Shi Z, Guo T, Li F, et al. VGLL4 functions as a new tumor suppressor in lung cancer by negatively regulat-

ing the YAP-TEAD transcriptional complex. Cell Res. 2014; 24(3): 331-343.
19. Zhou J, Wang H, Chu J, Huang Q, Li G, Yan Y, et al. Circular RNA hsa_circ_0008344 regulates glioblastoma cell proliferation, migration, invasion, and apoptosis. J Clin Lab Anal. 2018; 32(7): e22454.
20. Li X, Yang L, Chen LL. The biogenesis, functions, and challenges of circular RNAs. Mol Cell. 2018; 71(3): 428-442.
21. Dong Y, Xu T, Zhong S, Wang B, Zhang H, Wang X, et al. Circ_0076305 regulates cisplatin resistance of non-small cell lung cancer via positively modulating STAT3 by sponging miR-296-5p. Life Sci. 2019; 239: 116984.
22. Wang L, Tong X, Zhou Z, Wang S, Lei Z, Zhang T, et al. Circular RNA hsa_circ_0008305 (circPTK2) inhibits TGF-β-induced epithelial-mesenchymal transition and metastasis by controlling TIF1γ in non-small cell lung cancer. Mol Cancer. 2018; 17(1): 140.
23. Wang L, Tong X, Zhou Z, Wang S, Lei Z, Zhang T, et al. Circular RNA hsa_circ_0008305 (circPTK2) inhibits TGF-β-induced epithelial-mesenchymal transition and metastasis by controlling TIF1γ in non-small cell lung cancer. Mol Cancer. 2018; 17(1): 140.
24. Liang G, Meng W, Huang X, Zhu W, Yin C, Wang C, et al. miR-196b-5p-mediated downregulation of TSPAN12 and GATA6 promotes tumor progression in non-small cell lung cancer. Proc Natl Acad Sci U S A. 2020; 117(8): 4347-4357.
25. Khandelwal A, Seam RK, Gupta M, Rana MK, Prakash H, Vasquez KM, et al. Circulating microRNA-590-5p functions as a liquid biopsy marker in non-small cell lung cancer. Cancer Sci. 2020; 111(3): 826-839.
26. Li X, Chen SH, Zeng JW. MiR-421 is overexpressed and promotes cell proliferation in non-small cell lung cancer. Med Princ Pract. 2020; 29(1): 80-89.
27. Kim DH, Park S, Kim H, Choi YJ, Kim SY, Sung KJ, et al. Tumor-derived exosomal miR-619-5p promotes tumor angiogenesis and metastasis through the inhibition of RCAN1.4. Cancer Lett. 2020; 475: 2-13.
28. Wang Y, Xu R, Zhang D, Lu T, Yu W, Wo Y, et al. Circ-ZKSCAN1 regulates FAM83A expression and inactivates MAPK signaling by targeting miR-330-5p to promote non-small cell lung cancer progression. Transl Lung Cancer Res. 2019; 8(6): 862-875.
29. Li S, Niu X, Li H, Liang Y, Sun Z, Yan Y. Circ_0000003 promotes the proliferation and metastasis of non-small cell lung cancer cells via miR-338-3p/insulin receptor substrate 2. Cell Cycle. 2019; 18(24): 3525-3539.
30. Zhang E, Shen B, Mu X, Qin Y, Zhang F, Liu Y, et al. Ubiquitin-specific protease 11 (USP11) functions as a tumor suppressor through deubiquitinating and stabilizing VGLL4 protein. Am J Cancer Res. 2016; 6(12): 2901-2909.
31. Tajonar A, Maehr R, Hu G, Sneddon JB, Rivera-Feliciano J, Cohen DE, et al. Brief report: VGLL4 is a novel regulator of survival in human embryonic stem cells. Stem Cells. 2013; 31(12): 2833-2841.
32. Wu A, Wu Q, Deng Y, Liu Y, Lu J, Liu L, et al. Loss of VGLL4 suppresses tumor PD-L1 expression and immune evasion. EMBO J. 2019; 38(1): e99506.
33. Li H, Wang Z, Zhang W, Qian K, Liao G, Xu W, et al. VGLL4 inhibits EMT in part through suppressing Wnt/β-catenin signaling pathway in gastric cancer. Med Oncol. 2015; 32(3): 83.
34. Liu X, Kong C, Zhang Z. miR-130b promotes bladder cancer cell proliferation, migration and invasion by targeting VGLL4. Oncol Rep. 2018; 39(5): 2324-2332.
35. Deng X, Fang L. VGLL4 is a transcriptional cofactor acting as a novel tumor suppressor via interacting with TEADs. Am J Cancer Res. 2018; 8(6): 932-943.
36. Li N, Yu N, Wang J, Xi H, Lu W, Xu H, et al. miR-222/VGLL4/YAP-TEAD1 regulatory loop promotes proliferation and invasion of gastric cancer cells. Am J Cancer Res. 2015; 5(3): 1158-1168.
37. Shen S, Guo X, Yan H, Lu Y, Ji X, Li L, et al. A miR-130a-YAP positive feedback loop promotes organ size and tumorigenesis. Cell Res. 2015; 25(9): 997-1012.