Foxk2 inhibits non-small cell lung cancer epithelial-mesenchymal transition and proliferation through the repression of different key target genes

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Received August 4, 2016; Accepted December 28, 2016

DOI: 10.3892/or.2017.5461

Abstract. Increasing evidence suggests that numerous forkhead transcription factors are required to repress the mammalian cells phenotype. Among them, Foxk2 is a ubiquitously expressed family member, but the role of Foxk2 in mediating tumor metastasis in non-small cell lung cancer has not been explored. In this investigation reduced Foxk2 expression was found in lung adenocarcinoma tissues compared with the adjacent non-tumor tissues, and was associated with better overall survival. Low expression was also found in the NSCLC cell lines such as A549, NCI-H520, H1299, H358 and H460 cells. Recombinant lentivirus expressing Foxk2 constructs or ShFoxk2 were developed and transfected into A549 cells or NCI-H520 cells, immunofluorescence assay, qRT-PCR, and western blot analysis were used to measure the change of the epithelial markers, E-cadherin and α-catenin, and mesenchymal markers N-cadherin and vimentin. Wound healing assay and Transwell assay were used to measure the relative cell invasion ability. MTT assay, Edu assay, and cell cycle distribution analysis were used to confirm the effect of Foxk2 on cell proliferation. ChIP-seq, qChIP, as well as luciferase reporter gene assays were used to detect the target genes regulated by Foxk2, Bioinformatics predicated the potential miRNAs that could target Foxk2. Our study demonstrated that Foxk2 played major roles in NSCLC EMT by directly targeting N-cadherin and Snail, we found that Foxk2 regulated NSCLC cell growth by suppressing the expression of cyclin D1 and CDK4, which suggested that Foxk2 might be a multifunctional regulator in NSCLC. The expression of Foxk2 may be regulated by miR-1271, which could serve as a promising therapeutic target for NSCLC.

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Key words: Foxk2, epithelial-mesenchymal transition, proliferation, N-cadherin, Snail, cyclin D1, CDK4, miR-1271

Introduction

Non-small cell lung cancer (NSCLC) is a common form of lung adenocarcinoma, with a significant threat to public health and accounts for 80% of the total lung cancer cases all over the world (1). Although improvements in diagnosis and treatment have made significant strides, the 5-year survival rate still remains poor (2,3). Thus, it is crucial for research to expand our understanding of non-small cell lung cancer, to investigate the molecular mechanism on the NSCLC progression and identify novel diagnosis and therapeutic targets. Proliferation and epithelial-mesenchymal transition (EMT) in cancer are two critical malignant characteristics (4-6). Cell proliferation is considered as a complicated progress, which is regulated by a series of genes, among them, CCND1, was a classic oncogenic protein that could promote cell proliferation and the beginning of S phase in many cancers (7).

Epithelial-mesenchymal transition is a complicated progress, during which, epithelial cells lose their epithelial features and gain mesenchymal characteristics, and become invasive. As shown by many groups, loss of epithelial marker E-cadherin and gain of mesenchymal markers N-cadherin are usually considered as the molecular markers of EMT (8-10). Recent studies have shown that many transcription factors are involved in the progress of EMT, such as Snail (11,12). However, the regulation of Snail in NSCLC EMT and metastasis remains poorly understood.

There are over 40 members in the forkhead transcription factor family, which are characterized by a forkhead winged helix-turn-helix DNA binding domain, and regulate a diverse range of tumor progression, such as proliferation, apoptosis, metabolism and invasion. In mammals, Foxk2 is one of the two FOXK isoforms, however, little is known about the biological role and potential mechanisms of Foxk2 in tumorigenic driver pathways.

Materials and methods

Cell culture and transfection. Human non-small cell lung cancer cell lines A549, NCI-H520, H1299, H358, H460 cells and human lung fibroblast cell line WI-38 cells were purchased

from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI-1640 medium (Life Sciences, Corning, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), in humidified atmosphere of 5% CO_2 at 37˚C, with 100 units of penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA).

Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The relative recombinant lentivirus expressing Foxk2 or shRNA were purchased from Genechem Company (Shanghai, China). At 48-72 h after transfection, cells were analyzed as required.

Patients and specimens. The 50 samples of NSCLC and the adjacent non-tumor tissues were obtained from surgical specimens in Tongji Hospital of Tongji Medical College from the year of 2010-2012. The patients were collected with complete clinicopathologic information, and the patients who received chemotherapy and radiotherapy were excluded. The tissues were obtained immediately frozen and stored at -80˚C until use. This study was approved by the Ethics Committee of Tongji Medical College, and consent information was obtained from each patient, followed up for survival.

Cell proliferation assay. Cells were cultured in 24-well plates at a concentration of $1x10^4$ cells/well and incubated for various periods, 24 h after transfection, 0.5 ml methyl thiazolyl tetrazolium (MTT) solution (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and cells were further incubated for 4 h at 37˚C. Dimethyl sulfoxide (0.2 ml) was added to the supernatant medium to dissolve the purple crystals for 10 min. The optical density was determined at 490 nm with a SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Edu assay. Edu (5-ethynyl-2¢-deoxyuridine) assay was used to measure cell proliferation *in vitro*, according to the manufacturer's instructions. Cells ($1x10^4$) were seeded into a 96-well plate, after transfected with recombinant lentivirus expressing Foxk2 or shRNAs, cells were labeled with 50 μM Edu for 2 h before they were formalin fixed and processed. Then the stained cells were observed under a fluorescent microscope.

Cell cycle analysis. The relative cells were seeded in 10-cm diameter plates in 10% FBS. At 48 h after transfection, a total of $5x10^6$ cells were harvested, washed once with PBS and fixed with 70% ice-cold ethanol (diluted with PBS) for 24 h at 4˚C. Then the cells were rinsed with PBS again and incubated with 0.5 ml propidium iodide, 0.5 mg/ml ribonuclease A and 0.2% Triton X-100 for 30 min. Cell cycle distribution was measured and analyzed by BD C6 flow cytometry (BD Biosciences, San Jose, CA, USA). Each experiment was performed in triplicate.

Wound healing assay. For the wound healing assay, cells were seeded into 12-well plates and cultured in complete medium with 10% FBS to form a monolayer, 24 h after transfection, a uniform straight scratch was made by a pipette tip. The medium was replaced without FBS and the wound area was observed under the microscope (Olympus Corporation, Tokyo, Japan). Cells were further incubated at 37˚C with 5% CO_2 for 24 h. The speed of wound closure was measured. Each experiment was performed in triplicate.

Invasion assay. Cell invasion was measured using Transwell assay, Transwell chambers were from Corning Inc., Corning, NY, USA. Matrigel (30%) (BD Biosciences) was placed on the upper surface of the chamber. A total of $2x10^5$ cells/well without bovine serum were seeded into the upper chambers, and 1 ml RPMI-1640 with 10% FBS was added to the lower chamber. At 48 h after incubation at 37˚C, the penetrated cells were fixed with methanol, stained with crystal violet and counted, while the remaining cells in the upper chamber were removed. Three independent experiments were performed.

RNA extraction and quantitative real-time PCR analysis. Total RNA was extracted from tumor tissues or from the relative cells using TRIzol reagent (Thermo Fisher Scientific) following the manufacturer's protocol. Total RNA (1 μg) was used to transcribe to cDNA using with M-MLV reverse transcriptase.

Real-time PCR was carried out using the ABI 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The expression of TWIST1 was determined using SYBR green qPCR assay (Takara, Dalian, China) 2-ΔΔCt analysis method was used and normalized with GAPDH. Specific primers were obtained from Invitrogen. All reactions were performed in triplicate.

Western blotting. Cells were harvested using sodium dodecyl sulfate lysis buffer for 30 min at 4˚C. The lysates were centrifuged at 13000 rpm for 15 min at 4˚C, and the supernatants were harvested. An equal amount of protein was separated on 10% SDS-PAGE gels and then were transferred onto polyvinylidene difluoride membranes (Thermo Fisher Scientific). After blocked with 5% non-fat milk, the proteins were probed with primary antibodies against Foxk2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or antibodies from EMT antibody kit (Cell Signaling Technology, Danvers, MA, USA) or β-actin (cat. no. SC47778; Santa Cruz Biotechnology) overnight at 4˚C (1:1000 dilution). Followed by washing five times and incubated with respective secondary antibodies peroxidase-conjugated anti-IgG (Abcam), and were visualized using a chemiluminescent detection system (Western blot detection system; Thermo Fisher Scientific), according to the manufacturer's instructions. Representative data are shown from three individual experiments.

ChIP-seq and qChIP analysis. Approximately $5x10^7$ A549 cells were used for each ChIP-seq or qChIP assay. The chromatin DNA was precipitated by normal rabbit IgG (negative control) or polyclonal antibodies against Foxk2 at 4˚C overnight. After purified with the Qiagen PCR purification kit, qChIP was analyzed by quantitative PCR using specific primers. ChIP-seq was performed by the CapitalBio Corporation, Beijing, China.

Luciferase reporter analysis. For target gene reporter construction, the sequence of the target gene promoter

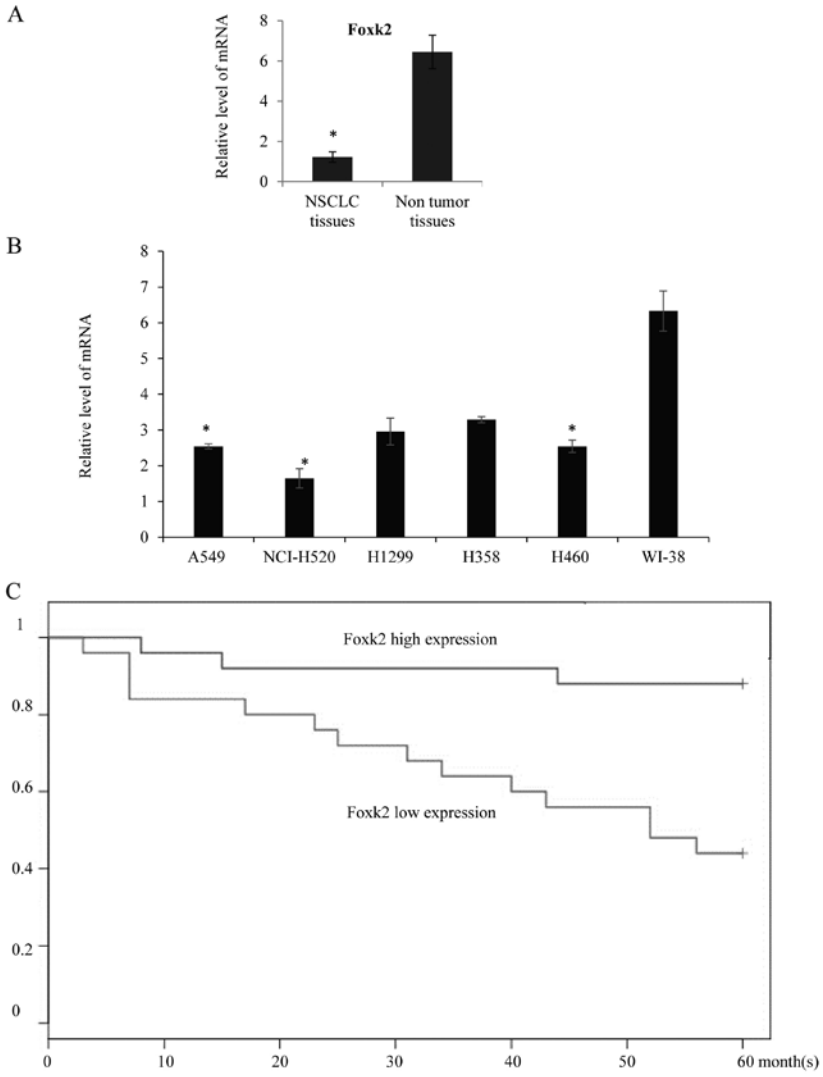

Figure 1. Foxk2 expression is downregulated in lung adenocarcinoma tissues and NSCLC cell lines. (A) Foxk2 was markedly decreased in NSCLC tissues compared to their adjacent non-tumor tissues, quantitative real-time PCR was performed, error bars represented as mean ± SD, of three independent experiments, and *P<0.05 was considered significant. (B) Quantitative real-time PCR was performed to measure the expression level of Foxk2 in the NSCLC cell lines A549 cells, NCI-H520 cells, H1299 cells, H358 cells and H460 cells compared with the human lung fibroblast cell line WI-38 cells. (C) Kaplan-Meier curves were used to measure the patient survival rate, Cox log-rank test was used to test the prognostic significance. The x-axis is the survival in months and the y-axis represents the survival probability (*P<0.05).

and partial first exon was obtained by PCR, these PCR products were digested and ligated into the pGL3 basic vector (Promega). A549 cells or NCI-H520 cells in 96-well plates were transfected with target gene luciferase reporter, *Renilla* plasmid, and Foxk2 constructs or shRNAs, using Lipofectamine LTX-Plus (Invitrogen).

The 3'UTR reporter plasmids (RL-Foxk2) were constructed by Jikai Gene Co. (Shanghai, China). Mutation of the Foxk2 3'UTR were generated by QuikChang Multi site-directed mutagenesis kit (Stratagene). The RL reporter plasmids (a firefly luciferase reporter was used for normalization), together with miR-1271 and pGL3-control were transfected into A549 cells or NCI-H520 cells, 48 h after transfection, luciferase reporter assay was performed.

Statistical analysis. Statistical analysis was carried out using SPSS software (version 17.0). All assays were performed at least three times, and the data were expressed as the means ± SD, unless otherwise mentioned, P<0.05 was considered to be significant. Survival time was analyzed using the Kaplan-Meier method with log-rank tests. Two-tailed Student's t-test was used to analyze the difference between two groups, one-way ANOVA was applied to evaluate the difference among different lung cancer tissues and adjacent non-tumor tissues. TargetScan Human (http://www.targetscan.org/vert_61/) was used to perform prediction as to which of the miRNA target Foxk2.

Results

Foxk2 expression was downregulated in lung adenocarcinoma tissues and NSCLC cell lines. In order to understand the role of Foxk2 during the progress of NSCLC, we first assayed the expression level of Foxk2 in NSCLC tissues, compared with the adjacent non-tumor tissues, qRT-PCR showed that the mean expression of Foxk2 in NSCLC tissues was lower

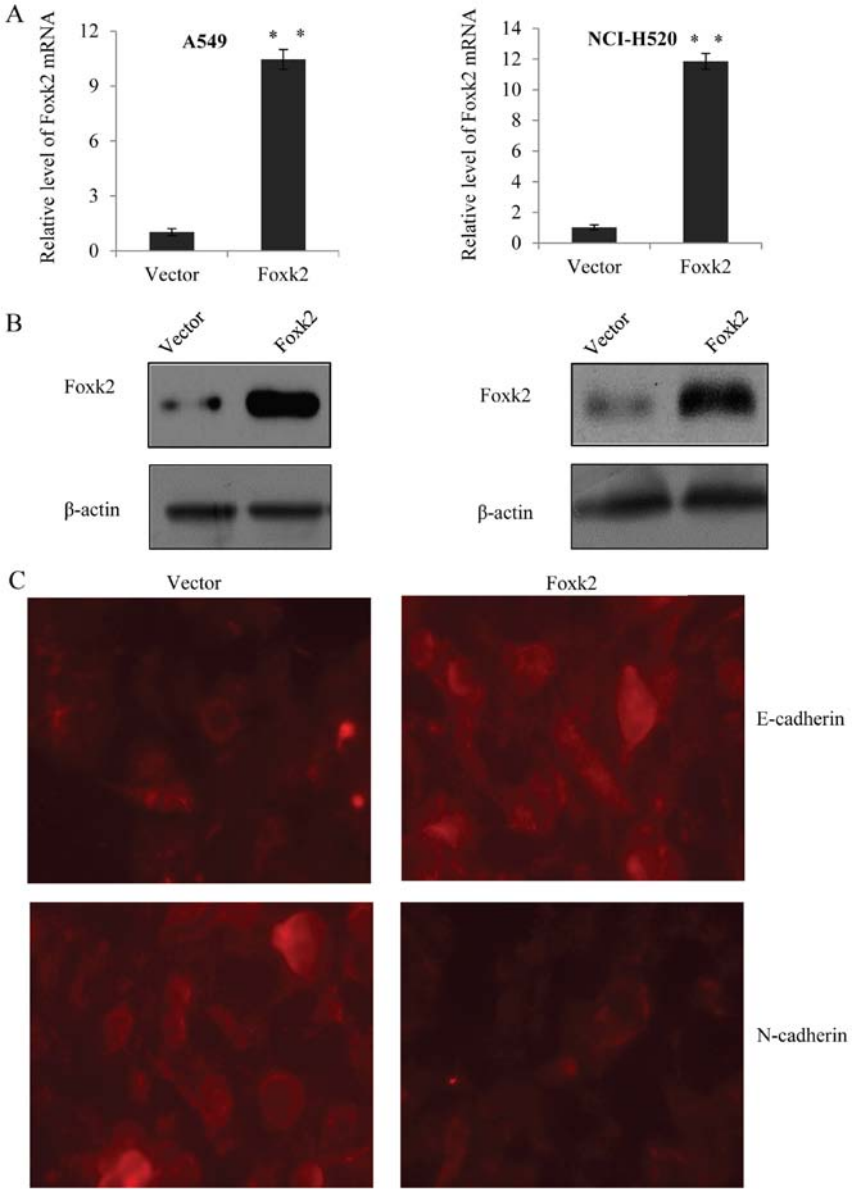

Figure 2. The overexpression of Foxk2 suppresses EMT in NSCLC cells. (A) qRT-PCR assay was performed in A549 cells (left panel) or NCI-H520 cells (right panel) transfected with lentivirus carrying Foxk2 constructs. β-actin was used as a normalization control. $^{**}P<0.01$. (B) Western blotting was performed in A549 cells (left panel) or NCI-H520 cells (right panel) transfected with lentivirus carrying Foxk2 constructs. β-actin was used as a normalization control. (C) Confocal microscopy was used to measure the morphological alterations in Foxk2 transfected A549 cells, E-cadherin and N-cadherin were immunofluorescent stained.

than control (Fig. 1A). In the NSCLC cell lines, qRT-PCR also revealed that the Foxk2 expression was lower in the NSCLC A549 cells, NCI-H520 cells, H1299 cells, H358 cells and H460 cells compared with the human lung fibroblast WI-38 cells (Fig. 1B). The relationship between Foxk2 expression and patient survival were investigated. Patients collected in the Tongji Hospital Hospital were divided in to two groups, depending on the median Foxk2 expression level, and found that high Foxk2 expression was correlated with better overall survival rate, and Kaplan-Meier curves were drawn (Fig. 1C, $P<0.05$).

Overexpression of Foxk2 suppresses EMT in NSCLC cells. In order to understand the biological significance of Foxk2 involved in non-small cell lung cancer, recombinant lentivirus expressing Foxk2 constructs were developed and transfected into A549 cells and NCI-H520 cells, respectively. qRT-PCR and western blotting was used to detect the transfection efficiency, β-actin was used as control. As expected, the Foxk2 lentivirus was successfully expressed in the two cell lines (Fig. 2A and B). To explore whether Foxk2 inhibits EMT in NSCLC cells, immunofluorescence assay was performed in A549 cells, and the results revealed that the overexpression of Foxk2 markedly promoted the expression of epithelial marker E-cadherin and markedly suppressed the expression of mesenchymal marker N-cadherin (Fig. 2C). Furthermore, qRT-PCR was used in A549 cells and NCI-H520 cells to detect the mRNA level of E-cadherin and α-catenin, as well as the level of N-cadherin and vimentin (Fig. 2D). Western blot assays were carried out in the above two cell lines, and the results showed that restoration of Foxk2 significantly increased the expression of epithelial markers (E-cadherin and α-catenin)

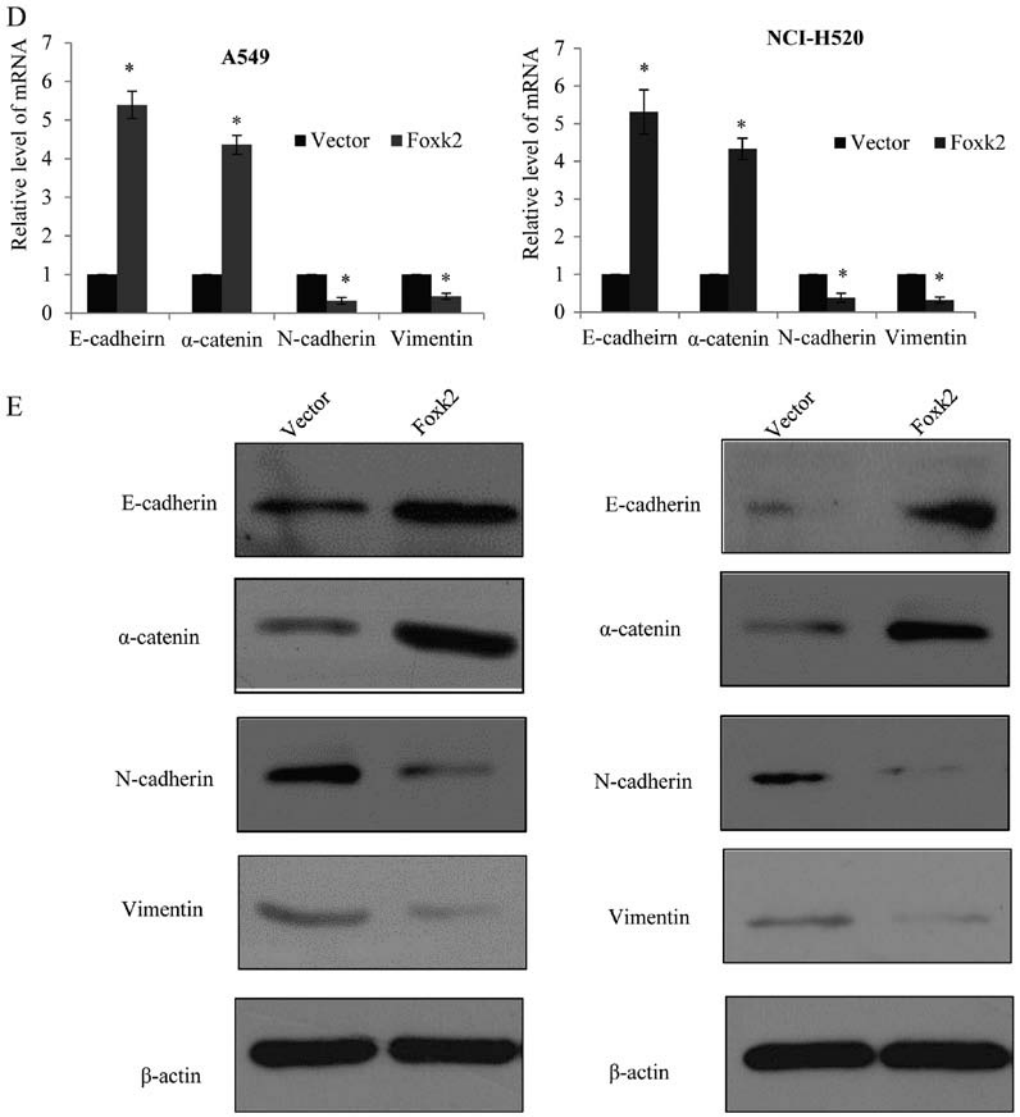

Figure 2. Continued. The overexpression of Foxk2 suppresses EMT in NSCLC cells. (D) The expression level of E-cadherin, α-catenin, N-cadherin and vimentin was measured using qRT-PCR assay in A549 cells (left panel) or NCI-H520 cells (right panel). *P<0.05. (E) The expression level of E-cadherin, α-catenin, N-cadherin and vimentin was measured using western blotting in A549 cells (left panel) or NCI-H520 cells (right panel).

and significantly inhibited the expression of mesenchymal markers (N-cadherin and vimentin) (Fig. 2E). These results suggested that Foxk2 was responsible for the inhibition of EMT in NSCLC cells.

Foxk2 inhibits NSCLC cell migration and invasion in vitro. As known, EMT affects tumors in an uncontrolled fashion and they acquire invasive ability, we supposed Foxk2 may have function in NSCLC cell migration and invasion. In support of our hypothesis, two different lentivirus stably silencing Foxk2 was developed and infected into A549 cells and NCI-H520 cells. Both mRNA level and protein level of Foxk2 in different groups were detected. As shown in Fig. 3A, mRNA level of Foxk2 was significantly reduced when cells were infected with ShFoxk2#1, or ShFoxk2#2 compared with the negative control lentivirus group (SCR). Concomitantly, in western blot analysis (Fig. 3B), the expression of Foxk2 was also remarkably downregulated in ShFoxk2-infected cells as compared with the control group. Both lentiviruses were successfully constructed, but the knockdown efficiency of ShFoxk2#2 was better than that of ShFoxk#1 and was chosen for further study.

We further proved the gain-of-function effect or loss-of-function effect of Foxk2 on the invasive potential by wound-healing assays in A549 cells and NCI-H520 cells. As shown in Fig. 3C and D, Foxk2 knockdown group was more efficient in wound healing, whereas cells treated with Foxk2 overexpression constructs, were resistant to wound healing to certain degrees, compared with the control groups, the overall tendency was shown. Further, when Foxk2 (ShFoxk2#2) was knocked down in A549 cells, there was an increase in the invasive potential, as measured by the Transwell analysis. To the contrary, when cells were transferred with Foxk2 lentivirus, there was a decrease in the invasive potential of A549 cells (Fig. 3E). Similar tendency was shown in the NCI-H520 cells transfected with the relative lentivirus (Fig. 3F).

Foxk2 modulates proliferation and tumorigenicity of NSCLC cells in vitro. In order to further understand the role of Foxk2 tumorigenicity in NSCLC cells, MTT assays was performed

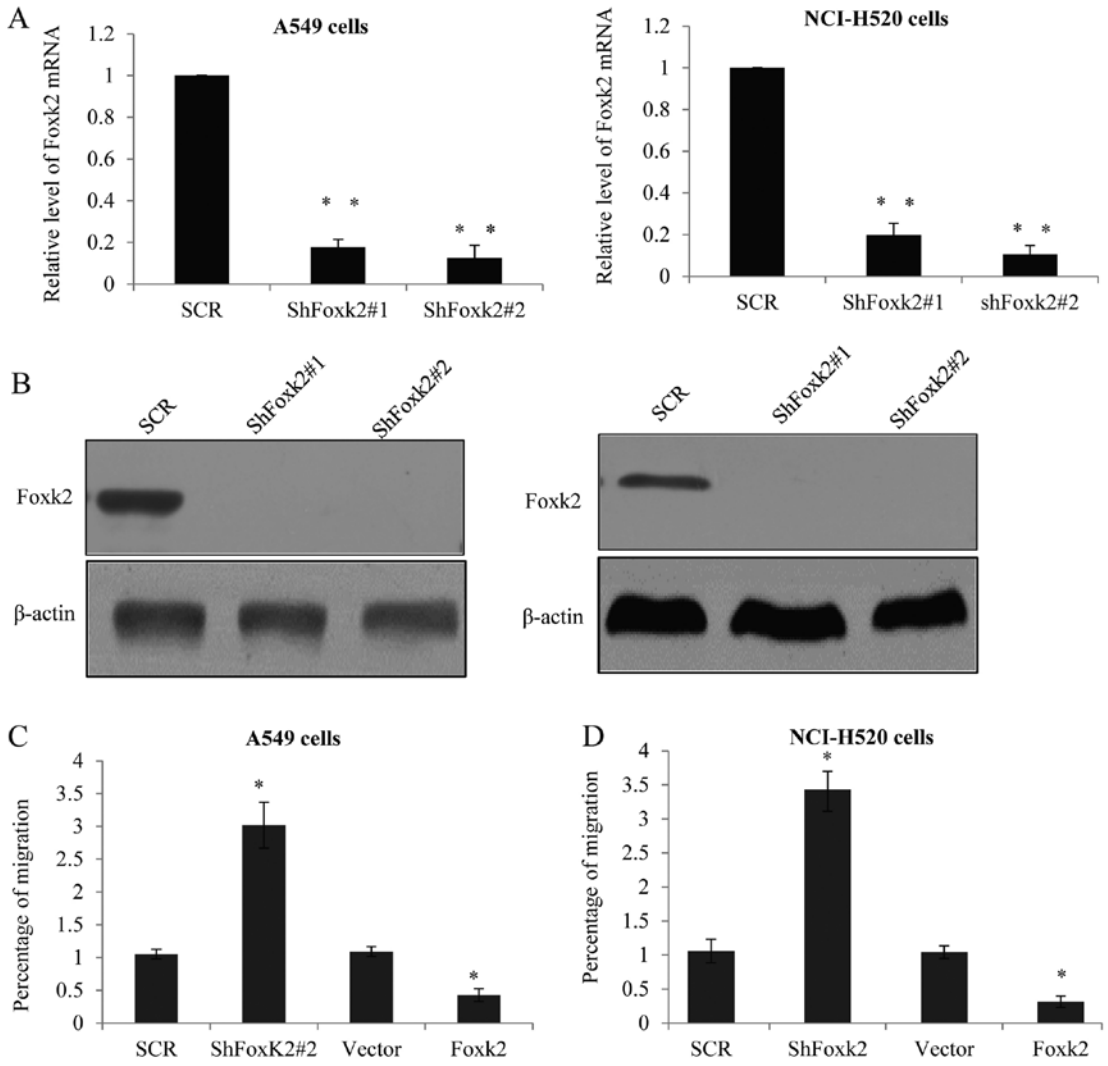

Figure 3. Foxk2 inhibits NSCLC cell migration and invasion *in vitro*. (A) The knock-down efficiencies of ShFoxk2#1, ShFoxk2#2 were confirmed by qRT-PCR in A549 cells or NCI-H520 cells, β-actin was used as a normalization control (left panel), experiments were repeated three times, $^{**}P<0.01$. (B) The protein level of Foxk2 was measured by western blotting in A549 cells or NCI-H520 cells. (C) A549 cells transfected with SCR, ShFoxk2#2, vector, Foxk2 were subjected to wound-healing analysis. Data are presented as fold change. (D) Wound-healing analysis was performed in NCI-H520 cells. $^{*}P<0.05$.

in A549 cells transfected with vector, Foxk2 gain-of-function, Foxk2 loss-of-function lentivirus. As shown in Fig. 4A, Foxk2 overexpression showed an evident growth inhibition, while Foxk2 was knocked down, there was an obvious growth promotion, compared with the vector group. Similar result was also observed in the NCI-H520 cells (Fig. 4B). Edu test further proved the function of Foxk2 in suppressing proliferation of A549 cells (Fig. 4C) and NCI-H520 cells (Fig. 4D) *in vitro*. To explore the mechanism of Foxk2 contribution to cell proliferation, we examined cell cycle distribution using flow cytometry in the above two cell lines, compared with the control group cells, ShFoxk2 transfected cells showed a substantial increase in S phase and a decrease in G1 phase populations, while overexpression of Foxk2 blocks the G1 phase to S phase (Fig. 4E and F). The above data suggested that Foxk2 might take part in the inhibition of osteosarcoma cell grow through induction of G1 arrest.

Identification of genome-wide transcriptional targets for Foxk2. As known, Foxk2 is a transcription factor which acts to repress gene transcription. In order to understand the molecular mechanism of Foxk2 in regulating NSCLC, ChIP-seq was performed using the Foxk2 antibody in A549 cells to identify the potential target genes, normal IgG was used as negative control. As shown in Fig. 5A, the Foxk2 ChIP-seq peak distribution and 806 different gene promoters were targeted by Foxk2. Those genes were further classified into various signaling pathways using KEGG, which included focal adhesion, PI3K-Akt signaling pathway, pathway in cancer, amoebiasis, cell cycle and TGF-β pathways that were critically involved in EMT, migration, invasion and proliferation (Fig. 5B). To validate the ChIP-seq result, different target genes involved in classified pathways were selected to perform qChIP assay, consistent with the ChIP-seq, on the promoter of CDH2 (N-cadherin), Snail, CCND1 and CDK4, there was obvious bindings of Foxk2 compared to the normal IgG (Fig. 5C). Real-time PCR was further carried out in A549 cells (Fig. 5D) with Foxk2 knocked down or Foxk2 overexpression, to detect the change of the target genes, as expected, in the Foxk2 knocked down group, the expression of CDH2

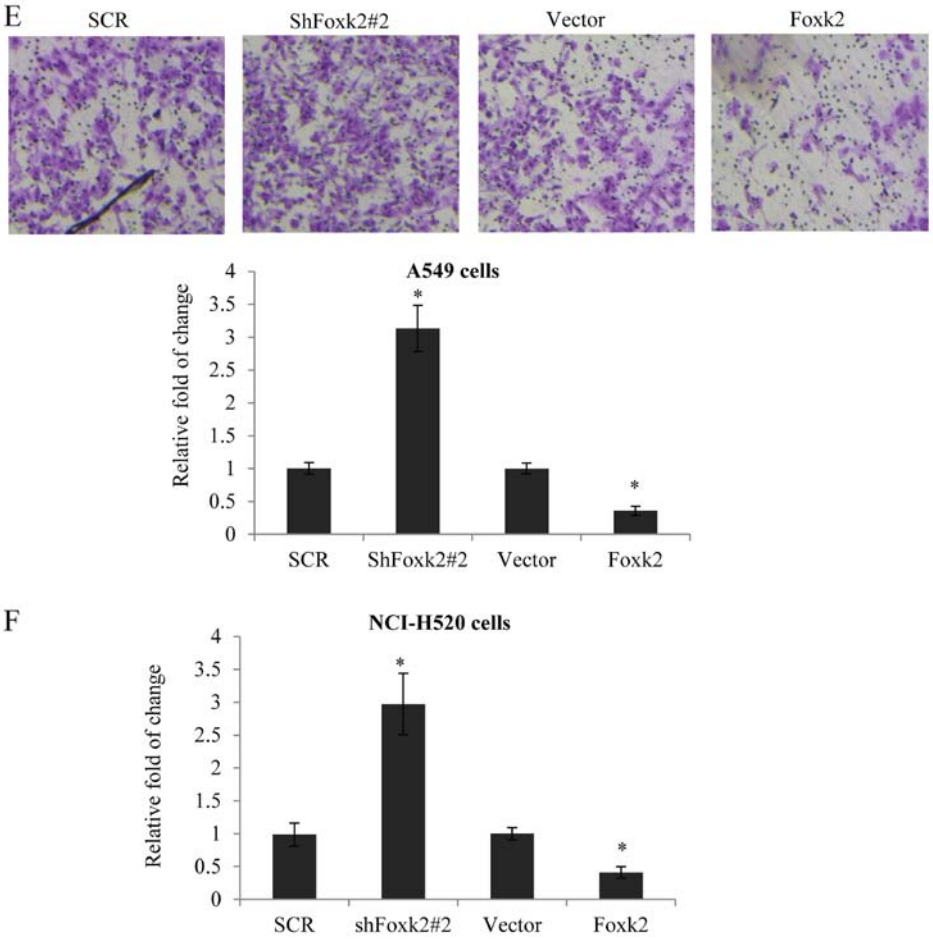

Figure 3. Continued. Foxk2 inhibited NSCLC cells migration and invasion *in vitro*. (E) A549 cells were transfected with SCR, ShFoxk2#2, vector, Foxk2, Transwell assay was performed, and invaded cells were stained and counted. Representative images were shown and statistically analyzed were represented. (F) Transwell assay was performed in NCI-H520 cells. *P<0.05.

(N-cadherin), Snail, CCND1 and CDK4 increased, while the overexpression of Foxk2 showed the opposite tendency, similar result was also observed in the NCI-H520 cells (Fig. 5E).

Molecular mechanism of Foxk2 inhibiting NSCLC cell EMT and invasion. As reported, CDH2 (N-cadherin) and Snail are usually considered to have crucial roles in the progress of EMT, to understand whether Foxk2 has a direct transcription repression on the two target genes, luciferase reporter activity assays were carried out. In Foxk2 depletion or overexpression of A549 cells, transfected with CDH2 or Snail promoter-driven luciferase reporter, a significant repression effect of Foxk2 on the indicated reporter activity was observed (Fig. 6A), similar result was also observed in the NCI-H520 cells (Fig. 6B). When Foxk2 was knocked down in A549 cells (Fig. 6C), in protein level, N-cadherin and Snail increased, while the overexpression of Foxk2 resulted in the decrease of N-cadherin and Snail, further supporting the notion that Foxk2 could repress target gene expression from transcription level, similarly to the NCI-H520 cells (Fig. 6D). In Transwell assay, A549 transfected with lentiviruses carrying ShFoxk2 plus ShSnail or ShFoxk2 plus ShN-cadherin could partially rescue the effect of Foxk2 knockdown on the invasive potential of A549 cells (Fig. 6E), also in the NCI-H520 cells (Fig. 6F).

Foxk2 suppresses the activity of PI3K/AKT/mTOR signaling pathway to inhibit NSCLC cell tumorigenicity. Attributed to the fact that Foxk2 suppressed NSCLC cell cycle progression, and as indicated from the ChIP-seq result, cell cycle G1 to S checkpoint proteins CCND1 and CDK4 were considered as the two target genes of Foxk2, and luciferase reporter activity assays were carried out. In Foxk2 depletion or overexpression of A549 cells, transfected with CCND1 or CDK4 promoter-driven luciferase reporter, Foxk2 has a significant repression effect on the reporter activity (Fig. 7A), similar result was also observed in the NCI-H520 cells (Fig. 7B).

Next, we examined the protein expression of cyclin D1 (CCND1) and CDK4, in both Foxk2-overexpressed or knocked down A549 cells. We found that introduction of Foxk2 blocked the expression of cyclin D1 and CDK4, while knocking down endogenous Foxk2 induced the target gene expression of cyclin D1 and CDK4 (Fig. 7C). Similar tendency was also shown in the NCI-H520 cells (Fig. 7D). Many studies have indicated the PI3K/Akt signaling pathway was responsible for the cell cycle, we also examined the effect of Foxk2 on PI3K/Akt signaling pathway, and found that introduction of Foxk2 reduced the expression of pPI3K (Tyr458) and pAkt (Ser473), while knockdown of Foxk2 increased the expression respectively (Fig. 7C and D), whereas their total levels remained unchanged (data not shown). MTT assay further proved that

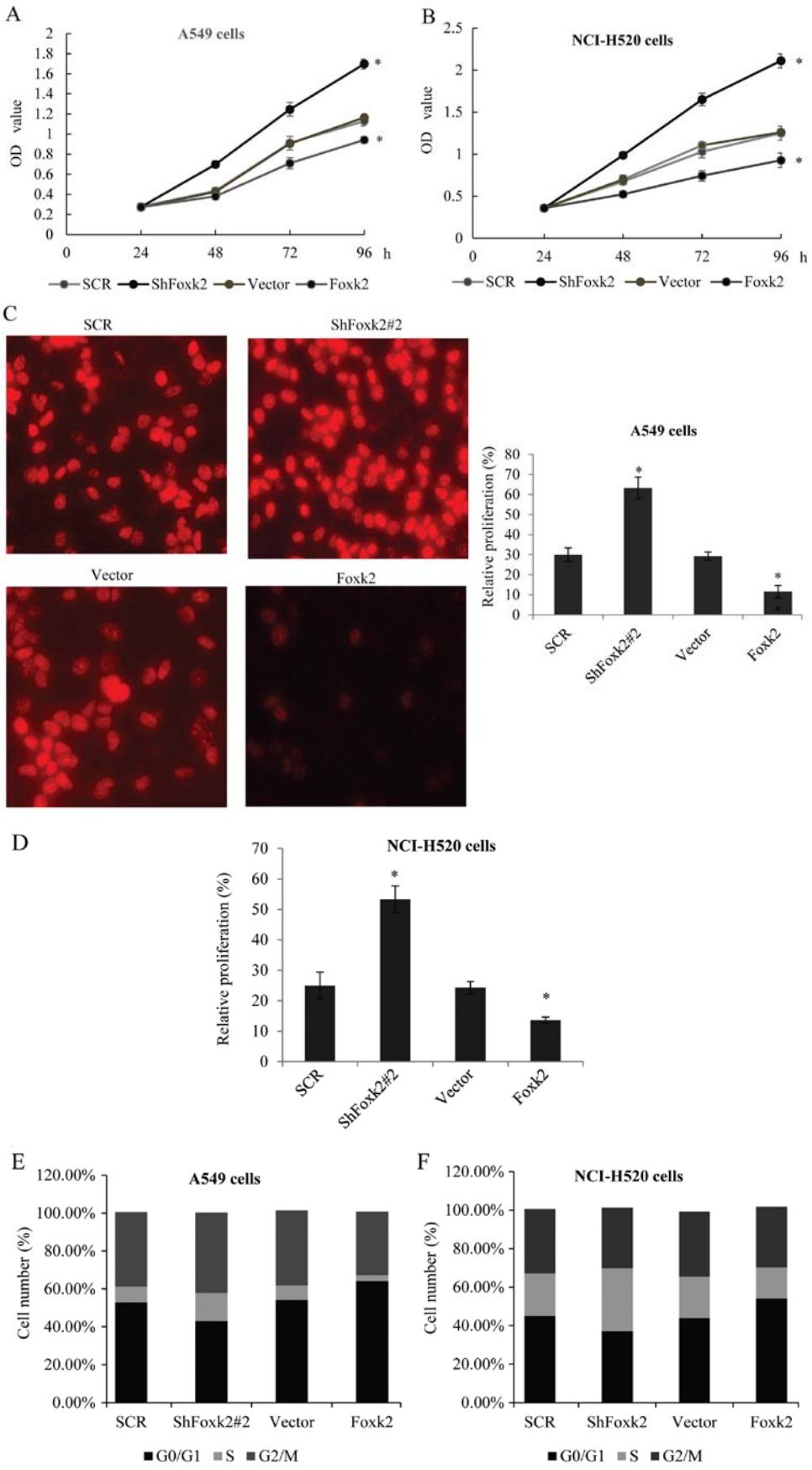

Figure 4. Foxk2 modulates proliferation and tumorigenicity of NSCLC cells *in vitro*. (A) MTT assay was used to measure the A549 proliferation. Equal numbers of cells transfected with SCR, ShFoxk2#2, vector, Foxk2 were cultured, MTT assay was performed every 24 h. (B) The above experiment was performed in NCI-H520 cells. (C) Edu assay was performed in A549 cells transfected with SCR, ShFoxk2#2, vector, Foxk2. Representative images are shown, statistical analysis are presented as fold change. (D) The above experiment was performed in NCI-H520 cells. (E) Cell cycle distribution was performed using flow cytometry (FCM) in A549 cells transfected with SCR, ShFoxk2#2, vector, Foxk2. The percentage of cells in the G0/G1, S, and G2/M phase are shown, experiments were repeated three times, $^{*}P<0.05$. (F) Cell cycle distribution was performed in NCI-H520 cells.

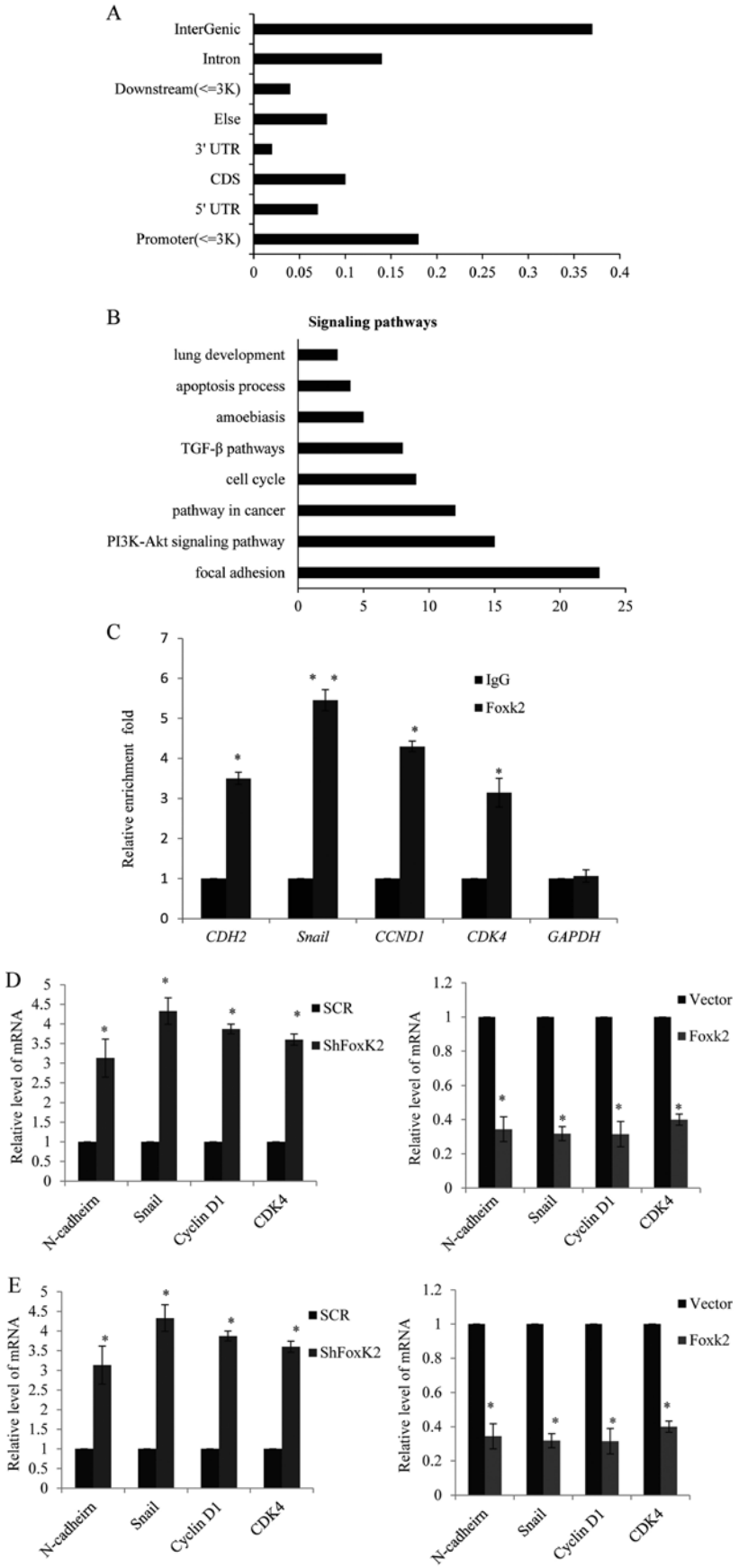

Figure 5. Identification of genome-wide transcriptional targets for Foxk2. (A) ChIP-seq assays were performed in A549 cells with Foxk2 or normal IgG as negative control, the peak distribution is shown. (B) KEGG was used to analyze the target genes pathways, the relative pathways are shown. (C) qChIP experiments were performed in A549 cells with Foxk2 or normal IgG, the enrichments on the promoter of CDH2 (N-cadherin), Snail, CCND1 and CDK4 were detected. Each bar indicates mean ± SD, of three independent experiments. **P<0.01. (D) Foxk2 overexpression led to decrease in N-cadherin, Snail, cyclin D1 or CDK4, mRNA level was measured by qPCR in A549 cells, while the knockdown of Foxk2 showed the opposite tendency. (E) The overexpression or knockdown of Foxk2 was performed in NCI-H520 cells.

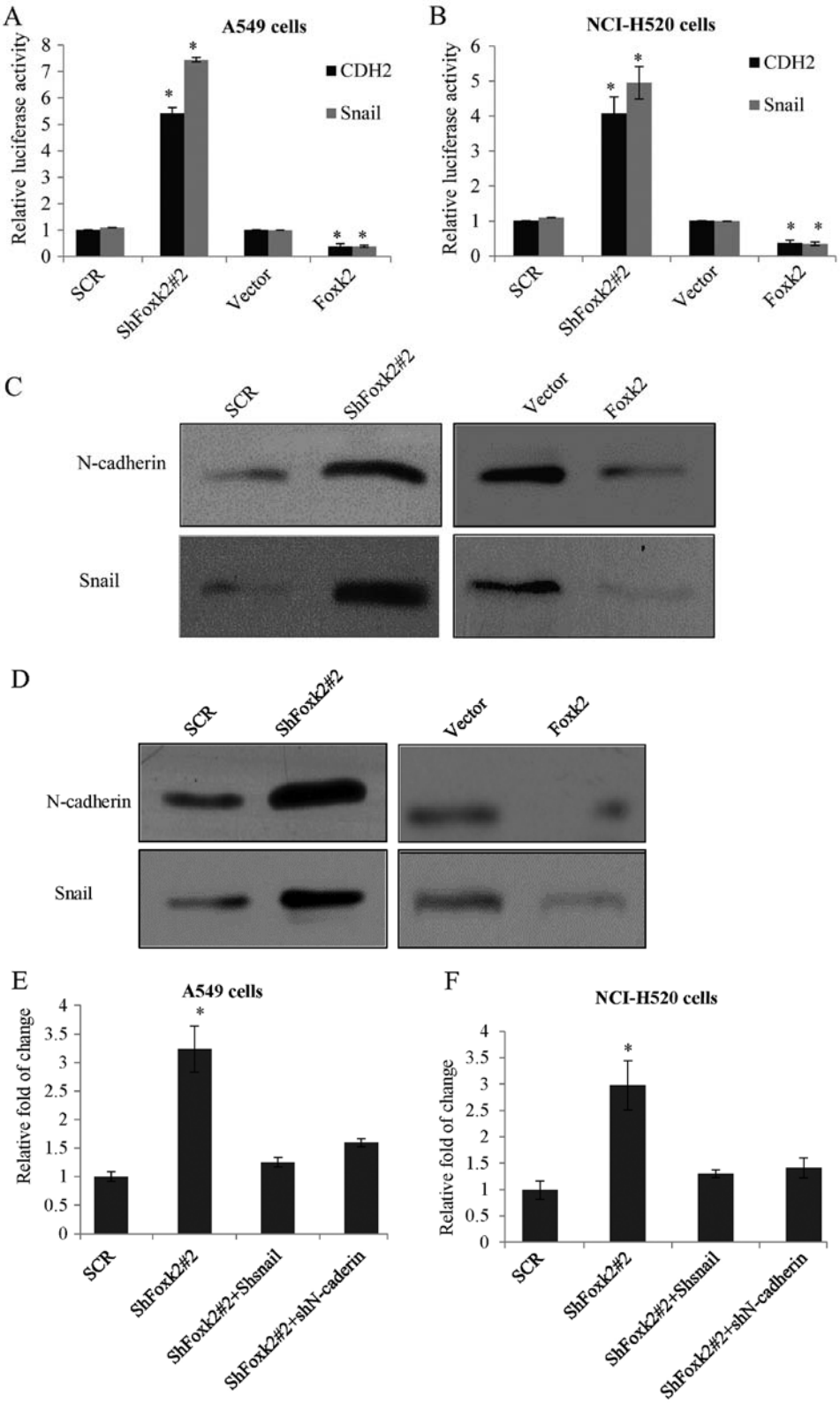

Figure 6. The molecular mechanism of Foxk2 inhibiting NSCLC cell EMT and invasion. (A) A549 cells were transfected with CDH2 promoter luciferase constructs, or Snail promoter together with Foxk2 construct or silencing molecules. Luciferase activities were measured and normalized to those of *Renilla*. (B) The luciferase report assay was performed in NCI-H520 cells. (C) Knockdown of Foxk2 led to increase in N-cadherin or Snail, protein level was measured by western blotting in A549 cells, while the Foxk2 overexpression showed the opposite tendency. (D) Western blotting was performed in NCI-H520 cells. (E) Transwell assay was performed in A549 cells transfected with SCR, ShFoxk2#2, ShFoxk2#2+ShSnail, or ShFoxk2#2+ShN-cadherin, statistical analysis are represented. (F) The above Transwell assay was performed in NCI-H520 cells. $^{*}P<0.05$; $^{**}P<0.01$.

A549 transfected with lentiviruses carrying ShFoxk2 plus shCyclinD1 or ShFoxk2 plus shCDK4 could partially rescue the effect of Foxk2 knockdown on the tumorigenesis potential of A549 cells (Fig. 7E), also in the NCI-H520 cells (Fig. 7F).

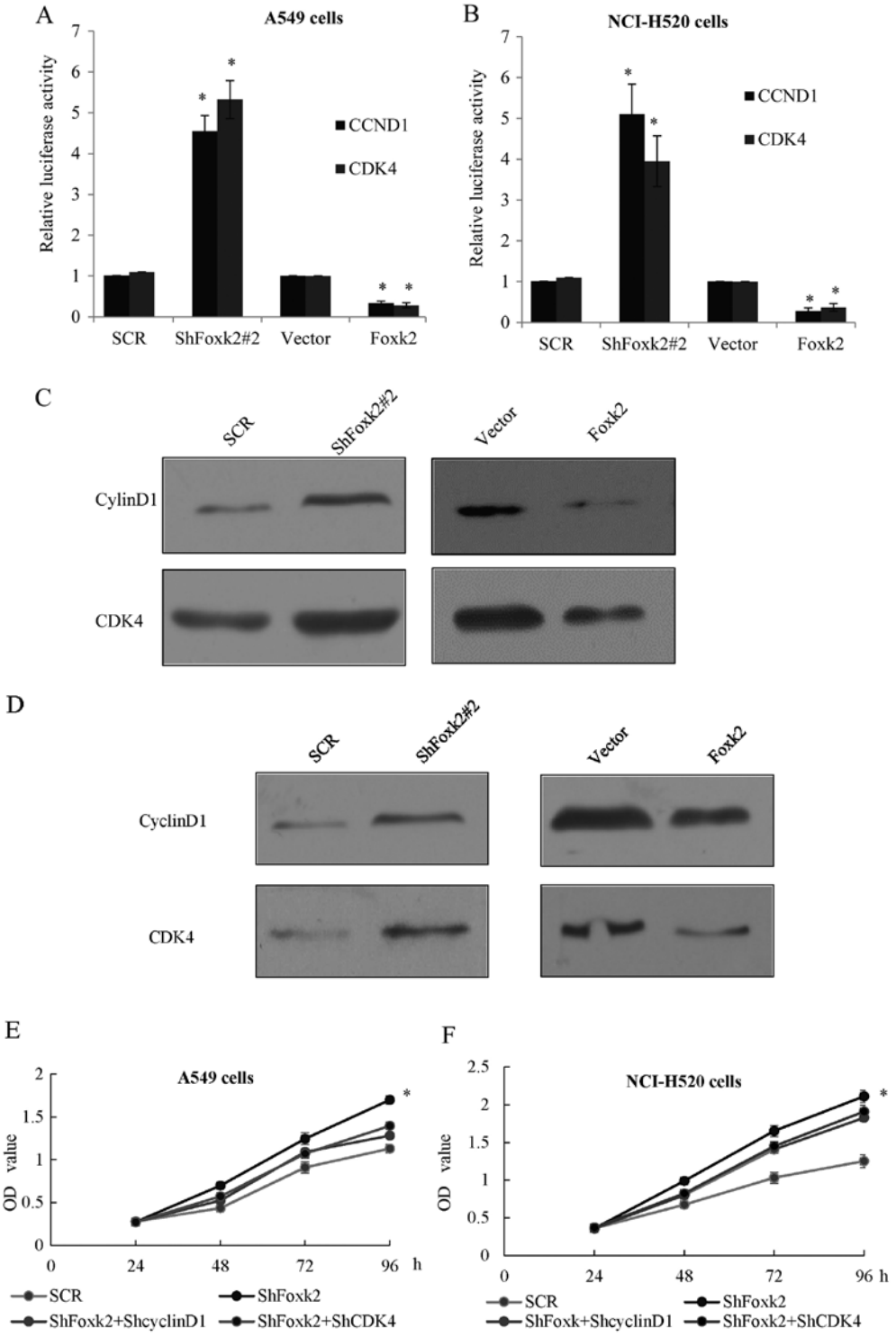

Figure 7. Foxk2 suppresses the activity of PI3K/AKT/mTOR signaling pathway to inhibit NSCLC cell tumorigenicity. (A) A549 cells were transfected with CCND1 promoter luciferase constructs, or CDK4 promoter together with Foxk2 construct or silencing molecules. Luciferase activities were measured and normalized to those of *Renilla*. (B) The luciferase report assay was performed in NCI-H520 cells. (C) Knockdown of Foxk2 led to increase in cyclin D1 or CDK4, protein level was measured by western blotting in A549 cells, while the Foxk2 overexpression showed the opposite tendency. (D) Western blotting was performed in NCI-H520 cells. (E) MTT assay was performed in A549 cells transfected with SCR, ShFoxk2#2, ShFoxk2#2+ShSnail, or ShFoxk2#2+ShN-cadherin, statistical analysis are represented. (F) The above MTT assay was performed in NCI-H520 cells. *P<0.05; **P<0.01.

Foxk2 is negatively targeted by miR-1271. Since Foxk2 was downregulated in the NSCLC cells, we tried to find which miRNAs target Foxk2. Bioinformatics was used to predicate the potential miRNAs. Based on their potential relevance to NSCLC and conservation, we found the Foxk2 3-UTR harbors potential miR-1271 target sites (position 40-47) (GUGCCAA), the site was complementary to the human miR-1271 heptamer motif (CACGGUU) and highly conserved regions in different species (Fig. 8A). Next, the 3'UTR of Foxk2 was cloned into luciferase reporter plasmids. miR-1271 and the reporter plasmids were co-transfected into A549 cells or NCI-H520 cells. We found that miR-1271 reduced mainly the Foxk2 luciferase

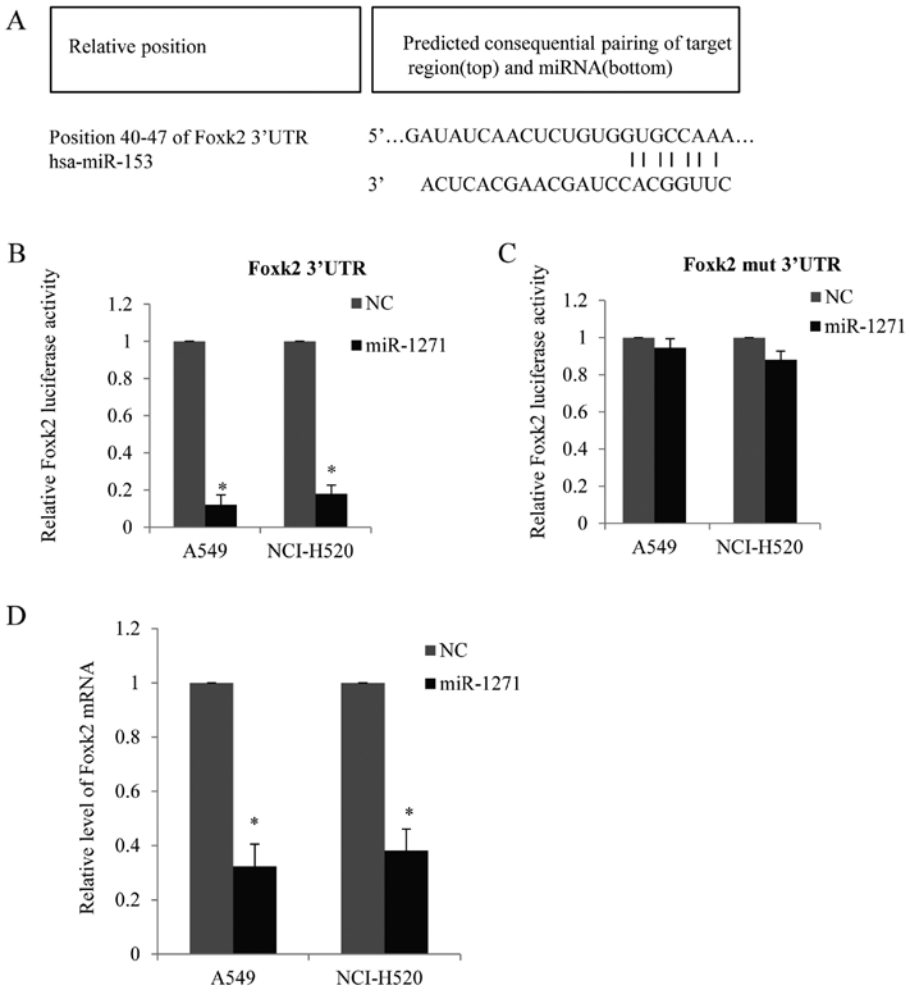

Figure 8. Foxk2 is negative targeted by miR-1271. (A) TargetScan representing the position 40-47 of Foxk2 3-UTR is a highly conserved sequence and considered to be one of the candidate targets for miR-1271. (B) Luciferase reporter assay was performed in A549 cells or NCI-H520 cells to identify the binding of miR-1271 to Foxk2 3'UTR. The relative cells were co-transfected with negative control (NC) or miR-1271 mimics (miR-1271) together with the luciferase gene driven Foxk2 3'UTR. (C) Luciferase reporter assay was performed using Foxk2 mutant 3'UTR (GUGCAAA). *P<0.05. (D) A549 cells or NCI-H520 cells were transfected with control or miR-1271 for 48 h. The endogenous Foxk2 mRNA were measured by qRT-PCR and normalized to β-actin.

activity (Fig. 8B). To further confirm the repression effect of miR-1271 on Foxk2, we mutated Foxk2 3'UTR as indicated (GUGCAAA) and the mutated UTR was cloned into luciferase reporter plasmids and co-transfected with miR-1271 into A549 cells or NCI-H520 cells (Fig. 8C). As shown, miR-1271 did not reduce the luciferase activity in cells transfected with mutated Foxk2 3'UTR. The mRNA level of Foxk2 was reduced in the A549 cells or NCI-H520 cells transfected with miR-1271 compared with the control groups (Fig. 8D). Thus, our data indicated that Foxk2 was negatively targeted by miR-1271.

Discussion

Non-small cell lung cancer is not only a disease with malignant proliferation, but also with high invasiveness and metastasis. Foxk2, a member of the forkhead transcription factor family has been reported to regulate a number of essential biological progresses (13,14). However, in NSCLC, the role and molecular mechanism of Foxk2 remain largely unknown.

In this study, the expression of Foxk2 was evaluated in NSCLC tissues and cell lines. We found that Foxk2 was significantly downregulated in NSCLC tissues compared with the corresponding adjacent paracarcinoma tissues. Foxk2 has also low expression in the NSCLC cell lines. Our data hinted that lost expression of Foxk2 was involved in the pathogenesis of NSCLC. Subsequently, we presented evidence that Foxk2 inhibited NSCLC cell EMT ability. As lentiviral-delivered Foxk2 markedly induced the expression of epithelial markers (E-cadherin and α-catenin) and significantly reduced the expression of mesenchymal markers (N-cadherin and vimentin) in A549 and NCI-H520 cells. Further, the overexpression of Foxk2 reduced cell invasion, growth, proliferation and blocked cell cycle progression. On the contrary, stably knocking down Foxk2 significantly elevated NSCLC cell malignancy phenotypes. ChIP-seq was used to identify genome-wide transcriptional targets for Foxk2.

Several classic oncogenic proteins were proved to be Foxk2 target genes and repressed by Foxk2. Such as N-cadherin, which was considered not only as a mesenchymal marker but also promoted cancer cell invasion. Snail was a crucial EMT inducer and contributed to the NSCLC metastasis (15). CCND1 is involved in promoting cell proliferation in many cancers (16,17). CDK4, a member of the cyclin-dependent

kinase family, which was responsible for cell cycle G1-S phase progression (18). By repressing the above target genes, we underlined the mechanism in which Foxk2 suppressed NSCLC tumor progression. MicroRNAs are short, non-coding RNAs (18-25 nucleotides) which negatively regulate numerous target genes by complementary recognized sequences of 3-UTRs at post-transcriptional level. MicroRNAs play important roles in the pathogenesis of various cancers, including lung cancer, particularly non-small cell lung cancer (NSCLC) (19,20). miR-1271 was reported to promote NSCLC proliferation and invasion via targeting HOXA5 (21). In our study, we found that Foxk2 was another target gene of miR-1271, and it is reasonable to suggest that the low expression of Foxk2 was possibly due to the repression function of miR-1271.

In conclusion, our study on the function and mechanism of Foxk2 in NSCLC indicated that miR-1271 may target Foxk2 in NSCLC. Our study indicates that Foxk2 could be a tumor suppressor and miR-1271 a potential target in NSCLC.

Acknowledgements

This study was financially supported by the National Natural Science Foundation of China (no. 81470252). We thank all our coworkers who contributed to this study.

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