

# (–)-Epigallocatechin-3-gallate inhibits human papillomavirus (HPV)-16 oncoprotein-induced angiogenesis in non-small cell lung cancer cells by targeting HIF-1 $\alpha$

Li He · Erying Zhang · Jingli Shi · Xiangyong Li ·  
Keyuan Zhou · Qunzhou Zhang · Anh D. Le ·  
Xudong Tang

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## Abstract

**Purpose** To investigate the effects of (–)-epigallocatechin-3-gallate (EGCG) on human papillomavirus (HPV)-16 oncoprotein-induced angiogenesis in non-small cell lung cancer (NSCLC) cells and the underlying mechanisms.

**Methods** NSCLC cells (A549 and NCI-H460) transfected with *EGFP* plasmids containing HPV-16 *E6* or *E7* oncogene were treated with different concentrations of EGCG for 16 h. The effects of EGCG on angiogenesis in vitro and in vivo were observed. The expression of HIF-1 $\alpha$ , p-Akt, and p-ERK1/2 proteins in NSCLC cells was analyzed by Western blot. The levels of HIF-1 $\alpha$  mRNA in NSCLC cells were detected by real-time RT-PCR. The concentration of VEGF and IL-8 in the conditioned media was determined by ELISA. HIF-1 $\alpha$ , VEGF, and CD31 expression in A549 xenografted tumors of nude mice was analyzed by immunohistochemistry.

**Results** HPV-16 *E6* and *E7* oncoproteins HIF-1 $\alpha$ -dependently promoted angiogenesis in vitro and in vivo, which

was inhibited by EGCG. Mechanistically, EGCG inhibited HPV-16 oncoprotein-induced HIF-1 $\alpha$  protein expression but had no effect on HIF-1 $\alpha$  mRNA expression in NSCLC cells. Additionally, 50 and 100  $\mu\text{mol/L}$  of EGCG significantly reduced the secretion of VEGF and IL-8 proteins induced by HPV-16 *E7* oncoprotein in NSCLC A549 cells. Meanwhile, HPV-16 *E6* and *E7* oncoproteins HIF-1 $\alpha$ -dependently enhanced Akt activation in A549 cells, which was suppressed by EGCG. Furthermore, EGCG inhibited HPV-16 oncoprotein-induced HIF-1 $\alpha$  and HIF-1 $\alpha$ -dependent VEGF and CD31 expression in A549 xenografted tumors.

**Conclusions** EGCG inhibited HPV-16 oncoprotein-induced angiogenesis conferred by NSCLC through the inhibition of HIF-1 $\alpha$  protein expression and HIF-1 $\alpha$ -dependent expression of VEGF, IL-8, and CD31 as well as activation of Akt, suggesting that HIF-1 $\alpha$  may be a potential target of EGCG against HPV-related NSCLC angiogenesis.

Li He and Erying Zhang contributed equally to this work.

L. He · E. Zhang · J. Shi · X. Li · K. Zhou · Q. Zhang ·  
X. Tang (✉)

Institute of Biochemistry and Molecular Biology,  
Guangdong Medical College, 2 Wenming Donglu, Xiashan,  
Zhanjiang 524023, Guangdong, People's Republic of China  
e-mail: txd2599@yahoo.com.cn; tangxudong2599@126.com

X. Li · K. Zhou · X. Tang  
Guangdong Key Laboratory of Medical Molecular Diagnosis,  
Guangdong Medical College, Zhanjiang 524023,  
People's Republic of China

Q. Zhang · A. D. Le  
Department of Oral Surgery and Pharmacology,  
University of Pennsylvania School of Dental Medicine,  
Philadelphia, PA 19104, USA

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## Introduction

Lung cancer, a leading cause of cancer-related mortality in the world, is classified into small cell lung cancer (SCLC, 15–18 % of incident cases) and non-small cell lung cancer (NSCLC, 82–85 % of incident cases) according to histopathology and differences in prognosis and treatment [1]. Cigarette smoking has been considered to be the most important risk factor for lung cancer, but a growing body of evidence has supported that non-smoking-related factors

may also contribute to the development of lung cancer among never-smokers [2, 3]. Recently, accumulating evidence has shown that the infection of human papillomavirus (HPV) may be related to non-smoking-associated lung cancer.

HPVs, a group of small non-enveloped DNA viruses, are divided into high- and low-risk types. Accumulating epidemiological evidence around world has shown that the positive rates of high-risk HPV-16/18 DNA and E6 and E7 oncoproteins in NSCLC are significantly higher than those in benign lung tumors [4–10], wherein HPV-16 is the most prevalent HPV genotype with frequent E6/E7 oncogene expression [4, 6, 9]. Most recently, we have demonstrated that the overexpression of HPV-16 E6 and E7 oncoproteins significantly promotes angiogenesis in NSCLC both in vitro and in vivo through the induction of hypoxia-inducible factor (HIF)-1 $\alpha$  protein accumulation and HIF-1 $\alpha$ -dependent vascular endothelial growth factor (VEGF) and interleukin (IL)-8 expression [11], suggesting that HIF-1 $\alpha$  may be developed as a potential molecular target in the treatment for HPV-related NSCLC.

HIF-1 $\alpha$  is highly regulated by oxygen concentration. Under normoxic conditions, HIF-1 $\alpha$  is hydroxylated at the proline residues Pro<sup>402</sup> and Pro<sup>564</sup> in the oxygen-dependent domain (ODD). The hydroxylated HIF-1 $\alpha$  interacts with the von Hippel–Lindau gene product (pVHL), triggering the ubiquitination and subsequent degradation via the 26S proteasome system. Under hypoxic conditions, due to the inhibition of hydroxylation, HIF-1 $\alpha$  accumulates rapidly. HIF-1 $\alpha$  has been found to overexpress in various tumors including lung cancer and its metastases, which is related to a more aggressive tumor phenotype and radiation-resistant lung cancer cells [12–14]. HIF-1 $\alpha$  also plays an important role in lung cancer tumorigenesis, metastasis, drug-resistance [15], prognosis [12, 16], and angiogenesis [17]. Recently, Liu et al. [15] have found that HIF-1 $\alpha$  contributes to cisplatin resistance in lung cancer by regulating the expression of XPA. Kuo et al. [16] have reported that NSCLC patients carrying a HIF-1 $\alpha$ -1772 T/T genotype or a HIF-1 $\alpha$ -1790 A/A have a tendency toward inferior prognosis compared with other patients. Moreover, HIF-1 $\alpha$  plays a crucial role in angiogenesis of NSCLC [17]. Therefore, these findings further demonstrate that HIF-1 $\alpha$  may be a novel therapeutic target of NSCLC.

Green tea is the most widely consumed beverage worldwide. The water extractable fraction of green tea contains various polyphenolic compounds known as catechins. Among these catechins, (-) epigallocatechin-3-gallate (EGCG) has been found to be the most effective component of anti-cancer [18]. A growing body of evidence has demonstrated that EGCG can inhibit growth [19, 20], induce apoptosis [21, 22], and suppress invasion [23]

in lung cancer cells. In recent years, EGCG has also been found to inhibit tumor angiogenesis in vitro and in vivo [24–27].

Accumulating evidence has demonstrated that EGCG exerts its anti-angiogenic effects through the intervention of VEGF/VEGFR axis [24, 25], Stat3 activity [26], VE-cadherin phosphorylation [27], and Akt [25, 28] and extracellular-signal-regulated kinase (ERK) activation [25]. Our previous studies further found that EGCG had significant inhibitory effect on hypoxia- and serum-induced HIF-1 $\alpha$  protein accumulation and VEGF expression in human cervical carcinoma (HeLa) and hepatoma (HepG2) cells [29]. Moreover, EGCG inhibited hypoxia-induced HIF-1 $\alpha$  protein accumulation in HeLa cells via blocking the activation of PI3K/Akt and ERK1/2 signaling pathways and substantially promoting HIF-1 $\alpha$  protein degradation through the proteasome system [29]. However, opposite reports showed that EGCG inhibited HIF-1 $\alpha$  degradation [30–32]. These different reports indicate that the effect of EGCG on HIF-1 $\alpha$  is not completely clear. Moreover, the effect of EGCG on HPV-16 oncoprotein-induced NSCLC angiogenesis has not been reported.

In this study, we have demonstrated, to our knowledge for the first time, that EGCG inhibited HPV-16 oncoprotein-induced NSCLC angiogenesis through suppressing HIF-1 $\alpha$  protein expression and HIF-1 $\alpha$ -dependent expression of VEGF, IL-8, and CD31 as well as activation of Akt. Therefore, EGCG can inhibit HPV-16 oncoprotein-induced NSCLC angiogenesis by targeting HIF-1 $\alpha$ -mediated pathways.

## Materials and methods

### Drug and reagents

EGCG was purchased from Sigma (St. Louis, MO, USA) and dissolved at a concentration of 100 mmol/L in distilled water and stored at  $-80^{\circ}\text{C}$  as a stock solution. Complete protease inhibitor cocktail was from Roche (Mannheim, Germany). Transfection reagent (Lipofectamine<sup>TM</sup> 2000) was obtained from Invitrogen Corporation (Carlsbad, CA). In vitro angiogenesis assay kit (ECM625) was obtained from Millipore (Temecula, CA, USA). BD Matrigel<sup>TM</sup> Basement Membrane Matrix (containing high protein concentration; 18–22 mg/mL) was obtained from BD Biosciences (Bedford, MA, USA). HiCN hemoglobin detection kit was from Shanghai Rongsheng Biotech Co., Ltd. (Shanghai, China). Mouse anti-human HIF-1 $\alpha$  monoclonal antibody was from BD Transduction Laboratories (San Diego, CA, USA). Total and phosphorylated ERK1/2 (Thr202/Tyr204) or Akt (Ser473) antibodies were

purchased from Cell Signaling Technology (USA). Rabbit anti-human VEGF and CD31 polyclonal primary antibodies were purchased from Beijing Biosynthesis Biotechnology Co., Ltd (Beijing, China). Antibody for  $\beta$ -actin was from Beyotime Biotechnology Corporation, Shanghai (Shanghai, China). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Cell Signaling Technology (USA). One Step SYBR<sup>®</sup> PrimeScript<sup>®</sup> RT-PCR (No. DRR086A) was purchased from TaKaRa Biotechnology (Dalian) Co., LTD (Dalian, China). Human VEGF and IL-8 ELISA reagent kits were 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 (HUVEC) 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 medium supplemented with 10 % FBS, penicillin (100 U/mL), and streptomycin (100  $\mu$ g/mL) (Invitrogen). All cultures were maintained at 37 °C in a humidified atmosphere with 5 % CO<sub>2</sub>.

#### Transient transfection and EGCG treatment

A series of plasmids including pEGFP-HPV-16 E6, E7, E6 mutant (E6-mut), and E7 mutant (E7-mut) were constructed by ourselves [11]. A549 and NCI-H460 cells at 70–80 % confluence were transiently transfected for 4 h with pEGFP-N1-HPV-16 E6 or E7 plasmids using Lipofectamine<sup>™</sup> 2000. The transfected cells and the conditioned media were harvested for further analysis, 24 h post-transfection. Transfection with empty vector or HPV-16E6 or 16E7 mutants served as controls. Cells exposed to Lipofectamine<sup>™</sup> 2000 or Oligofectamine<sup>™</sup> alone served as mock transfection controls. The transfection efficiency was evaluated by observing green fluorescence under a fluorescence microscope and flow cytometric analysis (Epics-XL, Coulter, USA). The expression of HPV-16 E6 and E7 oncoproteins in transfected cells was confirmed [11]. To observe the effect of EGCG on HPV oncoprotein-induced HIF-1 $\alpha$ , VEGF, and IL-8 expression, transfected cells were exposed to different concentrations of EGCG for 16 h. HIF-1 $\alpha$  protein expression in transfected cells was detected by Western blot analysis. VEGF and IL-8 concentration in the conditional media was determined by ELISA. HIF-1 $\alpha$  mRNA levels were analyzed by real-time RT-PCR.

#### shRNA plasmid transfection assay

SureSilencing<sup>™</sup> shRNA plasmid specific for human HIF-1 $\alpha$  (KH01361N for the Neomycin resistance marker) was from SuperArray Bioscience Corporation (Frederick, USA). The insert sequence is 5'-GGCCACATTCACGTA TATGAT-3'. The plasmid for non-specific target (SuperArray Bioscience Corporation, Frederick, USA) was used as controls. The transfection was performed using Lipofectamine<sup>™</sup> 2000 reagent according to the manufacturer's instructions (Invitrogen).

#### Western blot analysis

Transfected and non-transfected lung cancer cells in the presence or absence of EGCG were lysed with cell lysis buffer containing 20 mmol/L Tris (pH7.5), 150 mmol/L NaCl, 1 % Triton X-100, sodium pyrophosphate,  $\beta$ -glycerophosphate, EDTA, Na<sub>3</sub>VO<sub>4</sub>, leupeptin phenylmethylsulfonylfluoride (PMSF), and complete protease inhibitor cocktail (Beyotime Biotechnology Corporation, No. P0013), 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. 50–100  $\mu$ g of protein was separated on 10 % polyacrylamide-SDS gel and electro-blotted onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia, Piscataway, NJ). After blocking with 5 % fat-free milk, the membrane was incubated overnight at 4 °C with primary antibody against HIF-1 $\alpha$  (mouse anti-human antibodies), total-Akt (t-Akt), phosphorylated Akt (p-Akt), total-ERK1/2 (t-ERK1/2), or phosphorylated-ERK1/2 (p-ERK1/2), followed by incubation with HRP-conjugated secondary antibodies (1:1,000). As a loading control, the blots were stripped and re-probed with anti- $\beta$ -actin antibody (1:1,000).

#### Real-time RT-PCR analysis

Total RNA was isolated from cells using TRIZOL<sup>®</sup> Reagent (Invitrogen). Real-time RT-PCR analysis of HIF-1 $\alpha$  mRNA levels was performed using One Step SYBR<sup>®</sup> PrimeScript<sup>®</sup> RT-PCR (TaKaRa, China) according to the manufacturer's instructions. The following primers were designed for real-time RT-PCR: for HIF-1 $\alpha$ , forward 5'-TC TGGGTTGAAACTCAAGCAACTG-3' and reverse 5'-CA ACCGGT TTAAGGACACATTCTG-3'.  $\beta$ -Actin: forward 5'-TGGCACCCAGCACAATGAA-3' and reverse 5'-CTA AGTCATAGTCCGCCTAGAAGCA-3'. All the primers were synthesized by TaKaRa Biotechnology (Dalian, China) 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 31 s. The size of the PCR product of HIF-1 $\alpha$  and  $\beta$ -actin was

150 and 186 bp, respectively. The relative HIF-1 $\alpha$  mRNA levels were normalized to  $\beta$ -actin. The experiment was repeated in triplicate.

#### Enzyme-linked immunosorbent assay (ELISA)

The concentration of VEGF and IL-8 protein in the conditioned media derived from EGCG-treated or EGCG-untreated cells was determined using human VEGF and IL-8 ELISA reagent kits according to the manufacturer's instructions. Results were normalized to the cell number ( $2 \times 10^5$  cells) in 1 mL of culture medium ( $2 \times 10^5$  cells/mL). The experiments were repeated in triplicate.

#### In vitro angiogenesis assay

An in vitro angiogenesis assay kit was employed according to the manufacturer's instructions. Briefly, HUVECs ( $5 \times 10^3$  cells/well) were seeded onto the surface of 96-well cell culture plates pre-coated with polymerized ECMatrix<sup>TM</sup> and then incubated at 37 °C for 6–8 h in the conditioned media derived from transfected cells in the presence or absence of 100  $\mu$ mol/L of EGCG. The tubule formation was observed under a phase-contrast microscope. The total tube length in three random view-fields per well was measured by Scion Image software, and average value was calculated. The experiment was repeated in triplicate.

#### In vivo angiogenesis assay

Six- to eight-week-old male nude mice (BALB/C nu/nu) were from Animal Center of Guangdong Medical College. All experiments with animals were undertaken in accordance with the institution guidelines of the Institutional Animal Care and Use Committee of Guangdong Medical College. Transfected A549 cells in the presence or absence of 100  $\mu$ mol/L of EGCG were re-suspended in serum-free media at  $8.0 \times 10^6$  cells/mL, and 0.25 mL ( $2.0 \times 10^6$  cells in total) of cell suspension was mixed with the same volume of BD Matrigel Matrix (0.25 mL). Then, the BD Matrigel mixture was subcutaneously injected into both flanks of nude mice ( $n = 5$  each group). On day 11, the mice were euthanized and Matrigel plugs were harvested, with half of each Matrigel plug fixed with 10 % neutral formalin for immunohistochemical studies, and the other half weighed for the determination of hemoglobin content as described previously [33].

#### Immunohistochemistry

The expression of HIF-1 $\alpha$ , VEGF, and CD31 proteins in Matrigel plugs was analyzed by immunohistochemistry. Briefly, formalin-fixed Matrigel plugs were paraffin-

embedded and sectioned at a thickness of 4  $\mu$ m. All sections were then deparaffinized in xylene, rehydrated through serial dilutions of alcohol, and washed with PBS (pH 7.2). The sections were heated in a microwave oven twice for 5 min in citrate buffer (pH 6.0) and then incubated overnight at 4 °C with primary antibodies, followed by incubation with streptavidin/HRP-conjugated secondary antibodies. The conventional streptavidin/peroxidase method (Histostain<sup>TM</sup>-Plus Kits, SP0023, Beijing Biosynthesis Biotechnology Co., LTD) was used to develop signals, and the cells were counterstained with hematoxylin. The sections incubated with secondary antibodies in the absence of primary antibodies served as negative controls. Cells stained with brown color represent positive immunoreactivity signals, whereby signals for HIF-1 $\alpha$  and VEGF protein expression were interpreted in the nucleus and cytoplasm, respectively.

#### Statistical analysis

Data are presented as the mean  $\pm$  SD for three separate experiments. One-way ANOVA and Bonferroni were employed for statistical analysis using SPSS 19.0 for windows software.  $P < 0.05$  was considered to be statistically significant.

## Results

#### EGCG inhibited HPV-16 oncoprotein-induced HIF-1 $\alpha$ protein expression in A549 and NCI-H460 cells

Our previous studies have found that HPV-16 E6 and E7 oncoproteins enhanced HIF-1 $\alpha$  protein expression in NSCLC cells [11]. In this study, we further investigated the effect of EGCG on HPV-16 oncoprotein-induced HIF-1 $\alpha$  expression in NSCLC cells, A549 and NCI-H460. Our results showed that EGCG significantly inhibited HPV-16 E6 oncoprotein-induced HIF-1 $\alpha$  expression in both A549 and NCI-H460 cells (Fig. 1a, b). Moreover, EGCG treatment had a similar inhibitory effect on HPV-16 E7 oncoprotein-induced HIF-1 $\alpha$  expression (Fig. 1c, d). To rule out the possibility that the inhibitory effect of EGCG on HIF-1 $\alpha$  protein expression was due to its cellular toxicity, cell viability was determined using MTT assay. No apparent changes in cell viability were observed in transfected A549 (Fig. 1e) and NCI-H460 cells (Fig. 1f) following treatment with various concentrations of EGCG for 16 h. These results indicated that the inhibition of HIF-1 $\alpha$  protein expression by EGCG was not related to cellular toxicity.

To study whether the suppression of HPV-16 oncoprotein-induced HIF-1 $\alpha$  protein expression by EGCG was the result of transcriptional inhibition, HIF-1 $\alpha$  levels were

determined by real-time RT-PCR. No obvious changes in HIF-1 $\alpha$  mRNA levels were observed in HPV-16 E6- or E7-transfected A549 and NCI-H460 cells, and EGCG had no obvious effects on HIF-1 $\alpha$  mRNA expression in HPV-16 E6- or E7-transfected A549 (Fig. 2a, b) and NCI-H460 cells (Fig. 2c, d). These results suggested that EGCG inhibited HPV-16 oncoprotein-induced HIF-1 $\alpha$  protein expression through a posttranscriptional mechanism.

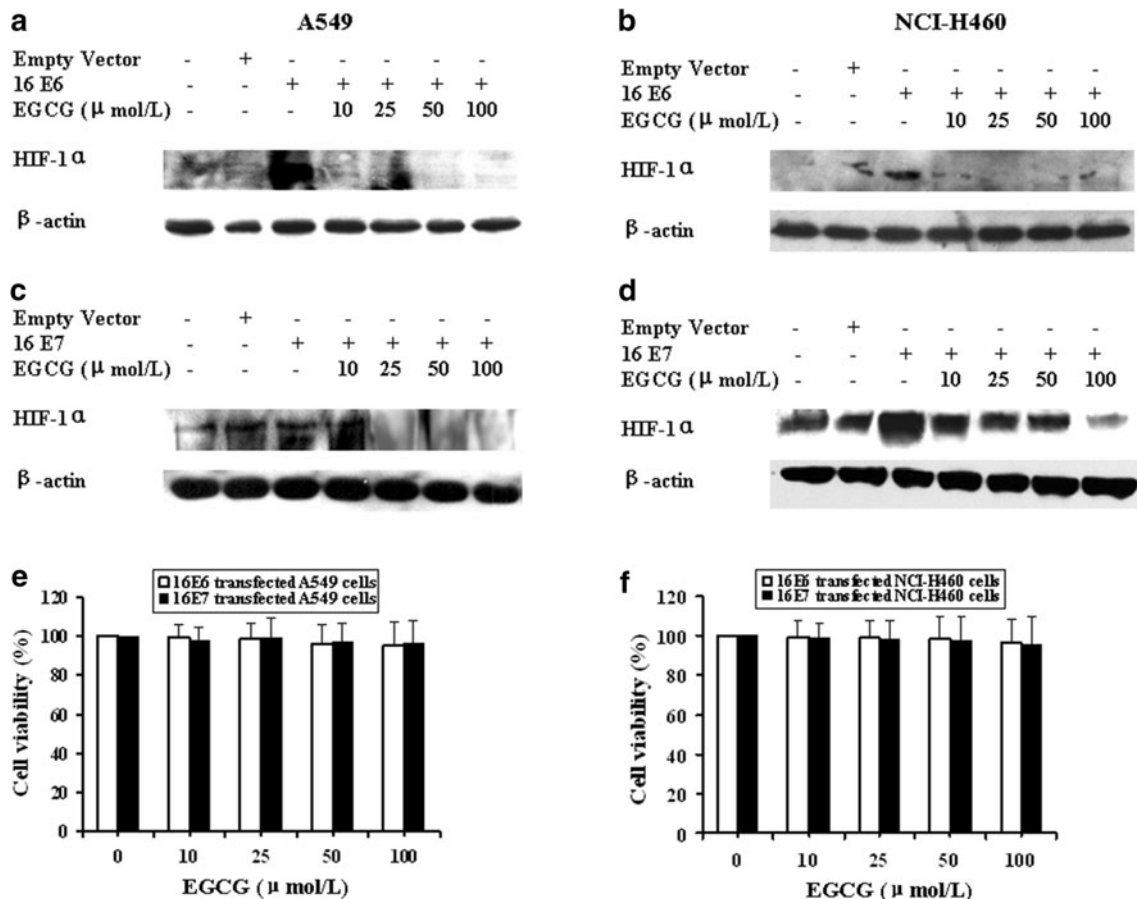
EGCG inhibited HPV-16 E7 oncoprotein-induced VEGF and IL-8 protein secretion in A549 cells

VEGF and IL-8 are important pro-angiogenic factors. Our previous studies have demonstrated that HPV-16 oncoproteins enhanced VEGF and IL-8 protein secretion in a HIF-1 $\alpha$ -dependent manner in A549 cells [11]. Herein, we further investigated the effect of EGCG on HPV-16 oncoprotein-induced VEGF and IL-8 protein secretion by ELISA. Our results showed that EGCG reduced HPV-16

E7 oncoprotein-induced VEGF (Fig. 3a) and IL-8 (Fig. 3b) protein secretion in A549 cells in a dose-dependent manner, while significant inhibitory effects were noticed for EGCG at 50 and 100  $\mu$ mol/L, respectively ( $P < 0.01$ ).

EGCG inhibited HIF-1 $\alpha$ -dependent activation of Akt mediated by HPV-16 oncoproteins in A549 cells

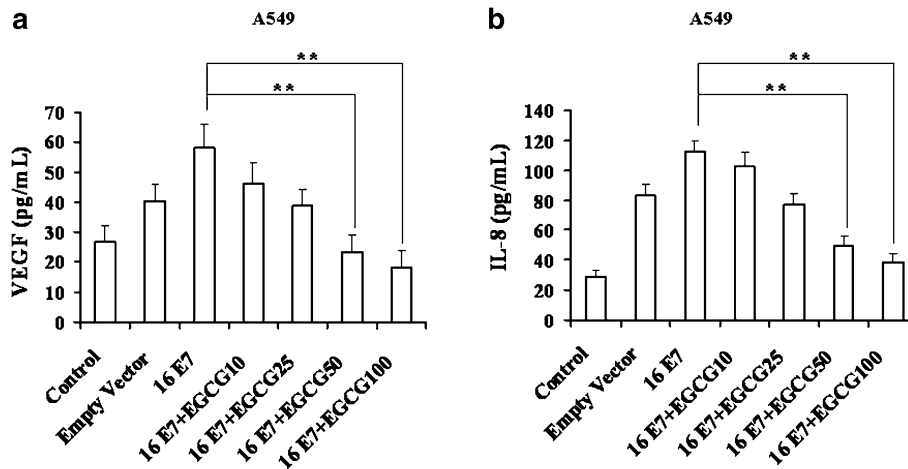
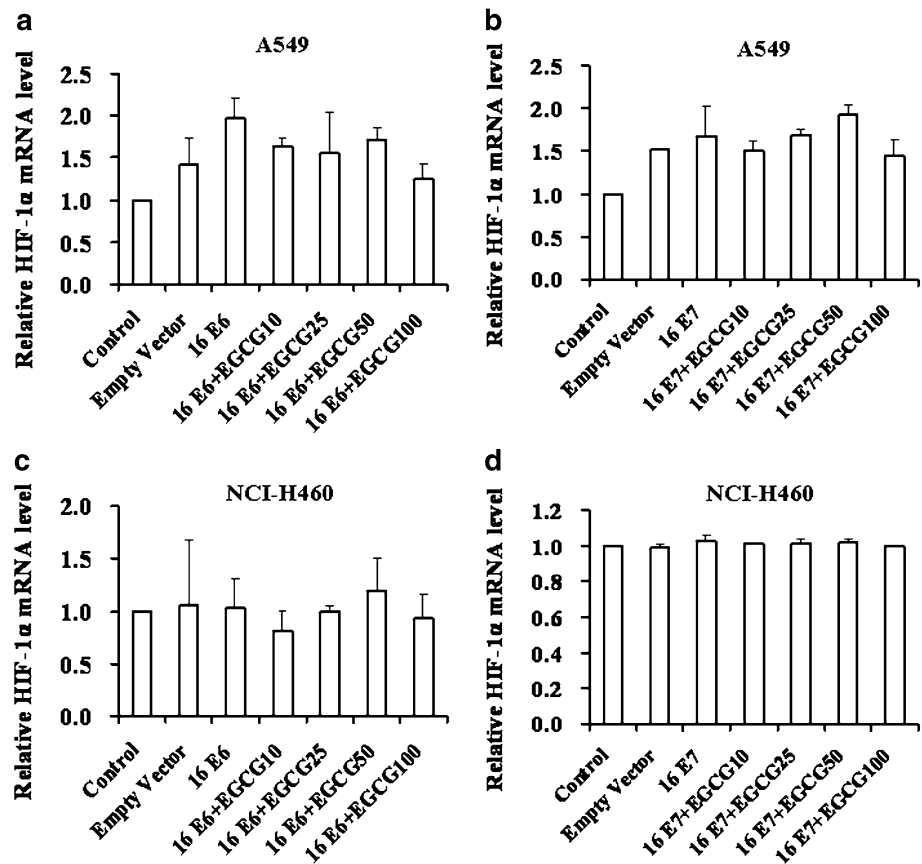
Previous studies have demonstrated that HPV-16 oncoproteins can upregulate Akt and ERK1/2 activity [34, 35]. To further explore whether EGCG can inhibit HPV-16 oncoprotein-mediated activation of Akt and ERK1/2 in A549 cells, HPV-16 E6- or E7-transfected cells were treated with various concentrations of EGCG for 16 h. Our results showed that EGCG treatment had no obvious effect on ERK1/2 activation, but led to a dose-dependent inhibition of HPV-16 E6- and E7-induced Akt activation (Fig. 4a, b), whereby 100  $\mu$ mol/L of EGCG almost



**Fig. 1** EGCG inhibited HPV-16 oncoprotein-induced HIF-1 $\alpha$  protein expression in A549 and NCI-H460 cells. A549 (**a, c**) and NCI-H460 (**b, d**) cells transfected with plasmid constructs harboring pEGFP-N1-HPV-16 E6 or E7 oncoprotein were treated with different concentrations of EGCG (10, 25, 50, and 100  $\mu$ mol/L) for 16 h. Western blot analysis was performed to detect the expression of HIF-1 $\alpha$  protein.

Transfected A549 (**e**) and NCI-H460 (**f**) cells were treated with various concentrations of EGCG for 16 h, and cell viability was assayed using MTT method. The percentage of viable cells represented the mean  $\pm$  SD from three replicate experiments. Results are representative of three independent experiments

**Fig. 2** EGCG had no effect on HIF-1 $\alpha$  mRNA expression. A549 (a, b) and NCI-H460 (c, d) cells transfected with plasmid constructs harboring pEGFP-N1-HPV-16 E6 or E7 oncoprotein were treated with different concentrations of EGCG (10, 25, 50, and 100  $\mu$ mol/L) for 16 h. Real-time RT-PCR was performed to determine the expression of HIF-1 $\alpha$  mRNA. The relative value of the mock transfection control (lane 1) was arbitrarily set as 1.0. The results represented the mean  $\pm$  SD from three replicate experiments

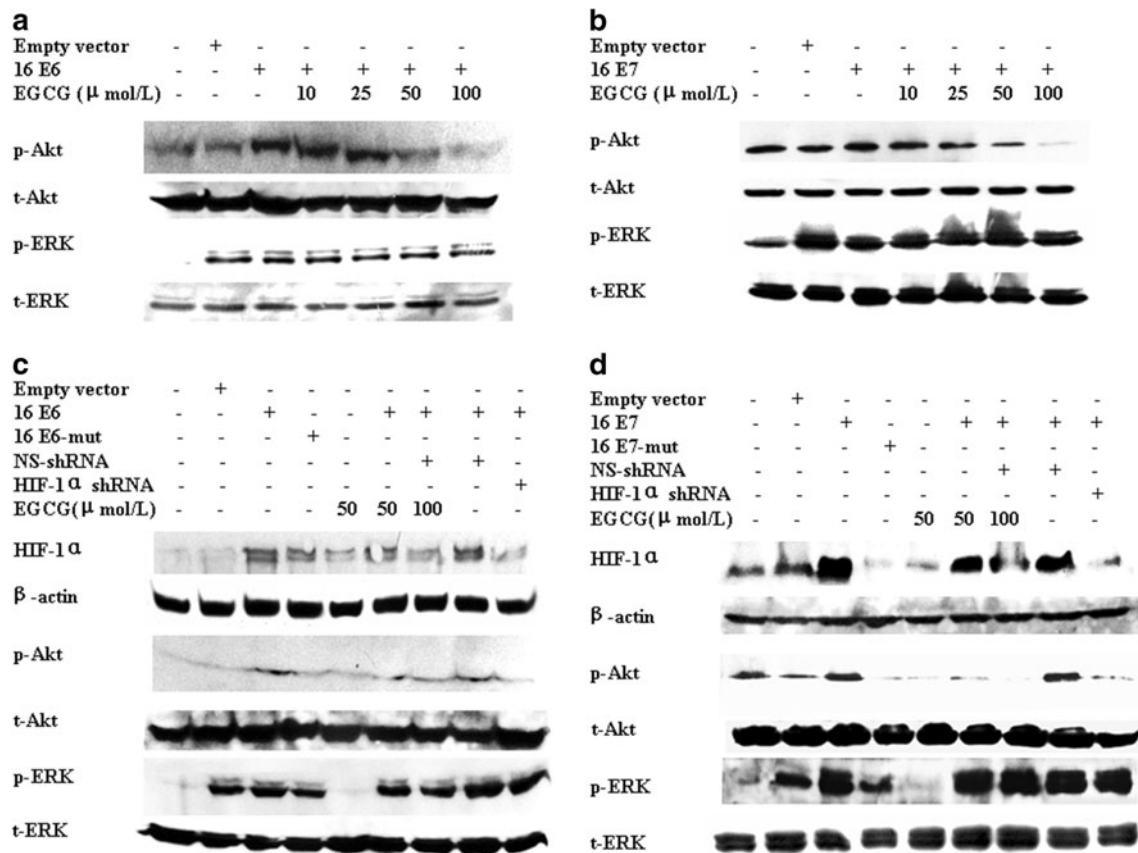


**Fig. 3** EGCG inhibited HPV-16 E7 oncoprotein-induced VEGF and IL-8 protein secretion in A549 cells. **a**, **b** A549 cells transfected with plasmid constructs harboring pEGFP-N1-HPV-16 E6 or E7 oncoprotein were treated with different concentrations of EGCG (10, 25, 50, and 100  $\mu$ mol/L) for 16 h. VEGF (**a**) and IL-8 (**b**) protein production

in the conditioned media was determined by ELISA. Results were normalized to the cell number ( $2 \times 10^5$  cells) in 1 mL of culture medium ( $2 \times 10^5$  cells/mL) and represented the mean  $\pm$  SD from three replicate experiments.  $**P < 0.01$

completely blocked HPV-16 E6- and E7-induced Akt activation (Fig. 4a, b, lane 7). Of note, such effects of EGCG on HPV oncoprotein-induced Akt activation comply with its inhibitory effects on HPV oncoprotein-induced HIF-1 $\alpha$  (Fig. 1a–d), VEGF (Fig. 3a), and IL-8 (Fig. 3b) protein expression in A549 cells.

To further confirm whether the activation of Akt induced by HPV-16 E6 and E7 oncoproteins is HIF-1 $\alpha$ -dependent, A549 cells were co-transfected with pEGFP-N1-HPV-16 E6 or 16 E7 constructs along with HIF-1 $\alpha$  shRNA plasmid (HIF-1 $\alpha$ -shRNA) or nonspecific shRNA plasmid (NS-shRNA). Our results showed that the



**Fig. 4** Effect of EGCG on HPV-16 oncoprotein-induced Akt and ERK1/2 activation in A549 cells. **a, b** A549 cells transfected with constructs harboring pEGFP-N1-HPV-16 E6 or E7 oncoprotein were treated with different concentrations of EGCG (10, 25, 50, and 100  $\mu$ mol/L) for 16 h. Western blot analysis was performed to detect the phosphorylated levels of Akt and ERK1/2. **c, d** A549 cells were

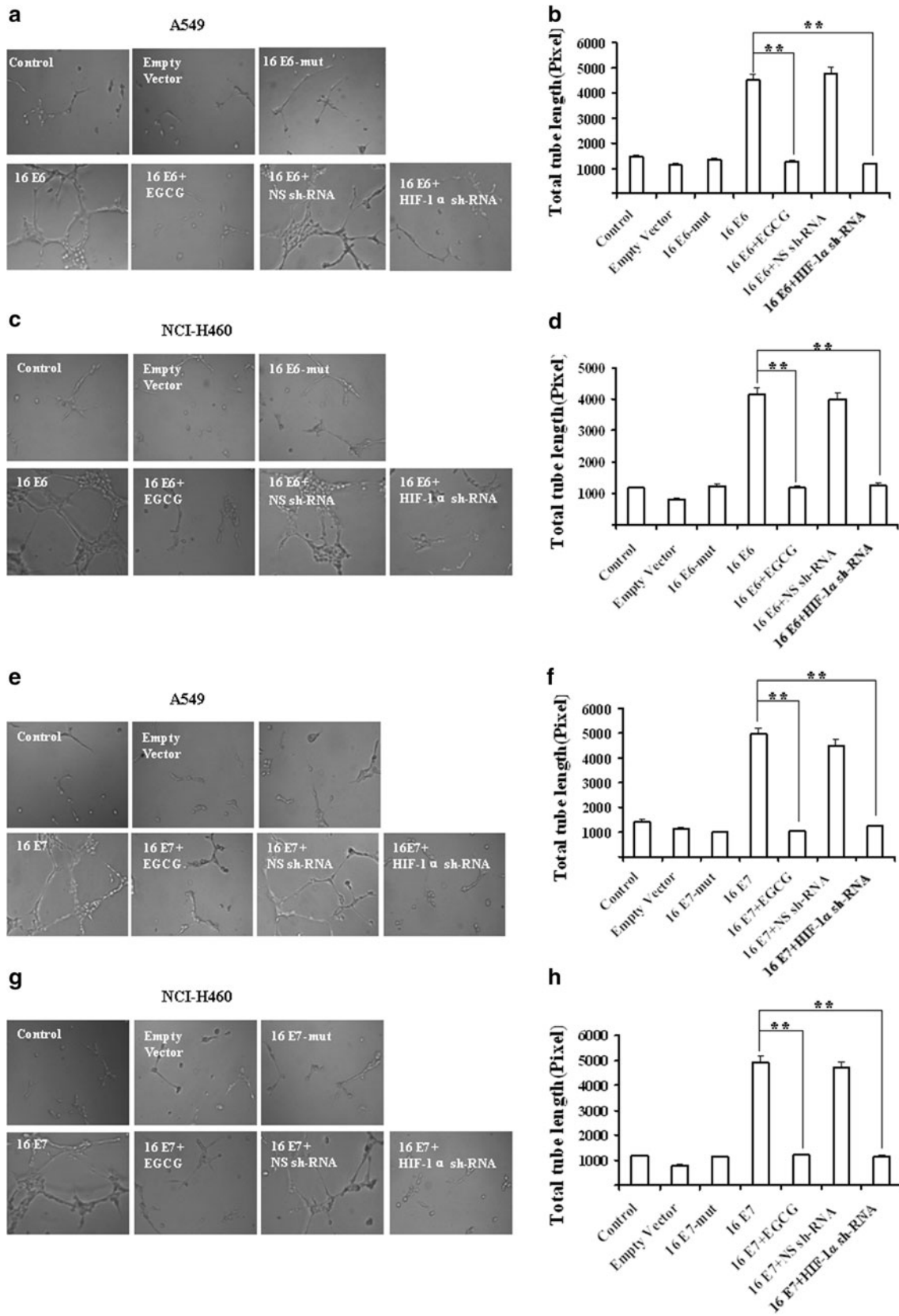
co-transfected with pEGFP-N1-HPV-16 E6 or 16 E7 constructs along with HIF-1 $\alpha$  shRNA plasmid (HIF-1 $\alpha$ -shRNA) or nonspecific shRNA plasmid (NS-shRNA). HIF-1 $\alpha$ , phosphorylated Akt (p-Akt), and phosphorylated ERK1/2 (p-ERK1/2) levels were analyzed by Western blot. Results presented are representative of three independent experiments

expression of HIF-1 $\alpha$  protein induced by HPV-16 E6 and E7 oncoproteins was inhibited by co-transfection with HIF-1 $\alpha$ -shRNA, but not by co-transfection with NS-shRNA (Fig. 4c, d, lanes 8, 9). Meanwhile, the activation of Akt induced by HPV-16 E6 and E7 oncoproteins was almost abrogated by co-transfection with HIF-1 $\alpha$ -shRNA, but not by co-transfection with NS-shRNA (Fig. 4c, d, lanes 8, 9). Taken together, these results suggested that the activation of Akt induced by HPV-16 E6 and E7 oncoproteins was HIF-1 $\alpha$ -dependent in NSCLC cells.

EGCG inhibited HIF-1 $\alpha$ -dependent angiogenesis in vitro stimulated by overexpression of HPV-16 oncoproteins in NSCLC cells

Our previous studies have demonstrated that overexpression of HPV-16 E6 and E7 oncoproteins in NSCLC cells significantly promotes tumor angiogenesis in vitro [11]. An increasing body of evidence has shown that EGCG can suppress tumor angiogenesis [24–27], so we further

investigated the effect of EGCG on HPV-16 oncoprotein-induced lung cancer angiogenesis in vitro. To this purpose, an in vitro angiogenesis model was employed to evaluate the capillary tube formation of HUVECs stimulated by the conditioned media derived from A549 or NCI-H460 cells transfected with pEGFP-N1-HPV-16 E6 or E7 plasmid in the presence or absence of 100  $\mu$ mol/L of EGCG. Our results showed that conditioned media from both A549 and NCI-H460 cells overexpressing HPV-16 E6 oncoproteins dramatically stimulated the formation of capillary tube-like structures by HUVECs (Fig. 5a, c), which was consistent with our previous findings [11]. Moreover, 100  $\mu$ mol/L of EGCG remarkably inhibited HPV-16 E6 oncoprotein-stimulated formation of capillary tube-like structures (Fig. 5a, c), which was further confirmed by the quantification of the total tube length pixel values ( $P < 0.01$ ; Fig. 5b, d). Additionally, EGCG had similar inhibitory effect on HPV-16 E7 oncoprotein-stimulated formation of capillary tube-like structures (Fig. 5e–h). These results indicated that EGCG can potentially inhibit the enhanced





**Fig. 5** EGCG inhibited HIF-1 $\alpha$ -dependent capillary tube formation in vitro stimulated by HPV-16 oncoproteins. HUVECs ( $5 \times 10^3$  cells/well) were seeded onto the surface of 96-well cell culture plates pre-coated with polymerized ECMatrix<sup>TM</sup> and then incubated at 37 °C for 6–8 h in the conditioned media derived from pEGFP-N1-HPV-16-transfected cells in the presence or absence of 100  $\mu$ mol/L of EGCG or pEGFP-N1-HPV-16 and HIF-1 $\alpha$  sh-RNA co-transfected cells. The tube formation in transfected A549 (a, e) and NCI-H460 (c, g) was observed under a phase-contrast microscope. The total tube length in three random view-fields per well was measured by Scion Image software, and average value was calculated (b, f: A549; d, h: NCI-H460). The results represented the mean  $\pm$  SD from three replicate experiments. \* $P < 0.05$

in vitro angiogenic activities stimulated by overexpression of HPV-16 oncoproteins in NSCLC cells.

To explore whether HIF-1 $\alpha$  is directly involved in HPV-16 oncoprotein-stimulated NSCLC angiogenesis in vitro, A549 cells and NCI-H460 cells were co-transfected with pEGFP-N1-HPV-16 E6 or E7 construct along with HIF-1 $\alpha$ -shRNA or NS-shRNA plasmid. Our results showed that the formation of capillary tube-like structures stimulated by overexpression of HPV-16 E6 and E7 oncoproteins in A549 cells and NCI-H460 cells was remarkably inhibited by co-transfection with HIF-1 $\alpha$ -shRNA, but not by co-transfection with NS-shRNA (Fig. 5a, c, e, g), which was further confirmed by the quantification of the total tube length pixel values ( $P < 0.01$ ; Fig. 5b, d, f, h). These results suggest that the enhanced in vitro angiogenic activity stimulated by overexpression of HPV-16 oncoproteins in NSCLC cells was, at least in part, in a HIF-1 $\alpha$ -dependent manner.

EGCG inhibited HPV-16 E6- and E7-promoted angiogenesis in vivo and expression of HIF-1 $\alpha$ , VEGF, and CD31 in A549 xenografts

Our previous studies have found that overexpression of HPV-16 E6 and E7 oncoproteins in NSCLC not only enhanced angiogenesis in vitro, but also promoted angiogenesis in vivo [11]. To further observe the effect of EGCG on HPV-16 E6 or E7 oncoprotein-promoted lung cancer angiogenesis in vivo, we performed Matrigel plug angiogenesis assay using BALB/C nude mice. Our results showed that Matrigel plugs mixed with HPV-16 E6- or E7-transfected cells significantly enhanced tumor angiogenesis in vivo (Fig. 6a-5, b-5) and showed much higher hemoglobin levels (Fig. 6c, d), which was consistent with our previous results [11]. Then, our results showed that EGCG had no obvious effect on basic angiogenesis in vivo (Fig. 6a-4, b-4) but significantly inhibited HPV-16 E6- and E7-stimulated angiogenesis in vivo (Fig. 6a-5 vs 6; Fig. 6b-5 vs 6), which was further confirmed by the quantification of the hemoglobin content in tumor xenografts ( $P < 0.01$ ; Fig. 6c, d). Additionally, our results also

showed that the in vivo angiogenesis promoted by overexpression of HPV-16 oncoproteins in A549 cells was significantly abrogated by co-transfection with HIF-1 $\alpha$  shRNA (Fig. 6a-8, b-8), but not by NS-shRNA (Fig. 6a-7, b-7), which further confirmed the critical role of HIF-1 $\alpha$  in HPV-16 oncoprotein-mediated angiogenic activities of NSCLC.

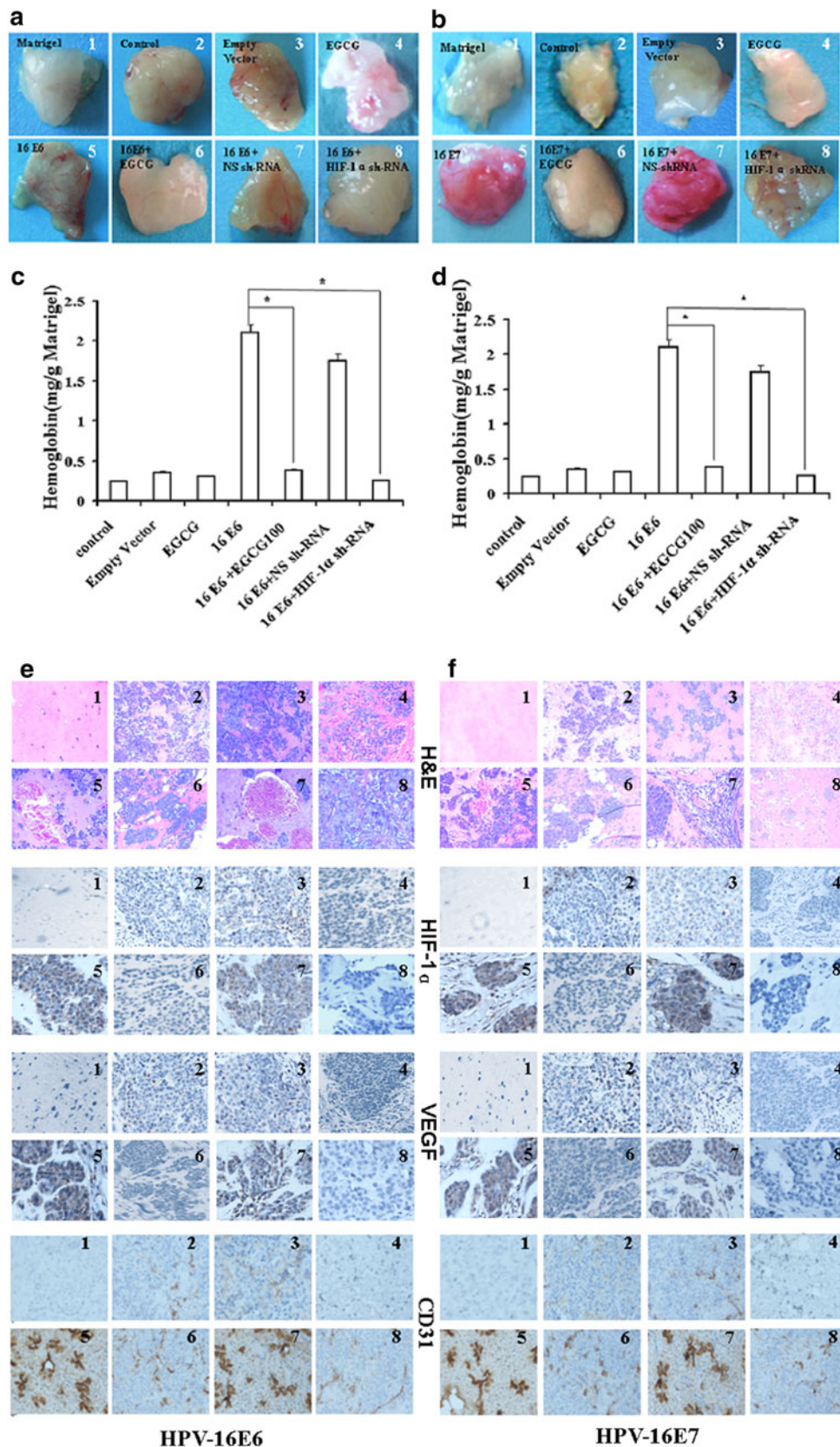
We then examined the histology and HIF-1 $\alpha$ /VEGF protein expression in tumor xenografts by H&E staining and immunohistochemical (IHC) studies, respectively. We also detected CD31 expression in the Matrigel as a marker of microvessel density. Our results showed that the location of immunoreactivity to HIF-1 $\alpha$  and VEGF was in nuclei and cytoplasm, respectively. Moreover, xenografts of A549 cells with overexpressed HPV-16 E6 or E7 oncoprotein displayed obviously enhanced HIF-1 $\alpha$ , VEGF, and CD31 (Fig. 6e, f) protein expression, while EGCG significantly suppressed the enhanced HIF-1 $\alpha$ , VEGF, and CD31 protein expression induced by HPV-16 E6 and E7 oncoproteins (Fig. 6e, f). As expected, the inhibition of HIF-1 $\alpha$  protein expression by co-transfection with HIF-1 $\alpha$  shRNA in xenografted A549 cells (Fig. 6e, f) led to a decreased expression of VEGF and CD31 proteins (Fig. 6e, f). Taken together, these results indicated that EGCG could also potentially inhibit the in vivo angiogenic activities of NSCLC induced by overexpression of HPV-16 oncoproteins through interfering with, at least in part, the HIF-1 $\alpha$ /VEGF axis.

## Discussion

Angiogenesis, the formation of new blood vessels from preexisting vascular network, is essential for tumor growth, migration, invasion, and metastasis. Specifically, angiogenesis is a key mediator of NSCLC progression [36], and anti-angiogenic therapy has been proven to be beneficial to patients with NSCLC [37]. Our previous results have shown that overexpression of HPV-16 E6 and E7 oncoproteins enhanced NSCLC angiogenesis [11]. Therefore, angiogenic inhibitors may be useful in the prevention and treatment for HPV-related NSCLC. Both in vitro and in vivo experimental studies have shown that EGCG has strong anti-angiogenic effects in different types of cancer [24–27]. In this study, we first demonstrate that EGCG can potentially inhibit HPV-16 E6 and E7 oncoprotein-induced NSCLC angiogenesis both in vitro (Fig. 5) and in vivo (Fig. 6), suggesting EGCG may be a potential agent for the prevention and treatment of HPV-related NSCLC.

HIF-1 $\alpha$ , a key transcriptional regulator, plays a central role in the adaptation of tumor cells to hypoxia by activating the transcription of targeting genes which regulate several biological processes including angiogenesis [38].

**Fig. 6** EGCG inhibited HIF-1 $\alpha$ -dependent angiogenesis in vivo and HIF-1 $\alpha$ , VEGF, and CD31 protein expression in A549 xenografts induced by overexpression of HPV-16 oncoproteins. The transfected A549 cells were re-suspended in serum-free media at  $2.0 \times 10^6$  cells/mL. Aliquots of transfected or non-transfected A549 cell suspension (0.25 mL) were mixed with BD Matrigel Matrix (0.25 mL). The BD Matrigel mixture was subcutaneously injected into both flanks of nude mice. On day 11, mice were killed and the Matrigel plugs were removed and photographed. The hemoglobin levels and the expression of HIF-1 $\alpha$ , VEGF, and CD31 proteins in A549 xenografted tumors were analyzed. **a, b** Matrigel plugs. **c, d** The hemoglobin levels in Matrigel plugs. Hemoglobin content was expressed as (mg/g) of Matrigel plug. \* $P < 0.05$ . **e, f** Immunohistochemical studies on the expression of HIF-1 $\alpha$ , VEGF, and CD31 proteins in A549 xenografted tumors. The results are representative of five independent experiments



Our previous studies have demonstrated that EGCG inhibited hypoxia- and serum-induced HIF-1 $\alpha$  expression in cervical cancer cells and hepatoma cells [29]. Domingo et al. [39] also found that HIF-1 $\alpha$  expression was decreased in EGCG-treated sites after they conducted a small randomized, double-blind, and split-face trial using a cream containing 2.5 % w/w of EGCG. Most recently, Zhu et al. [40] further reported that the attenuation of HIF-1 $\alpha$  and the consequently reduced P-gp could contribute to the inhibitory effects of EGCG on the proliferation of human pancreatic carcinoma cell line PANC-1 cells. In the present study, we found that EGCG inhibited HPV-16 oncoprotein-induced HIF-1 $\alpha$  protein expression in A549 and NCI-H460 NSCLC cells (Fig. 1). Meanwhile, our immunohistochemical results further confirmed that EGCG obviously inhibited HPV-16 oncoprotein-induced HIF-1 $\alpha$  expression in the xenografts of A549 cells (Fig. 6e, f). Moreover, the results in vitro and in vivo angiogenesis all showed that HPV-16 oncoprotein-induced NSCLC angiogenesis is HIF-1 $\alpha$ -dependent. Specially, HPV-16 oncoprotein-induced expression of CD31, a marker of microvessel density, is also HIF-1 $\alpha$ -dependent in the xenografts of A549 cells (Fig. 6e, f). Taken together, our results indicate that HIF-1 $\alpha$  may be a key molecular target for EGCG against angiogenesis of HPV-related NSCLC.

In this study, we showed that HPV-16 E6 and E7 oncoproteins had no obvious effects on HIF-1 $\alpha$  mRNA expressions, and treatment with EGCG did not affect HIF-1 $\alpha$  mRNA levels in both A549 and NCI-H460 cells (Fig. 2), suggesting that EGCG inhibited HIF-1 $\alpha$  protein expression through post-transcriptional mechanisms, for example, by inhibiting HIF-1 $\alpha$  protein synthesis and/or promoting its degradation. However, in human prostate cancer cells, EGCG has been reported to induce a dose-dependent increase in HIF-1-mediated transcription and HIF-1 $\alpha$  protein levels under normoxia, and to inhibit HIF-1 $\alpha$  degradation [30]. In rat kidneys, EGCG has been found to inhibit prolyl hydroxylase (PHD) activity, essential for HIF-1 $\alpha$  degradation in vivo and in vitro, thus stabilizing HIF-1 $\alpha$  protein [31]. Even in lung cancer cell lines, CL13, H1299, and H460, EGCG has been demonstrated to suppress lung cancer cell growth through upregulating miR-210 expression caused by stabilizing HIF-1 $\alpha$ . These controversial results may be due to the dual roles of HIF-1 $\alpha$  in tumorigenesis and/or the different experimental conditions used [32]. Therefore, more studies in the various experimental conditions are needed to confirm the effect of EGCG on HIF-1 $\alpha$ .

A large body of evidence has demonstrated that HIF-1 regulates the expression levels of more than 200 kinds of target hypoxia responsive genes [38] including VEGF and IL-8. VEGF is generally regarded as one of the most potent and specific angiogenic factors, and HIF-1 $\alpha$  is now widely

accepted to be a key regulator of VEGF expression since it can bind to VEGF promoter [36]. Our previous results, along with other reports, have shown that EGCG can inhibit VEGF expression in several sorts of cancer cells [24–27]. In the present study, we also found that EGCG suppressed HPV-16 E7 oncoprotein-induced VEGF protein secretion in A549 lung cancer cells (Fig. 3a), which was further confirmed in the xenografts of A549 cells (Fig. 6e, f). Additionally, another angiogenic factor IL-8 has been found to be modulated by EGCG in pancreatic cancer model in vivo [41] and in normal human bronchial epithelial cells [42]. In the present study, we found that EGCG inhibited HPV-16 E7 oncoprotein-induced IL-8 protein secretion in A549 cells, specially EGCG at 50 and 100  $\mu$ mol/L (Fig. 3b). Our previous study has demonstrated that overexpression of HPV-16 E6 and E7 oncoproteins in A549 cells increased the levels of VEGF and IL-8 expression in a HIF-1 $\alpha$ -dependent manner [11]. In this study, we further confirmed that VEGF protein expression in the xenografts of A549 cells with the overexpression of HPV-16 E6 and E7 oncoproteins is mediated by HIF-1 $\alpha$  (Fig. 6e-7 vs 8; Fig. 6f-7 vs 8). Collectively, these results suggest that EGCG inhibits HIF-1 $\alpha$ -dependent VEGF and IL-8 protein expression induced by HPV-16 oncoproteins in NSCLC.

HPV-16 oncoproteins have been found to activate Akt and ERK1/2 pathways [34, 35]. Our previous studies have further demonstrated that PI3K/Akt and ERK1/2 signaling pathways are involved in the regulation of HIF-1 $\alpha$  protein expression induced by HPV-16 E6 and E7 oncoproteins in C-33A and Hela cervical cancer cells [43]. In this study, we found that HPV-16 E6 and E7 oncoproteins promoted the activation of Akt in NSCLC A549 cells (Fig. 4, lane 3). To further clarify the signaling mechanisms by which EGCG inhibited HPV-16 oncoprotein-induced HIF-1 $\alpha$  protein expression, we next analyzed the effect of EGCG on the activation of Akt pathways in A549 cells in response to HPV-16 E6 and E7 oncoproteins. Our findings indicated that EGCG significantly inhibited HPV-16 oncoprotein-mediated activation of Akt in A549 cells (Fig. 4a, b), which was consistent with its inhibitory effects on HPV-16 oncoprotein-induced HIF-1 $\alpha$  (Fig. 1a, c), VEGF (Fig. 3a), and IL-8 (Fig. 3b) protein expression. To deeply determined whether Akt activation mediated by HPV-16 oncoproteins is HIF-1 $\alpha$ -dependent, A549 cells were co-transfected with pEGFP-N1-HPV-16 E6 or 16 E7 constructs along with HIF-1 $\alpha$ -shRNA or NS-shRNA plasmid. Our results showed that co-transfection with pEGFP-N1-HPV-16 E6 or 16 E7 construct along with HIF-1 $\alpha$ -shRNA abrogated Akt activation induced by HPV-16 oncoproteins (Fig. 4c, d), indicating that Akt activation mediated by HPV-16 oncoproteins is HIF-1 $\alpha$ -dependent. Taken together, these results indicate that EGCG inhibits HPV-16

oncoprotein-induced Akt activation through targeting HIF-1 $\alpha$ .

In summary, in this study, we have demonstrated, to our knowledge for the first time, that EGCG possesses potent inhibitory effects on HPV-16 oncoprotein-induced NSCLC angiogenesis both in vitro and in vivo, which may potentially attribute to the inhibition of HIF-1 $\alpha$ -governed expression of VEGF, IL-8, and CD31 as well as activation of Akt. These findings have provided substantial evidence that HIF-1 $\alpha$  might be a potential target of EGCG against HPV-related NSCLC angiogenesis.

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**Conflict of interest** None declared.

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