



Roles of PI3K/Akt and c-Jun Signaling Pathways in Human Papillomavirus Type 16 Oncoprotein-Induced HIF-1 α , VEGF, and IL-8 Expression and *In Vitro* Angiogenesis in Non-Small Cell Lung Cancer Cells

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Abstract

Background and Objectives: Human papillomavirus (HPV)-16 infection may be related to non-smoking associated lung cancer. Our previous studies have found that HPV-16 oncoproteins promoted angiogenesis *via* enhancing hypoxia-inducible factor-1 α (HIF-1 α), vascular endothelial growth factor (VEGF), and interleukin-8 (IL-8) expression in non-small cell lung cancer (NSCLC) cells. In this study, we further investigated the roles of PI3K/Akt and c-Jun signaling pathways in it.

Methods: Human NSCLC cell lines, A549 and NCI-H460, were stably transfected with pEGFP-16 E6 or E7 plasmids. Western blotting was performed to analyze the expression of HIF-1 α , p-Akt, p-P70S6K, p-P85S6K, p-mTOR, p-JNK, and p-c-Jun proteins. VEGF and IL-8 protein secretion and mRNA levels were determined by ELISA and Real-time PCR, respectively. The *in vitro* angiogenesis was observed by human umbilical vein endothelial cells (HUVECs) tube formation assay. Co-immunoprecipitation was performed to analyze the interaction between c-Jun and HIF-1 α .

Results: HPV-16 E6 and E7 oncoproteins promoted the activation of Akt, P70S6K, P85S6K, mTOR, JNK, and c-Jun. LY294002, a PI3K inhibitor, inhibited HPV-16 oncoprotein-induced activation of Akt, P70S6K, and P85S6K, expression of HIF-1 α , VEGF, and IL-8, and *in vitro* angiogenesis. c-Jun knockdown by specific siRNA abolished HPV-16 oncoprotein-induced HIF-1 α , VEGF, and IL-8 expression and *in vitro* angiogenesis. Additionally, HPV-16 oncoproteins promoted HIF-1 α protein stability *via* blocking proteasome degradation pathway, but c-Jun knockdown abrogated this effect. Furthermore, HPV-16 oncoproteins increased the quantity of c-Jun binding to HIF-1 α .

Conclusions: PI3K/Akt signaling pathway and c-Jun are involved in HPV-16 oncoprotein-induced HIF-1 α , VEGF, and IL-8 expression and *in vitro* angiogenesis. Moreover, HPV-16 oncoproteins promoted HIF-1 α protein stability possibly through enhancing the interaction between c-Jun and HIF-1 α , thus making a contribution to angiogenesis in NSCLC cells.

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Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, and mortality rates continue to increase among older women with lung cancer in many countries [1]. Non-small cell lung cancer (NSCLC) comprises the majority of lung cancer. Cigarette smoking is considered the major risk factor for NSCLC. However, approximately 25% of all lung cancer cases have been

observed in never-smokers [2,3]. Moreover, it was reported that there are different epidemiologic evidences, clinicopathologic features, and survival rates between ever-smoking and never-smoking NSCLC patients [4–6], implying that never-smoking NSCLC might be a different disease and have different risk factors [5,7]. Therefore, other non-smoking risk factors might contribute to never-smoking NSCLC.

In the early 1980s, Syrjanen first suggested the possibility of human papillomavirus (HPV) involvement in bronchial squamous cell carcinoma [8]. Afterwards, a growing body of epidemiological evidence from different countries has shown that the positive rate of high-risk HPV-16/18 DNA and *E6* and *E7* oncogenes in NSCLC was much higher than that in benign lung neoplasms [9–16], wherein HPV-16 was the most prevalent HPV genotype with frequent *E6/E7* oncogene expression [10,13,16]. It is worth noting that the prevalence of HPV infection in clinical specimens of bronchial carcinomas is widely divergent in different geographic regions and histological tissue types, ranged from 0.0 to 100% [17,18]. But high-risk HPV infection, especially HPV-16, in NSCLC patients has a higher prevalence in Asia, especially in China [9,11,12,15]. Recently, high levels of IgG against HPV-16 and 18 *E7* in 16% of NSCLC patients were also detected [18]. With the progress of the studies, high-risk HPV infection has been proposed as a potential cause for NSCLC [17,18].

Angiogenesis is required for invasive tumor growth and metastasis and plays an important role in the development and progression of cancer including NSCLC [19–21]. Angiogenesis, inflammation, and coagulation markers were found to increase in NSCLC patients [21]. Increased levels of vascular endothelial growth factor (VEGF), a key angiogenic factor, correlated with a poor prognosis in NSCLC patients [21,22]. Hypoxia inducible factor-1 α (HIF-1 α) was suggested to be an important upstream molecule mediating VEGF expression and angiogenesis. It was reported that there was an association of HIF-1 α polymorphisms with susceptibility to NSCLC [23]. Additionally, interleukin-8 (IL-8), a pro-inflammatory chemokine, has also been found to be associated with NSCLC risk [24,25]. Therefore, HIF-1 α , VEGF, and IL-8 play key roles in the development of NSCLC. Interestingly, our previous study has demonstrated that HPV-16 *E6* and *E7* oncoproteins promoted HIF-1 α protein accumulation and HIF-1 α -dependent VEGF and IL-8 expression in NSCLC cells [26]. However, the underlying mechanisms by which HPV-16 oncoproteins enhanced HIF-1 α , VEGF, and IL-8 expression in NSCLC cells remain unclear.

Previous studies have demonstrated that multiple signaling pathways including phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) signaling pathways mediate HIF-1 α and VEGF expression induced by hypoxia or insulin-like growth factor-1 (IGF-1) in various cancer cells [27–30]. PI3K/Akt/mTOR signaling pathway has been well characterized and recognized to play essential roles in lung cancer cell proliferation and survival [31]. There are three major MAPK signaling pathways, namely, signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK pathways. Targets of JNK pathway include the activator protein 1 (AP-1) group of transcription factors, such as Jun. c-Jun contributes to transformation and cancer development and JNK activation has been demonstrated to be involved in the control of the tumor-initiating capacity of NSCLC cells [32]. Therefore, PI3K/Akt/mTOR and MAPK signaling pathways play crucial roles in the initiation and development in NSCLC. Moreover, our previous studies have demonstrated that PI3K/Akt and ERK1/2 signaling pathways were involved in HPV-16 *E6*- and *E7*-induced HIF-1 α protein accumulation in C-33A and HeLa cervical cancer cell lines [33]. However, the roles of PI3K/Akt and MAPK signaling pathways in HPV-16 oncoprotein-induced HIF-1 α , VEGF, and IL-8 expression in NSCLC cells have not been reported.

In this study, we investigated the roles of PI3K/Akt and JNK/c-Jun signaling pathways in HIF-1 α , VEGF, and IL-8 expression, and *in vitro* angiogenesis induced by HPV-16 *E6* and *E7*

oncoproteins in NSCLC cells. We found for the first time to our knowledge that PI3K/Akt signaling pathway and c-Jun were involved in HPV-16 *E6*- and *E7*-induced HIF-1 α , VEGF, and IL-8 expression in NSCLC cells, leading to angiogenesis *in vitro*. HPV-16 *E6* and *E7* oncoproteins promoted HIF-1 α protein stability through enhancing the interaction between HIF-1 α and c-Jun, thus triggering HIF-1 α -mediated angiogenesis in NSCLC cells.

Materials and Methods

Reagents

Transfection reagent (Lipofectamine 2000) was obtained from Invitrogen Corporation (Carlsbad, CA). *In vitro* angiogenesis assay kit (ECM625) was from Millipore (Temecula, CA, USA). Mouse anti-human HIF-1 α monoclonal antibody was from BD Transduction Laboratories (San Diego, CA, USA). Mouse anti-human β -actin antibody was purchased from Beyotime Biotechnology Corporation, Shanghai (Shanghai, China). One Step SYBR PrimeScript RT-PCR (No.DRR086A) was purchased from TaKaRa Biotechnology (Dalian) Co., LTD (Dalian, China). Total and phosphorylated Akt (Ser⁴⁷³), P70S6K (Thr³⁸⁹ and Thr⁴²¹), P85S6K, mTOR (Ser²⁴⁸¹), JNK (Thr¹⁸³/Tyr¹⁸⁵), and c-Jun (Ser⁶³) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-VHL antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). G418 was from Sigma (St. Louis, MO, USA). MG132, Cycloheximide (CHX), LY294002, SP6002125, normal rabbit IgG, protein A garose beads, and Immunol Fluorecence Staining Kit were from Beyotime Biotechnology Corporation, Shanghai (Shanghai, China). Human VEGF and IL-8 Enzyme-linked immunosorbent assay (ELISA) reagent kits were purchased from Wuhan Boster Bio-engineering limited company (Wuhan, China).

Cell lines and Cell Culture

Human NSCLC cell line A549 (adenocarcinoma cell line) and human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC; Rockville, MD). Human NSCLC cell line NCI-H460 (a large cell lung cancer cell line) was purchased from Chinese Academy of Sciences Cell Bank of Type Culture Collection (CBTCCCAS; Shanghai, China). All cells were cultured in RPMI-1640 media supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μ g/mL) (Invitrogen). All cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂.

Stable transfection and establishment of stable-transfected cells

The method was as described previously [34]. Briefly, A549 and NCI-H460 cells at 70% to 80% confluence were respectively transfected with pEGFP empty vector, pEGFP-16 *E6*, *E6* mutant (*E6m*), *E7*, and *E7* mutant (*E7m*) plasmids using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. G418 (400 μ g/mL in RPMI 1640) combined with flow cytometry was used to screen transfected cells. The expression of HPV-16 *E6* or *E7* oncoprotein was detected by Western blotting every other week [34].

RNA interference

The target sequences of RNA interference (RNAi) against c-Jun were previously confirmed [35] and synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China). The sequences of sense strand-directed siRNA against c-Jun are as follows: c-Jun siRNA-1 (Si-1) 5'-GAUGGAAACAGC CUUCUAUTT -3'; c-Jun

siRNA-2(Si-2) 5'-CCUCAGCAACUUCAACCCATT-3' [Genbank:NC_000001.10] [35]. HPV-16 E6 or E7-transfected NSCLC cells were cultured in 35 mm plates with growth media without antibiotics. The cells were transiently co-transfected for 4 h with c-Jun siRNA or non-specific control siRNA (NS-siRNA) *via* lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 48 h incubation at 37°C, the cells were harvested and subjected to Western blot analysis, and the conditioned media were used to ELISA and *in vitro* angiogenesis assay.

Protein extraction and Western blotting

The method was as described previously [26]. Briefly, total proteins were extracted from treated and untreated cells with lysis buffer containing 20 mmol/L Tris (pH 7.5), 1 mmol/L EDTA, 150 mmol/L NaCl, 1% Triton X-100, leupeptin, sodium pyrophosphate, EDTA, leupeptin, β -glycerophosphate, Na_3VO_4 , phenylmethylsulfonyl fluoride (PMSF), and complete protease inhibitor cocktail (Sigma), followed by incubation at 4°C for 1 h. The lysates were ultra-sonicated and centrifuged at 12,000 \times g for 10 min. Protein concentrations were determined by BCA methods. The proteins were separated on 10% polyacrylamide-SDS gel and electro-blotted onto polyvinylidene difluoride (PVDF) membrane. After blocking with TBS/5% skim milk, the membrane was incubated overnight at 4°C with primary antibodies, followed by incubation with HRP-conjugated secondary antibodies. As a loading control, the blots were stripped and re-probed with anti- β -actin antibody.

Co-immunoprecipitation

The cells were lysed with IP lysis buffer containing 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, sodium pyrophosphate, β -glycerophosphate, EDTA, Na_3VO_4 , and leupeptin, followed by centrifugation. Total lysate (0.5 mg) was incubated with 2 μ g anti-c-Jun antibody overnight at 4°C. The lysate incubated with normal rabbit IgG served as a negative control. Protein A agarose (40 μ L) was added into the mixture and incubated with agitation for an additional 4 h at 4°C. The beads were washed thrice with cell lysis buffer for 10 min each time. The precipitants were dissolved with the SDS loading buffer for the analysis of HIF-1 α and c-Jun protein by Western blotting.

Cell immunofluorescence

The cells (2×10^6) were seeded onto coverslips in 6-well plates. 12 h later, the cells were rinsed with PBS, fixed with immunostaining fix solution overnight at 4°C, and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Next, the cells were washed with PBS and 3% bovine serum albumin (blocking reagent) for 1 h. Afterwards, the cells were sequentially incubated with diluted primary antibodies (1:1000) for 1 h, washed thrice with wash buffer, and incubated with fluorescent secondary antibodies for 1 h in the dark. Finally, the coverslips were washed with wash buffer for analysis under confocal microscope.

RNA isolation and quantitative real-time PCR (qRT-PCR)

The method was as described previously [26]. Briefly, total RNA was extracted by homogenization in 1 mL TRIZOL Reagent (Invitrogen), followed by chloroform extraction and isopropanol precipitation. The analysis of HIF-1 α , VEGF, and IL-8 mRNA relative levels was performed using One Step SYBR PrimeScript RT-PCR (TaKaRa, China) according to the manufacturer's instructions. A 50 ng sample of total RNA from A549 or NCI-H460 cells was used. The primers used were as follows: for

human HIF-1 α : forward 5'-TCTGGGTTGAAACTCAAGCAACTG-3' and reverse 5'-CAACCGGTTTAAGGACACATTCTG-3' [Genbank: NM_001243084.1]; VEGF: forward 5'-TCTACCTCCACCATGCCAAGT-3' and reverse 5'-GATGATTCTGCCCTCCTCCTT-3' [Genbank: NM_001025366.2]; IL-8: forward 5'-TTGCCAAGGAGTGCTAAAGAA-3' and reverse 5'-GCCCTC TTCAAAAACCTTCTCC-3' [Genbank: NM_000584.3]; β -actin: forward 5'-TCAAGATCATTGCTCCTCCTG-3' and reverse 5'-CTGCTTGCTGATCCACATCTG-3' [Genbank: NM_001017992.3]. All the primers were synthesized by TaKaRa Biotechnology (Dalian, Co., LTD (Dalian, China). The thermocycling conditions were as follows: 42°C for 5 min, 95°C for 10 s, followed by 40 cycles at 95°C for 5 s, and 60°C for 31s. The relative HIF-1 α , VEGF, and IL-8 mRNA levels were normalized to β -actin. The experiment was repeated in triplicate.

ELISA

The concentration of VEGF and IL-8 in the conditioned media derived from treated and untreated cells was measured using ELISA reagent kits (Wuhan Boster Bio-engineering limited company, Wuhan, China) according to the manufacturer's instructions. The experiment was repeated in triplicate.

In vitro angiogenesis assay

An *in vitro* angiogenesis assay kit was employed according to the manufacturer's instructions (Millipore). 96-well plates were coated with 50 μ L of cold liquid ECMatrix each well and incubated at 37°C for 1 h to promote solidification. Afterwards, HUVECs were seeded at a density of 5×10^3 cells/well into 96-well plates pre-coated with polymerized ECMatrix and incubated with conditioned media at 37°C for 6 to 8 h. The tubule formation was observed under a phase-contrast microscope, and the total tubule length in 3 random view-fields per well was measured by Scion image software and average value was calculated. The experiment was repeated in triplicate.

Statistical analysis

For all experiments, data were presented as mean \pm SD for three separate experiments. ANOVA and LSD-test were employed for statistical analysis using SPSS version 19.0. $P < 0.05$ was considered to be statistically significant.

Results

HPV-16 E6 and E7 oncoproteins promoted the activation of PI3K/Akt/mTOR signaling pathway

We previously established stable-transfected NSCLC cell lines (A549 and NCI-H460) using enhanced green fluorescent protein (EGFP) plasmid vectors harboring HPV-16 E6 or E7 gene, and the expression of HPV-16 E6 or E7 oncoprotein in the stable-transfected cells was confirmed [34]. HIF-1 α protein and mRNA expression levels were analyzed in the stable-transfected A549 cells. Our results showed that HIF-1 α protein levels were up-regulated by HPV-16 oncoproteins but HIF-1 α mRNA levels had no significant difference (Figure 1A, B), which was the same as our previous results using transient transfection method [33]. PI3K/Akt/mTOR signaling pathway plays an important role in the development of NSCLC [31]. Moreover, our previous study has demonstrated that HPV-16 oncoproteins promoted the activation of PI3K/Akt in cervical cancer cells [33]. To investigate the effect of HPV-16 oncoproteins on PI3K/Akt/mTOR activation in NSCLC cells, we determined the phosphorylated levels of Akt, P70S6K, P85S6K, and mTOR in stable-transfected A549 cells.

Our results showed that over-expression of HPV-16 E6 and E7 oncoproteins enhanced the phosphorylated levels of Akt, P70S6K, P85S6K, and mTOR in A549 NSCLC cells (Figure 1C). Similar results were found in another NSCLC cell line NCI-H460 (Figure 1D). These results indicated that HPV-16 oncoproteins activated PI3K/Akt/mTOR signaling pathway in NSCLC cells.

PI3K/Akt signaling pathway was involved in HPV-16 E6- and E7-induced HIF-1 α , VEGF, and IL-8 expression and *in vitro* angiogenesis

The inhibition of PI3K activity has been reported to block HIF-1 α activation and estrogen receptor recruitment to the VEGF promoter [36,37]. Therefore, we further analyzed whether PI3K/Akt signaling pathway is involved in the expression of HIF-1 α induced by HPV-16 oncoproteins in NSCLC cells. As shown in Figure 2 and 3, the pretreatment with different concentrations of LY294002, a specific PI3K inhibitor, significantly inhibited HPV16 E6- (Figure 2) and E7- (Figure 3) induced HIF-1 α protein expression in both A549 and NCI-H460 cells (Figure 2A, 3A). Our previous study has shown that the expression of VEGF and IL-8 induced by HPV-16 oncoproteins in NSCLC cells is HIF-1 α -dependent [26]. In this study, we further explored the role of PI3K/Akt signaling pathway in the expression of VEGF and IL-8 induced by HPV-16 E6 and E7 oncoproteins in NSCLC cells. Our results showed that the pretreatment with LY294002 remarkably inhibited HPV-16 oncoprotein-induced VEGF and IL-8 protein secretion ($P < 0.05$, Figure 2B and 3B) and mRNA expression ($P < 0.05$, Figure 2C and 3C) in both A549 and NCI-H460 cells. These findings suggested that PI3K/Akt signaling pathway was involved

in HPV-16 E6- and E7-induced HIF-1 α , VEGF, and IL-8 expression.

HIF-1 α , VEGF, and IL-8 are important angiogenic factors. To deeply analyze the role of PI3K/Akt signaling pathway in angiogenesis, an *in vitro* angiogenesis model was employed to evaluate the capillary tube formation of HUVECs stimulated by the conditioned media derived from stable-transfected A549 cells. Our results showed that LY294002 significantly blocked HPV-16 oncoprotein-stimulated formation of capillary tube-like structures ($P < 0.05$, Figure 2D and 3D), indicating that PI3K/Akt signaling pathway was involved in angiogenesis *in vitro* induced by HPV-16 oncoproteins.

HPV-16 oncoproteins promoted HIF-1 α protein stability via blocking 26S proteasome degradation pathway

Our results showed that HPV-16 E6 and E7 oncoproteins enhanced HIF-1 α protein accumulation but had no effect on HIF-1 α mRNA expression in A549 cells (Figure 1A and B). Our previous studies have demonstrated that HIF-1 α protein accumulation induced by hypoxia or IGF-1 was through enhancing HIF-1 α protein stability [28–30]. To analyze the effect of HPV-16 oncoproteins on HIF-1 α protein stability, we used cycloheximide (CHX) to prevent further synthesis of HIF-1 α protein in A549 and NCI-H460 cells. We found that HPV-16 E6 and E7 oncoproteins obviously inhibited HIF-1 α protein degradation as compared with empty vector or mutant controls in A549 and NCI-H460 cells (Figure 4A and B), suggesting that HPV-16 E6 and E7 oncoproteins enhanced HPV-16 oncoprotein-induced HIF-1 α protein expression possibly by inhibiting its degradation. To further explore whether HPV-16 oncoproteins inhibited HIF-1 α degra-

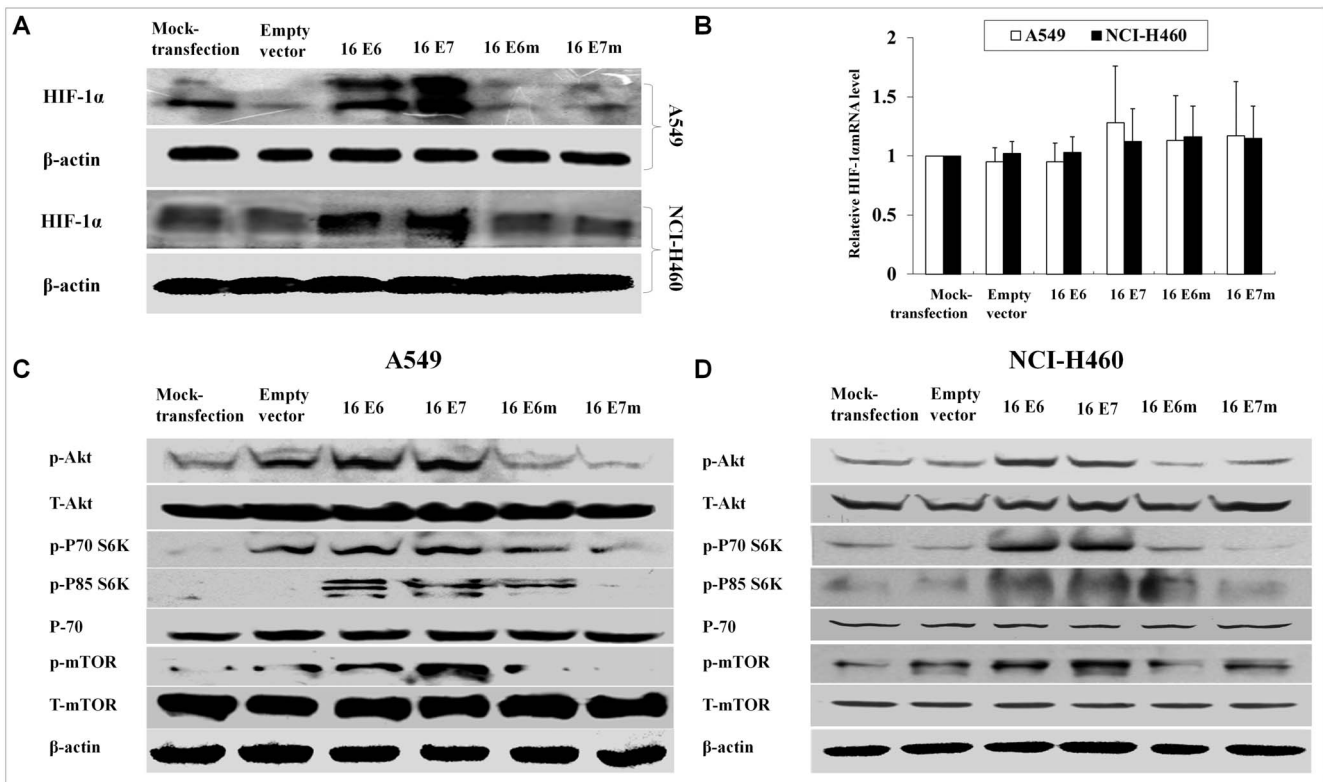


Figure 1. Effects of HPV-16 oncoproteins on HIF-1 α expression and PI3K/Akt/mTOR signaling pathway activation in NSCLC cells. (A) Western blot analysis of HIF-1 α protein levels in stable-transfected A549 cells. (B) Real-time PCR analysis of HIF-1 α mRNA levels in stable-transfected A549 cells. (C and D) Western blot analysis of p-Akt, p-P70S6K, p-P85S6K, and p-mTOR protein levels in transfected A549 (C) and NCI-H460 (D) cells. doi:10.1371/journal.pone.0103440.g001

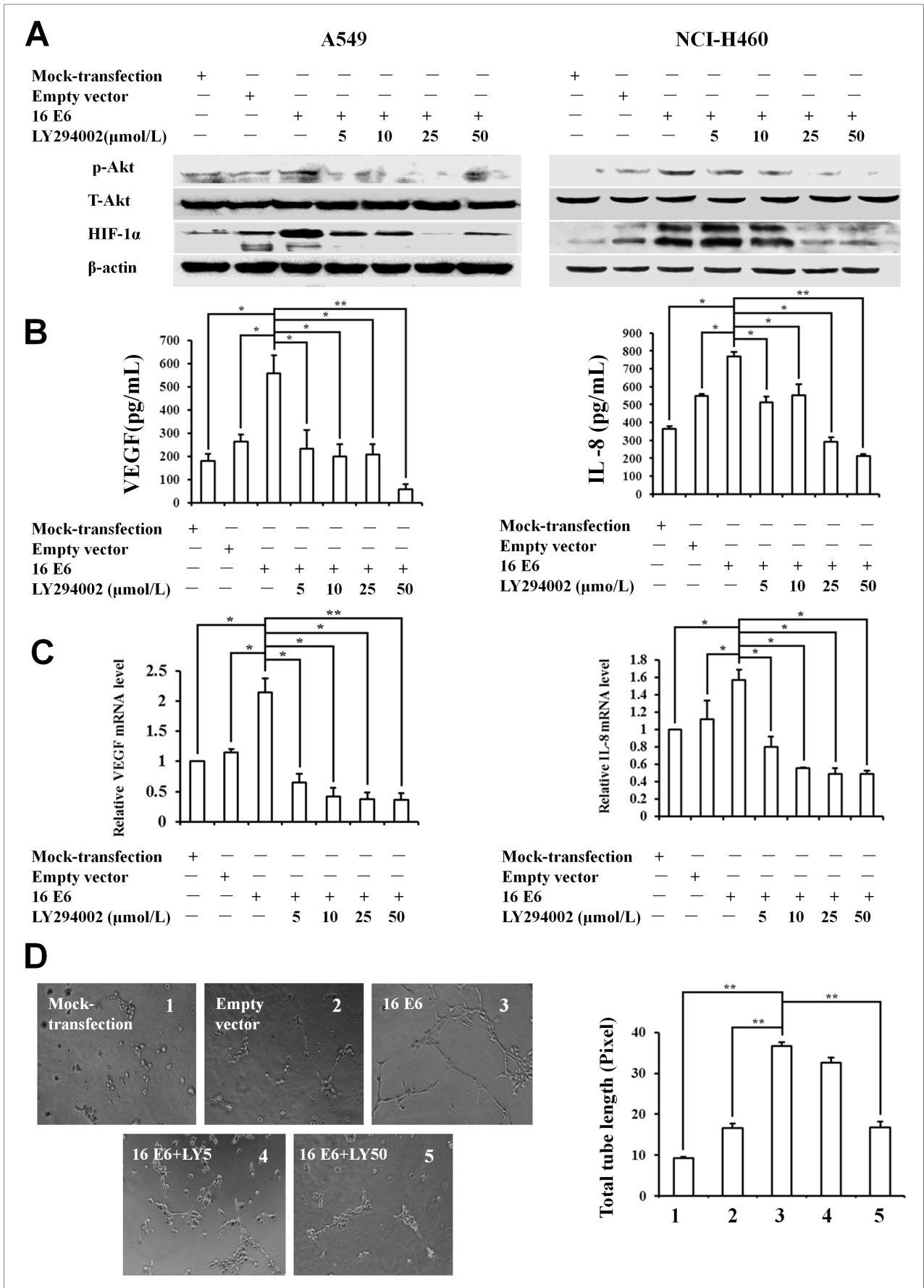


Figure 2. Effect of LY294002 on HPV-16 E6-induced HIF-1 α , VEGF, and IL-8 expression and *in vitro* angiogenesis in NSCLC cells. HPV-16 E6-transfected NSCLC cells were pretreated for 24 h with different concentrations of LY294002. (A) HIF-1 α and p-Akt protein levels in transfected NSCLC cells (Left: A549, Right: NCI-H460) were analyzed by Western blotting. (B) VEGF and IL-8 protein concentration in the conditioned media derived from transfected A549 cells was determined by ELISA. (C) VEGF and IL-8 mRNA levels in transfected A549 cells were determined by real-time PCR. (D) HUVECs (5×10^3 cells/well) were seeded onto the surface of 96-well cell culture plates pre-coated with polymerized ECMatrix and then incubated at 37°C for 6 to 8 h in the conditioned media derived from HPV-16 E6-transfected A549 cells in the absence or presence of LY294002. Left: The tube formation was observed under a phase-contrast microscope (20 \times). Right: The total tube length in three random view-fields per well was by Scion image software measured and average value was calculated. All data are expressed as mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$.

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dation is through interfering with 26S proteasome degradation pathway, A549 and NCI-H460 cells were treated with MG132, a potent and specific 26S proteasome inhibitor. Our results showed that HPV-16 E6 and E7 oncoproteins decreased ubiquitinated HIF-1 α levels in both A549 and NCI-H460 cells (Figure 4C), suggesting that HPV-16 oncoproteins promoted HIF-1 α stability may be *via* blocking 26S proteasome degradation pathway. To deeply study the effect of HPV-16 oncoproteins on ubiquitination, we analyzed the protein expression of von Hippel-Lindau (VHL), an important component of the E3 ubiquitin ligase complex. Our results showed that HPV-16 E7 oncoprotein partly inhibited VHL protein expression as compared with empty vector or HPV-16 E7 mutant, but E6 oncoprotein had no obvious effect on VHL protein expression (Figure 4D).

c-Jun was involved in HPV-16 oncoprotein-induced HIF-1 α , VEGF, and IL-8 expression and *in vitro* angiogenesis

Previous studies have suggested that phosphorylated c-Jun (p-c-Jun) may be associated with HIF-1 α degradation and accumulation [35]. To validate the role of p-c-Jun in HIF-1 α degradation inhibited by HPV-16 oncoproteins in NSCLC cells, we analyzed p-c-Jun and its upstream protein JNK expression using Western blotting. The data confirmed that HPV-16 oncoproteins, especially E7 oncoprotein, enhanced p-c-Jun and p-JNK protein expression in A549 and NCI-H460 cells (Figure 5A), suggesting that JNK/c-Jun signaling pathway could be activated by HPV-16 oncoproteins. To further explore whether JNK/c-Jun signaling pathway is involved in HIF-1 α protein accumulation induced by HPV-16 oncoproteins, two types of NSCLC cell lines were pretreated with SP600125, a specific JNK inhibitor, and HIF-1 α protein expression was detected by Western blotting. The results showed that SP600125 down-regulated p-c-Jun protein levels, but HIF-1 α protein levels had no significant changes when p-c-Jun was inhibited (Figure 5B). We also determined VEGF and IL-8 concentration in the conditional media derived from the cells pretreated with SP600125. We also found that SP600125 had no obvious effect on VEGF and IL-8 protein secretion induced by HPV-16 oncoproteins (Figure 5C). These results suggested that HIF-1 α expression and HIF-1 α -mediated VEGF and IL-8 expression were JNK-independent. To explore whether c-Jun makes a contribution to HPV-16 oncoprotein-induced HIF-1 α , VEGF, and IL-8 expression, HPV-16-transfected NSCLC cells were co-transfected with c-Jun siRNA (Si-1 or Si-2). As shown in Figure 5D, HPV-16 oncoprotein-induced HIF-1 α protein expression was down-regulated when c-Jun was inhibited by c-Jun siRNA. Expectantly, HPV-16 oncoprotein-induced VEGF and IL-8 protein secretion in NSCLC cells was also decreased in response to c-Jun knockdown (Figure 5E). Accordingly, we further found that c-Jun siRNAs (Si-1 and Si-2) dramatically inhibited angiogenesis *in vitro* stimulated by over-expression of HPV-16 E6 and E7 oncoproteins in A549 cells (Figure 6).

HPV-16 oncoproteins promoted HIF-1 α protein stability possibly through enhancing the interaction between c-Jun and HIF-1 α

To analyze the role of c-Jun in the stability of HIF-1 α protein enhanced by HPV-16 oncoproteins, HPV-16-transfected NSCLC cells were co-transfected with c-Jun siRNA (Si-1 or Si-2) or nonspecific (NS)-siRNA, followed by treatment with 10 μ g/mL CHX. Our results showed that the increased HIF-1 α protein stability induced by HPV-16 oncoprotein was abrogated by c-Jun siRNA (Si-1 or Si-2) co-transfection, but not by NS-siRNA co-transfection (Figure 7A, B), indicating that HPV-16 oncoproteins enhanced HIF-1 α protein stability in NSCLC cells was c-Jun-dependent. To further examine whether the role of c-Jun is *via* interfering with 26S proteasome-dependent degradation pathway, HPV-16-transfected NSCLC cells were co-transfected with c-Jun siRNA (Si-1 or Si-2) or NS-siRNA, followed by treatment with MG132. As shown in Figure 7C, the ubiquitination of HIF-1 α protein was increased in HPV-16- and c-Jun siRNA-co-transfected cells as compared with controls. Taken together, these findings suggested that c-Jun may play an important role in the enhancement of HIF-1 α protein stability induced by HPV-16 oncoproteins *via* blocking 26S proteasome-dependent degradation pathway in NSCLC cells.

Previous studies have demonstrated that c-Jun can interact with HIF-1 α *via* oxygen-dependent degradation (ODD) domain, thus preventing HIF-1 α from 26S proteasome-dependent degradation [35]. According to our results and previous studies, we hypothesize that HPV-16 oncoproteins inhibit HIF-1 α protein degradation *via* enhancing the interaction between HIF-1 α and c-Jun. To verify the hypothesis, co-immunoprecipitation was performed to determine the quantity of c-Jun binding to HIF-1 α . As shown in Figure 7D, HPV-16 E6 and E7 oncoproteins obviously increased the quantity of c-Jun-HIF-1 α complex in A549 cells. Furthermore, the results from cell immunofluorescence showed that c-Jun and HIF-1 α proteins were co-localized in the nuclei (Figure 7E). Therefore, our results confirmed the hypothesis.

Discussion

Accumulating evidence has demonstrated that HPV-16 oncoproteins can promote angiogenesis by up-regulating the expression of a variety of pro-angiogenic factors including fibroblast growth factor binding protein, basic fibroblast growth factor, transforming growth factor- β , tumor necrosis factor- α , angiopoietin-1, hepatocyte growth factor, and placental growth factor in cervical cancer cells [33,38–40]. Our previous studies have also found that HPV-16 E6 and E7 oncoproteins enhanced angiogenesis by up-regulating HIF-1 α protein accumulation and HIF-1 α -dependent VEGF and IL-8 expression in NSCLC cells [26], but the underlying mechanisms are not known. An increasing body of evidence has demonstrated that multiple signaling pathways including PI3K/Akt and JNK/c-Jun are involved in the up-regulation of HIF-1 α , VEGF, and IL-8 expression stimulated by different factors [27–30]. In this study, we first demonstrated that

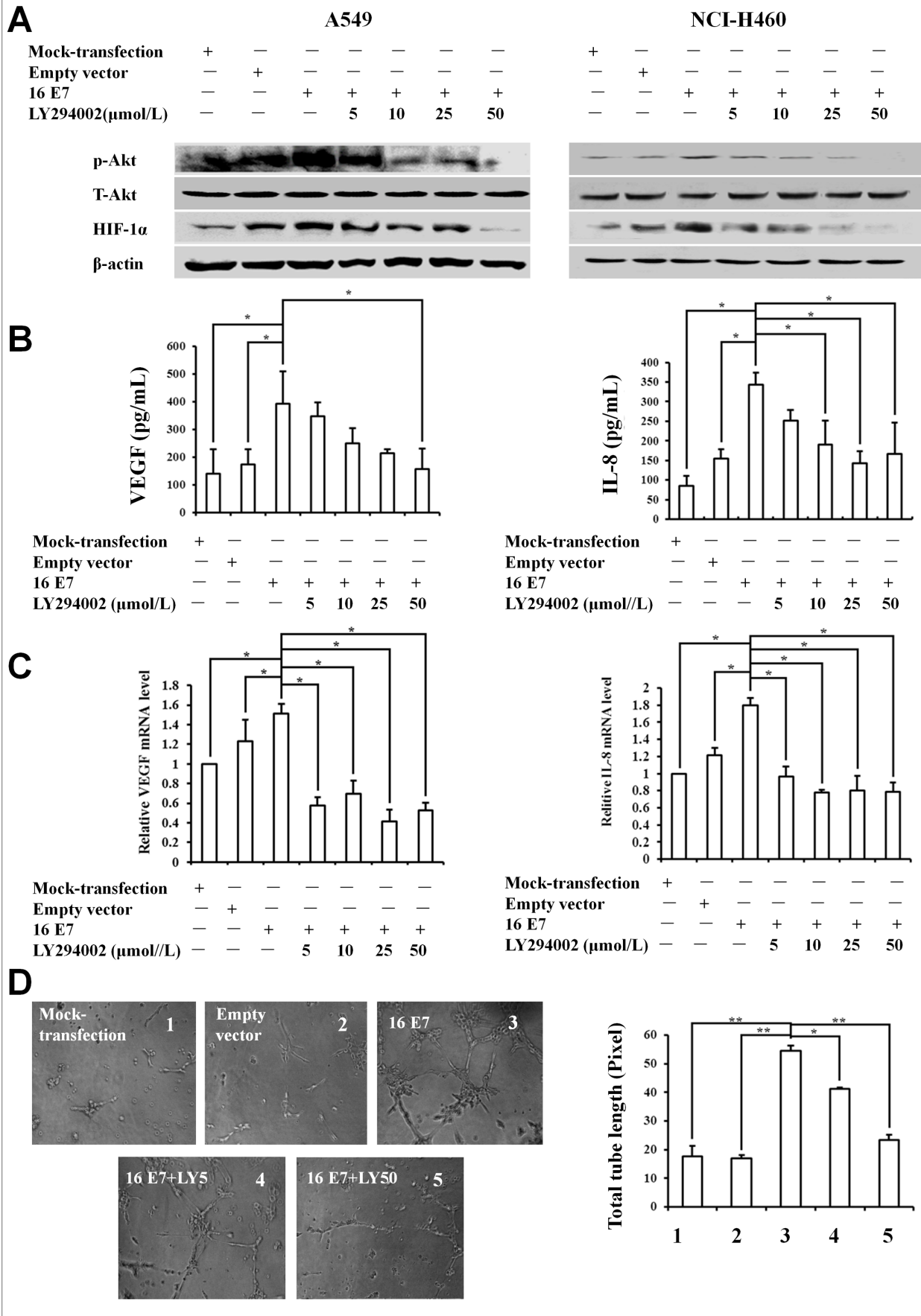


Figure 3. Effect of LY294002 on HPV-16 E7-induced HIF-1 α , VEGF, and IL-8 expression and *in vitro* angiogenesis in NSCLC cells. HPV-16 E7-transfected NSCLC cells were pretreated for 24 h with different concentrations of LY294002. (A) HIF-1 α and p-Akt protein levels in transfected NSCLC cells (Left: A549, Right: NCI-H460) were analyzed by Western blotting. (B) VEGF and IL-8 protein concentration in the conditioned media derived from transfected A549 cells was determined by ELISA. (C) VEGF and IL-8 mRNA levels in transfected A549 cells were determined by real-time PCR. (D) Results *in vitro* angiogenesis (A549 cells). HUVECs (5×10^3 cells/well) were seeded onto the surface of 96-well cell culture plates pre-coated with polymerized ECMatrix and then incubated at 37°C for 6 to 8 h in the conditioned media derived from HPV-16 E7-transfected A549 cells in the absence or presence of LY294002. Left: The tube formation was observed under a phase-contrast microscope (20 \times). Right: The total tube length in 3 random view-fields per well was by Scion image software measured and average value was calculated. All data are expressed as mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$.
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the roles of PI3K/Akt and c-Jun in the expression of HIF-1 α , VEGF, and IL-8 stimulated by HPV-16 oncoproteins in NSCLC cells.

PI3K/Akt signaling pathway is well known to play a key role in regulating angiogenesis in various cancers including NSCLC [31,41,42]. Especially, our previous study has demonstrated that

HPV-16 E6 and E7 induced HIF-1 α protein accumulation and VEGF expression *via* PI3K/Akt signaling pathway in human cervical cancer cells [33]. In the present study, we also detect the effect of HPV-16 E6 and E7 oncoproteins on the activation of PI3K/Akt signaling pathway in NSCLC cells. We found that HPV-16 E6 and E7 oncoproteins activated PI3K/Akt signaling

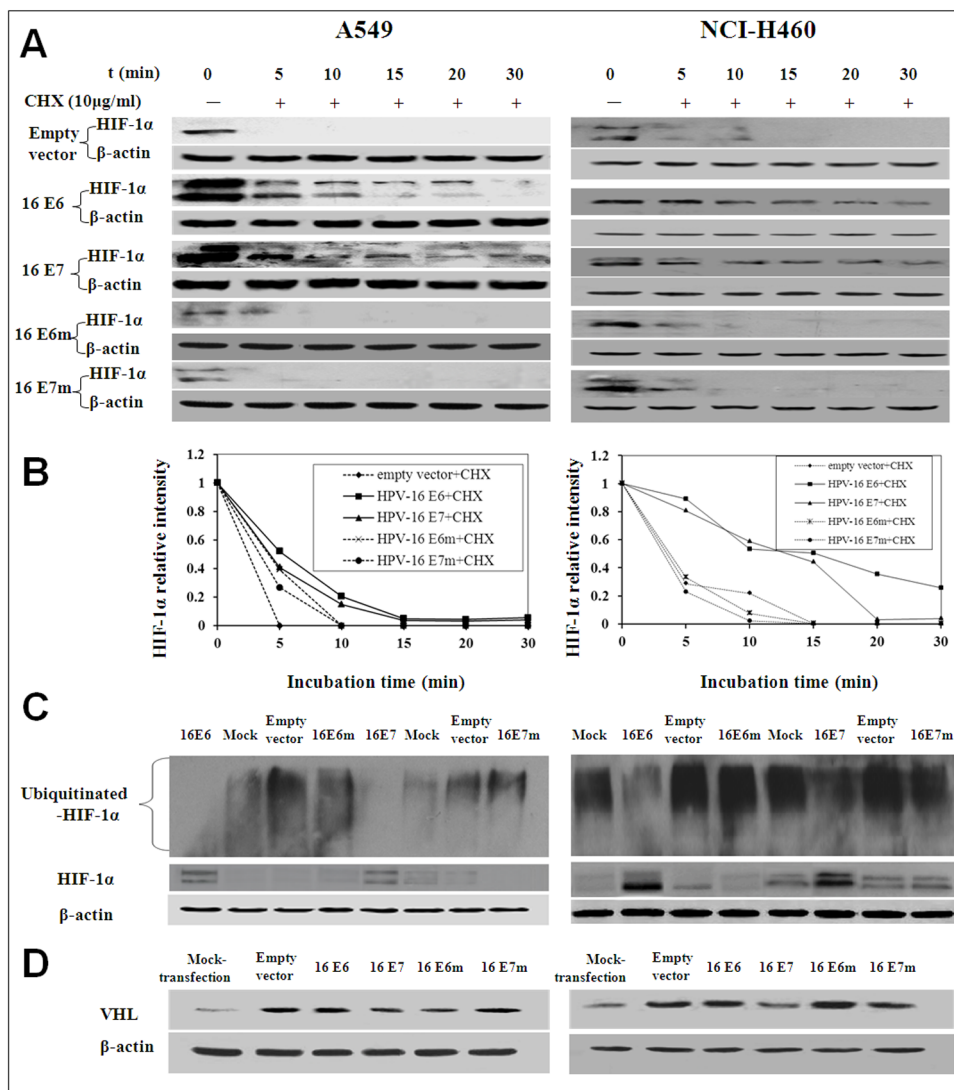


Figure 4. Stability of HIF-1 α protein in HPV-16 E6 or E7-transfected NSCLC cells. (A) HPV-16 E6- or E7-transfected NSCLC cells (Left: A549, Right: NCI-H460) were treated with 10 μ g/mL of cycloheximide (CHX) for different time periods. HIF-1 α protein levels were determined by Western blotting. (B) Quantitative densitometric analysis of results from A. (C) HPV-16 E6- or E7-transfected NSCLC cells (Left: A549, Right: NCI-H460) were treated with 20 μ mol/L of MG-132 for 6 h. Western blotting was performed to determine HIF-1 α protein levels. (D) VHL protein levels in transfected NSCLC cells (Left: A549, Right: NCI-H460) were analyzed by Western blotting. Data presented are representative of results from three independent experiments.
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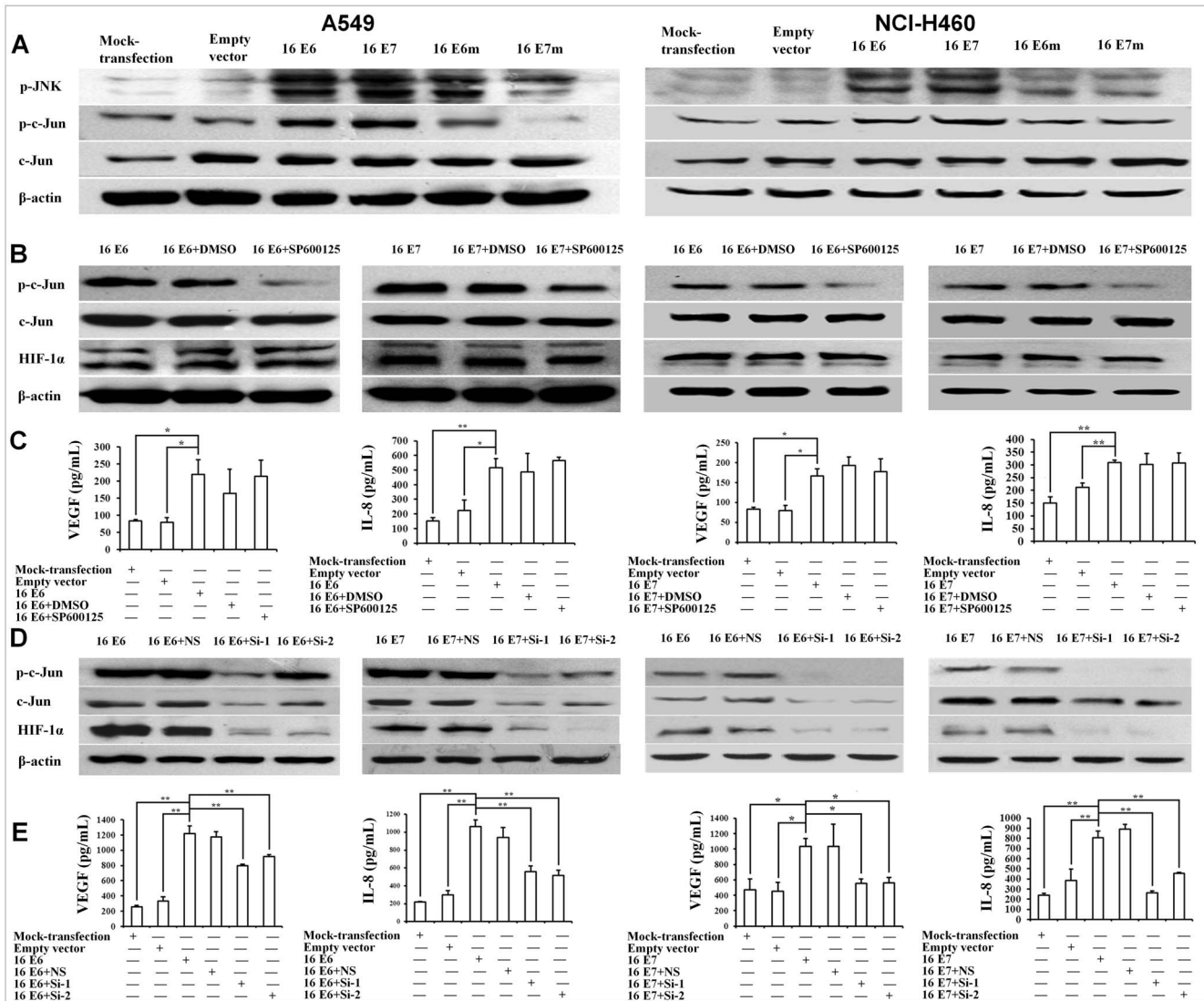


Figure 5. Role of JNK/c-Jun signaling pathway in HIF-1 α , VEGF, and IL-8 expression induced by HPV-16 oncoproteins in NSCLC cells. (A) Western blot analysis of p-JNK, p-c-Jun, and c-Jun protein levels in stable-transfected NSCLC cells (Left: A549, Right: NCI-H460). (B and C) HPV-16 E6- or E7-transfected NSCLC cells (Left: A549, Right: NCI-H460) were pretreated with SP600125. HIF-1 α , p-c-Jun, and c-Jun protein levels were analyzed by Western blotting (B), and VEGF and IL-8 protein concentration in the conditioned media was determined by ELISA (C). (D and E) HPV-16 E6- or E7-transfected NSCLC cells (Left: A549, Right: NCI-H460) were co-transfected with c-Jun siRNA (Si-1 or Si-2) or non-specific siRNA (NS-siRNA). HIF-1 α , p-c-Jun, and c-Jun protein levels were analyzed by Western blotting (D), and VEGF and IL-8 protein concentration in the conditioned media was determined by ELISA (E). All data are expressed as mean \pm SD of three independent experiments. * P <0.05, ** P <0.01. doi:10.1371/journal.pone.0103440.g005

pathway in two types of NSCLC cell lines, A549 and NCI-H460 cells. Moreover, the expression of HIF-1 α , VEGF, and IL-8 and angiogenesis *in vitro* were inhibited by LY294002, a specific PI3K inhibitor. Taken together, our results suggest that PI3K/Akt signaling pathway may be involved in the expression of HIF-1 α , VEGF, and IL-8 induced by HPV-16 E6 and E7 oncoproteins in NSCLC cells, leading to angiogenesis *in vitro*. However, our results from Western blotting showed that the highest dose of LY294002 reversed HIF-1 α protein expression, but VEGF expression was not consistent with the data of western blotting, which could reflect the possibility that PI3K/Akt signaling pathway may be not fully responsible for the expression of HIF-1 α , VEGF, and IL-8.

mTOR, the downstream effector of Akt, assembles into two complexes with distinct inputs and downstream effects: mTOR

complex 1 (mTORC1) and mTORC2. Akt activity can lead to the activation of mTORC1, an important regulator of cellular growth and protein synthesis [42]. Under favorable growth conditions, activated mTOR regulates the phosphorylation of its downstream targets, eIF4E-binding protein 1 (4E-BP1) and S6 kinase (S6K), resulting in protein translation initiation and elongation. Interestingly, in this study, we found that over-expression of HPV-16 E6 and E7 oncoproteins activated mTOR, P70S6K, and P85S6K in NSCLC cells. Therefore, whether mTOR signaling pathway mediated HPV-16 oncoprotein-induced HIF-1 α , VEGF, and IL-8 expression in NSCLC cells is worthy of further investigation.

HIF-1 α , an upstream regulator of VEGF, triggers angiogenesis through VEGF in various types of cancer. HIF-1 α expression is highly regulated by oxygen concentration. Under normoxic conditions, HIF-1 α is hydroxylated in its oxygen-dependent

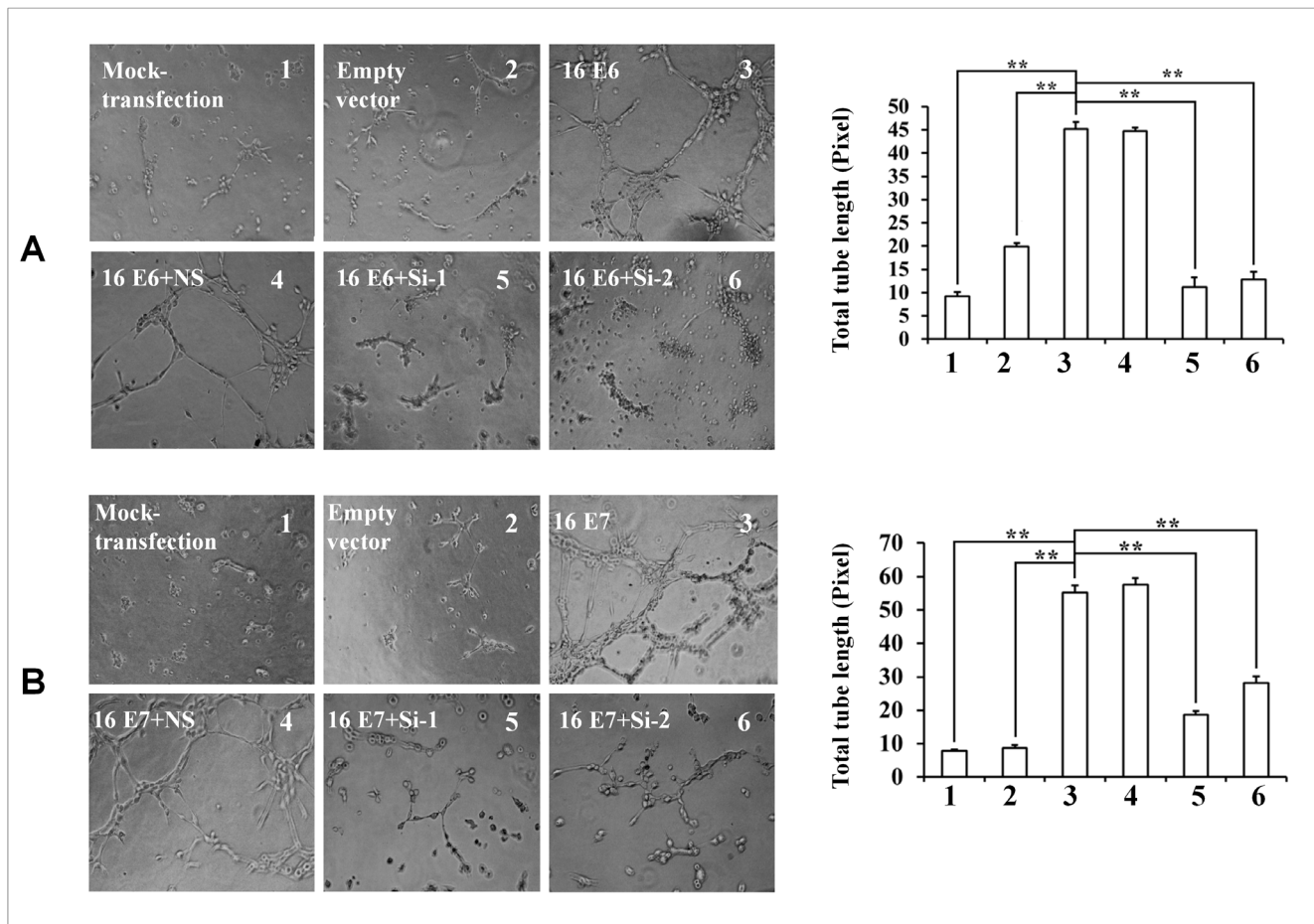


Figure 6. Effect of c-Jun siRNA on angiogenesis *in vitro* stimulated by over-expression of HPV-16 E6 or E7 in A549 cells. HPV-16 E6- (A) or E7- (B) transfected A549 cells were co-transfected with c-Jun siRNA (Si-1 or Si-2). Left: The tube formation was observed under a phase-contrast microscope (20 \times). Right: The total tube length in three random view-fields per well was by Scion image software measured and average value was calculated. All data are expressed as mean \pm SD of three independent experiments. ** $P < 0.01$. doi:10.1371/journal.pone.0103440.g006

degradation domain by propyl hydroxylases (PHD) [43]. Afterwards, hydroxylated HIF-1 α is recognized by a protein complex combining with VHL, leading to poly-ubiquitination and degradation. Under hypoxic conditions, HIF-1 α is unable to be hydroxylated by PHD, allowing it to escape from ubiquitination and degradation. Besides hypoxia, other non-hypoxic factors can also be found to up-regulate HIF-1 α protein levels [28–30,33]. In our previous and present studies, we found that over-expression of HPV-16 E6 and E7 oncoproteins up-regulated HIF-1 α protein expression both in transiently [26] and stably transfected NSCLC cells (Figure 1A). HPV-16 E6 and E7 oncoproteins seem to create the same “protein microenvironment” like hypoxic conditions to up-regulate HIF-1 α protein levels. However, our previous and present results showed that over-expression of HPV-16 E6 and E7 had no effect on HIF-1 α mRNA expression in NSCLC cells [26], indicating that HPV-16 oncoproteins enhanced HIF-1 α protein accumulation *via* a post-transcriptional mechanism, *e.g.* affecting HIF-1 α protein stability. As expected, in this study, our results showed that HPV-16 E6 and E7 oncoproteins significantly suppressed HIF-1 α protein degradation in NSCLC cells (Figure 4A, B). Moreover, we further found that HPV-16 E6 and E7 oncoproteins decreased ubiquitinated HIF-1 α levels in NSCLC cells (Figure 4C), suggesting that HPV-16 oncoproteins inhibited HIF-1 α degradation is possibly, at least in part, through interfering

with 26S proteasome degradation pathway, thus triggering HIF-1 α protein accumulation in NSCLC cells. VHL, the substrate recognition subunit of an E3 ligase, is well known to contribute to HIF-1 α degradation. However, in this study, we found that over-expression of HPV-16 oncoproteins, especially E6, had no obvious effect on VHL protein expression, indicating that HPV-16 E6 oncoprotein inhibited HIF-1 α degradation *via* VHL-independent pathways and the other underlying mechanisms possibly contribute to HIF-1 α protein stability mediated by HPV-16 E6 oncoprotein in NSCLC cells.

c-Jun and its upstream protein JNK have long been considered to be associated with angiogenesis. When c-Jun was suppressed in human endothelial cells, the cells no longer form new blood vessels *in vitro* or *in vivo* [44]. Additionally, c-Jun is an essential for high production of VEGF under hypoxic conditions, and the involvement of c-Jun can enhance VEGF transcription [45,46]. Our results showed that when c-Jun was inhibited by its specific siRNA, the increase of HIF-1 α , VEGF, and IL-8 protein expression (Figure 5D and E) induced by HPV-16 oncoproteins in NSCLC cells was also blocked (Figure 5D and E), suggesting that HIF-1 α , VEGF, and IL-8 protein expression induced by HPV-16 oncoproteins was c-Jun-dependent. Furthermore, we also found the knockdown of c-Jun remarkably inhibited HPV-16 E6 and E7 oncoprotein-stimulated angiogenesis *in vitro* in A549 cells (Fig-

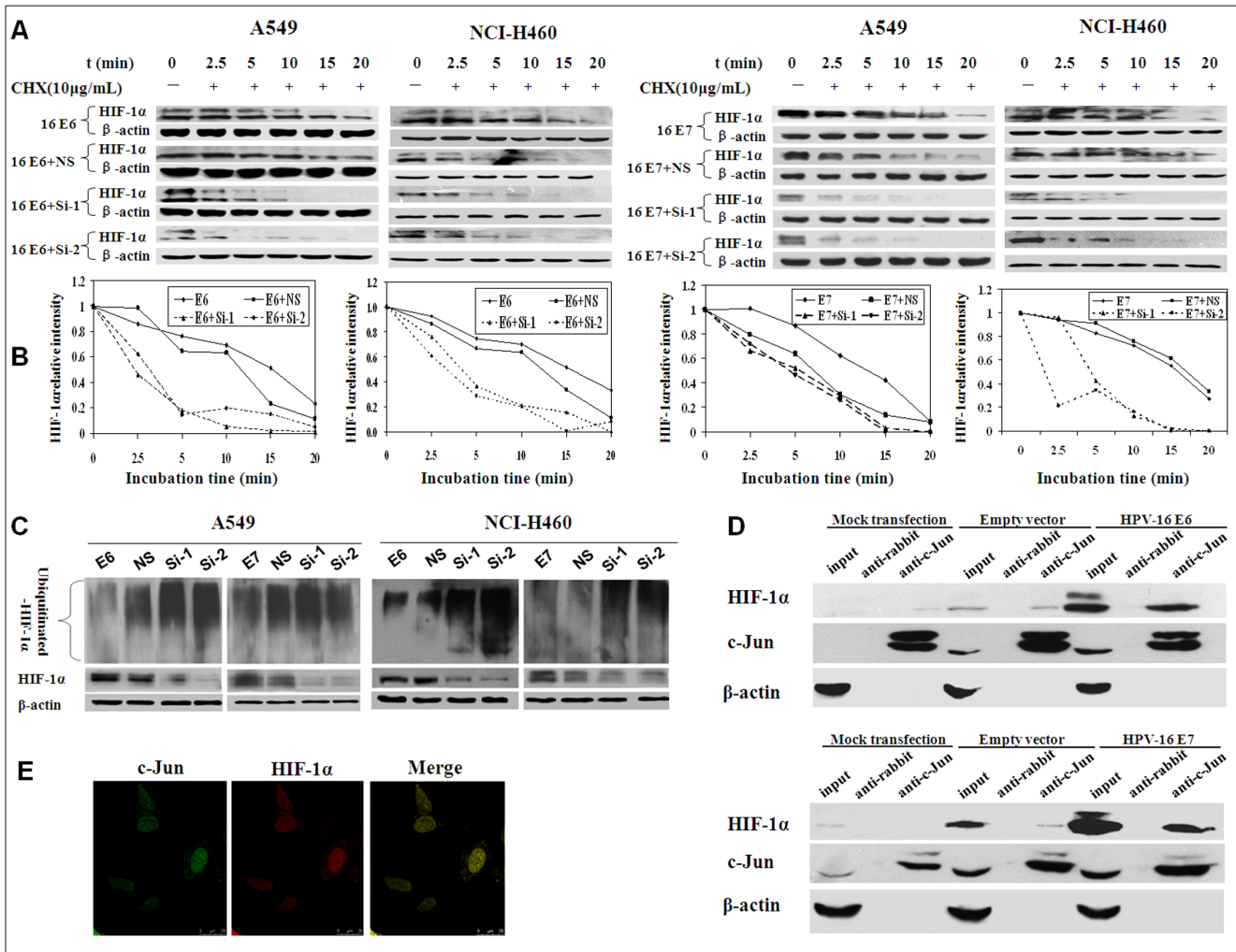


Figure 7. HPV-16 oncoproteins promoted HIF-1 α protein stability through enhancing the interaction between c-Jun and HIF-1 α . (A) HPV-16 E6-(Left) or E7-(Right) transfected A549 and NCI-H460 cells were co-transfected with c-Jun siRNA (Si-1 or Si-2) or NS-siRNA, followed by treatment with 10 μ g/mL CHX for different time periods. HIF-1 α protein levels were determined by Western blotting. (B) Quantitative densitometric analysis of results from A. (C) HPV-16 E6- or E7-transfected A549 (Left) and NCI-H460 cells (Right) were co-transfected with c-Jun siRNA (Si-1 or Si-2) or NS-siRNA, followed by treatment with 20 μ mol/L of MG-132 for 6 h. Western blotting was performed to determine HIF-1 α protein levels and ubiquitination. (D) Co-immunoprecipitation results of HIF-1 α and c-Jun in A549 cells. Upper: HPV-16 E6; Lower: HPV-16 E7. (E) Cell immunofluorescence results in A549 cells (40 \times). The green area showed the position of c-Jun expression in A549 cells. The red area showed the position of HIF-1 α expression in A549 cells. The yellow area showed the position of HIF-1 α and c-Jun co-expression. Data presented are representative of results from three independent experiments. doi:10.1371/journal.pone.0103440.g007

ure 6). These data indicated that c-Jun-mediated pathway may be, at least in part, involved in HPV-16 oncoprotein-induced HIF-1 α , VEGF, and IL-8 expression in NSCLC cells, thus leading to angiogenesis *in vitro*.

Generally, the phosphorylation of c-Jun at serine^{63/73} activates c-Jun-dependent transcription. Moreover, previous reports have demonstrated that phosphorylated c-Jun (p-c-Jun) binds to the VEGF promoter and regulates VEGF transcription directly [47]. Our results showed that the levels of p-c-Jun and its upstream p-JNK protein in NSCLC cells were up-regulated by HPV-16 oncoproteins, especially E7 (Figure 5A). However, the decrease of p-c-Jun levels by SP600125, a specific JNK inhibitor, had no obvious effects on HIF-1 α protein expression in HPV-16-transfected NSCLC cells (Figure 5B and C), suggesting HIF-1 α protein accumulation induced by HPV-16 oncoproteins was JNK/c-Jun-independent in NSCLC cells. To explore whether c-Jun can

mediate HPV-16 oncoprotein-induced HIF-1 α protein accumulation in NSCLC cells *via* other pathways, the effect of c-Jun on HIF-1 α protein stability was analyzed. Our results showed that c-Jun enhanced HIF-1 α protein stability by inhibiting its degradation through 26S proteasome-dependent ubiquitination pathway (Figure 7A–C). Regularly, the reduction of VHL protein levels can decrease HIF-1 α ubiquitination. However, our results showed that VHL protein levels had no significant changes in HPV-16 E6-transfected cells. Previous studies have demonstrated that c-Jun can interact with HIF-1 α through ODD domain, thus preventing HIF-1 α from 26S proteasome-dependent degradation [35]. Therefore, according to previous reports and our results, it can be hypothesized that HPV-16 oncoproteins may create a sort of “protein microenvironment” that can promote the combination between c-Jun and HIF-1 α proteins and block the combination between VHL and HIF-1 α ODD domain, leading to the

inhibition of HIF-1 α protein degradation. Interestingly, our results from co-immunoprecipitation and cell immunofluorescence verified this hypothesis. Our results showed that HPV-16 E6 and E7 oncoproteins obviously increased the quantity of c-Jun-HIF-1 α complex in A549 cells (Figure 7D). Furthermore, c-Jun and HIF-1 α proteins were co-localized in the nuclei (Figure 7E). Taken together, our findings suggest that HPV-16 E6 and E7 oncoproteins may inhibit HIF-1 α protein degradation *via* enhancing the interaction between HIF-1 α and c-Jun, thus contributing to HIF-1 α -mediated angiogenesis in NSCLC.

In this study, we did not make further research on the relationships between PI3K/Akt and c-Jun signaling pathways. Different signaling pathways have a complex cross-talk network in cells. It may be a coordination between PI3K/Akt signaling pathway and c-Jun, along with other signaling pathways, thus making a contribution to HPV-16 oncoprotein-induced angiogenesis in NSCLC, which needs to be further studied.

Conclusions

In this study, we demonstrated to our knowledge for the first time that PI3K/Akt signaling pathway and c-Jun are involved in

HPV-16 oncoprotein-induced HIF-1 α protein accumulation and VEGF and IL-8 expression in NSCLC cells. Moreover, HPV-16 oncoproteins promoted HIF-1 α protein stabilization possibly by enhancing the interaction between c-Jun and HIF-1 α , thus contributing to angiogenesis.

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Author Contributions

Conceived and designed the experiments: XDT. Performed the experiments: EYZ XWF FL PHZ JL XDT. Analyzed the data: EYZ XWF XDT. Contributed reagents/materials/analysis tools: PHZ JL. Contributed to the writing of the manuscript: EYZ XWF XDT.

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